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STUDIES ON CELLULOLYTIC ENZYMES

I. ISOLATION OF A LOW-MOLECULAR-WEIGHT CELLULASE FROM *POLYPORUS VERSICOLOR*

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SUMMARY

A cellulolytic enzyme with a molecular weight of 11 400 has been isolated from culture filtrates of the wood-destroying Basidiomycete, *Polyporus versicolor*. The enzyme was purified in 3 steps using dextran gels (Sephadex). The culture filtrate contained a β-glucosidase and at least 2 additional cellulolytic components that have not yet been separated.

INTRODUCTION

The enzymes that hydrolyze cellulose in nature play a major role in the carbon cycle and impose a considerable tax on the economies of the world by destroying useful cellulosic materials. The cellulolytic enzymes, particularly of organisms that destroy cotton fabrics, have been studied intensively by many investigators[1]. But comparatively little research has been done on the cellulases of wood-destroying fungi[2] and only limited progress has been achieved in the purification and characterization of cellulolytic enzymes in general[3,4].

This limited progress in part is due to the nature of the substrate and the cellulolytic enzymes themselves: (a) Cellulose is an insoluble, partially crystalline substance and is associated in nature with a variety of non-cellulosic polysaccharides and lignin. These factors tend to reduce the accessibility, and therefore the susceptibility, of cellulose to enzymic degradation[5]. As a result, only fairly well purified, modified and often poorly characterized forms of cellulose can be used as substrates for enzyme assays. (b) Many cellulolytic organisms produce multiple-component cellulase systems[6-12]. These components cannot be separated readily by the classical precipitation methods of protein fractionation[3] and must be resolved by physicochemical methods. (c) Cellulolytic enzymes have a specific affinity for cellulose so that excessive losses are involved when purification is attempted by such common procedures as: filtration through filter paper or membranes of cellulose derivatives, dialysis except in animal membranes, and chromatography or electrophoresis on paper or in cellulose columns.

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In recent years, an intensive effort has been made at this Institute to perfect new methods for the isolation and purification of proteins, particularly by column electrophoresis and filtration through gels of cross-linked dextran. The former technique separates proteins according to their charge whereas the latter does so mainly according to their molecular size[13,14]. These methods appeared to offer advantages for purification of cellulolytic enzymes particularly in separating possible multiple components and avoiding excessive losses in activity due to selective adsorption effects. They were therefore applied in an attempt to purify the cellulase of the wood-destroying Basidiomycete, *Polyporus versicolor* (L. ex Fries) Fries. The effects of decay by this organism on certain chemical and physical properties of wood polysaccharides have been determined earlier[5].

METHODS AND RESULTS

Production of cellulolytic culture filtrates

The cellulolytic enzymes of fungi are adaptive and extracellular. Filtrates from fungus cultures grown in synthetic media with cellulose as sole source of carbon, therefore, are a very suitable source of cellulolytic enzymes for purification.

Cellulolytic culture filtrates of *Polyporus versicolor* were obtained by cultivating* the organism in aerated flasks similar to those used by WHITAKER[3] and shown in Fig. 1. The medium was adapted from one used by NORKRANS[15] and contained the reagent grade chemicals listed in Table I.

4 l of this medium were placed in each culture flask and the entire unit sterilized in steam at 121° for 50 min. After cooling, each flask was inoculated with approx. 2 g (dry wt.) of a suspension of washed mycelial fragments. This suspension was prepared from a culture grown on 2% malt extract for 7 days by vigorous shaking

TABLE I

COMPOSITION OF MEDIUM USED TO OBTAIN CELLULOLYTIC CULTURE FILTRATES FROM *Polyporus versicolor*

Munktells' powdered cellulose for chromatography*	5.0 g
$NH_4H_2PO_4$	2.0 g
KH_2PO_4	0.6 g
K_2HPO_4	0.4 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g
Ferric citrate**	10.0 mg
$ZnSO_4 \cdot 7\ H_2O$	4.4 mg
$MnSO_4 \cdot 4\ H_2O$	5.0 mg
$CaCl_2$	55.0 mg
$CoCl_2 \cdot 6\ H_2O$	1.0 mg
Thiamine · HCl	100 µg
Distilled water to give 1 l. Final pH of medium about 5.8.	

* Available from Grycksbo Pappersbruk, Grycksbo (Sweden).

** The stock solution contained 2.5 g of ferric citrate and 1.6 g of citric acid in 250 ml of solution.

* Laboratory space was kindly made available by Dr. B. NORKRANS at the Royal Farmaceutical Institute, Stockholm (Sweden).

with 2-mm glass beads until the fragments were reasonably homogeneous (shaking for 60 sec usually was adequate). The suspension was then washed with several changes of distilled water and aseptically poured into the culture flasks. The inoculated cultures were incubated at 28° and aerated at a rate of 1 l of unhumidified air/l of medium/min. After incubation for 7–9 days the contents of each flask were filtered through fritted glass of porosity G-3. The resulting culture filtrate was preserved by adding 0.1 g of merthiolate per liter of filtrate and stored at 4°.

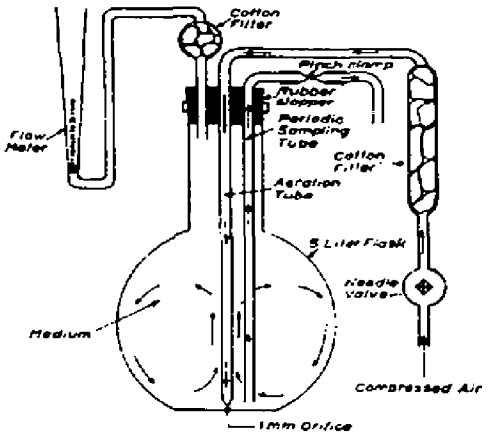

Fig. 1. Aerated culture flask used to obtain cellulolytic culture filtrate of *Polyporus versicolor*. Bubbles rising in the medium from the aeration tube cause circulation of the medium as shown.

Concentration of the culture filtrate

Preliminary experiments showed that concentration of culture filtrates of *Polyporus versicolor* by acetone precipitation or evaporation under vacuum lead to drastic losses in cellulase activity. To avoid these losses, the dextran gel concentration method of FLODIN *et al.*[16] was tried and found to give essentially quantitative recovery of cellulase activity.

By this method, dry Sephadex (a cross-linked dextran product of Pharmacia, Uppsala (Sweden)) is mixed with the solution to be concentrated. Dissolved substances of high molecular weight can not penetrate the gel particles and therefore remain in solution. A portion of the water and low-molecular-weight solutes, however, diffuse freely into the particles. When the water outside the gel particles is removed by filtration, an increase in concentration of the high-molecular-weight materials is achieved while the pH and ionic strength of the solution is maintained nearly constant. The degree of concentration achieved is a function of the water-holding capacity of the gel.

The details of the concentration procedure used in this study are as follows: approx. 300 g of Sephadex G-25 were added to each liter of culture filtrate. The resulting soft paste was filtered in a basket-type filtering centrifuge (MSE model 3000) 3 l in each batch at approx. 4500 rev./min through a sheet of Vyon (a product of Porous Plastics Limited, Dagenham Docks, Essex (England)) and the filtrate collected. The packed gel in the centrifuge basket was washed by spraying with an amount of

distilled water equal to one-tenth of the original filtrate volume. The washing water was removed by centrifugation and added to the original filtrate. By this procedure a 3-fold increase in concentration was achieved in a single step. By repeated application of the procedure 85 l of culture filtrate were concentrated to 315 ml without substantial loss in cellulase activity. The concentrated solution contained 20 mg of high-molecular-weight solutes (proteins plus carbohydrates)/ml. The solution showed no measurable loss in activity after storage for 6 months.

Enzyme assays

Cellulase activity was determined by the amount of soluble reducing sugars formed when the enzyme was incubated with carboxymethyl cellulose or a regenerated cellulose sol prepared from cotton linters according to NORKRANS[16] and MCBETH[17]. The carboxymethyl cellulose assay medium contained 9 ml of a 1% (w/v) aqueous solution of carboxymethyl cellulose of degree of substitution 0.7 (kindly provided by Mr. B. NAUCLER, Uddeholm Aktiebolag, Uddeholm (Sweden)), 1 ml of 0.5 *M* sodium acetate buffer (pH 5.0) and 1 mg of merthiolate. The cellulose sol assay medium contained 4.5 ml of a 1% (w/v) aqueous suspension of cellulose sol, 0.5 ml of 0.5 *M* sodium acetate buffer (pH 5.0) and 0.5 mg of merthiolate. Immediately upon addition of 50–200 μl of enzyme solution, and again after incubation for 15 min at 40°, the reducing sugars present in 1 ml of the enzyme-incubation mixture were determined spectrophotometrically by the method of SOMOGYI[18] and NELSON[19]. The difference in reducing power (expressed as glucose) at zero time and after 15 min incubation gave a relative estimate of cellulase activity.

β-Glucosidase activity was determined by a modification of the method of NORKRANS[20] using *p*-nitrophenyl-β-D-glucoside as the substrate. The assay mixture contained 2 ml of 0.001 *M* *p*-nitrophenyl-β-D-glucoside in 0.05 *M* sodium acetate buffer at pH 5.0 and 100 μl of enzyme solution. After zero time and again after incubation at 40° for 10 min, 0.5 ml of the mixture was placed in a test tube containing 1 ml of 1 *M* Na_2CO_3. 10 ml of distilled water were added and the amount of nitrophenol liberated was determined from the absorbancy at 400 mμ in a Beckman B spectrophotometer. The difference in percent *p*-nitrophenyl-β-D-glucoside split at zero time and after 10 min incubation gave a relative estimate of β-glucosidase activity.

Fractionation by gel filtration

Two columns of Sephadex G-75 (water regain 8 g/g dry gel, particle size 170 to 300 mesh) were prepared for use in this study by the method of FLODIN[21]. Column I was prepared by suspending 50 g of Sephadex in about 2 l of 0.1 *M* pyridine–acetate buffer (pH 5.0) and allowed to swell for 15 h. The fine particles that remained suspended 30 min after resuspending the gel in the buffer were removed by decantation. The gel suspension was then trasferred through a packing tube 3 cm in diameter into a cylindrical chromatography tube 3 × 100 cm in length. The chromatography tube was closed at the bottom with pyrex glass wool above a 2-cm layer of glass beads (Ballotini No. 12). A 70-cm-high column was obtained by sedimentation. The void volume of the column was 120 ml as determined using phycoerythrin as a test sub-

stance. Column II was 1.5 by 42 cm in length, had a void volume of 35 ml, and was prepared in a similar fashion.

Preliminary tests showed that the recovery and distribution of enzyme activity on the gel columns were essentially identical with 0.1 and 0.01 *M* pyridine–acetate buffer (pH 5.0) and 0.2 *M* barium acetate buffer (pH 5.6). Thus the following desalting and fractionation procedure was adopted: 5 ml of concentrated filtrate were placed on top of column I and filtered down. The column was then eluted at 3.0 ml/h with 0.1 *M* pyridine–acetate buffer (pH 5.0). 3-ml fractions were collected and their absorbancy at 280 mμ determined in a Beckman DU spectrophotometer to give an estimate of protein concentration. After filtration of each 5-ml aliquot of culture filtrate, the column was eluted for 15–17 h with the same buffer to remove previously uneluted contaminants.

A typical distribution of protein and enzyme activities after gel filtration on column I is shown in Fig. 2. Two protein peaks are evident and β-glucosidase and cellulase activities have been separated almost completely. The active fractions were pooled as shown in Fig. 2 to give 4 components—A, B, C and D—which were each freeze dried. This gel-filtration procedure was repeated batchwise until all of the concentrated culture filtrate was fractionated into these 4 major components.

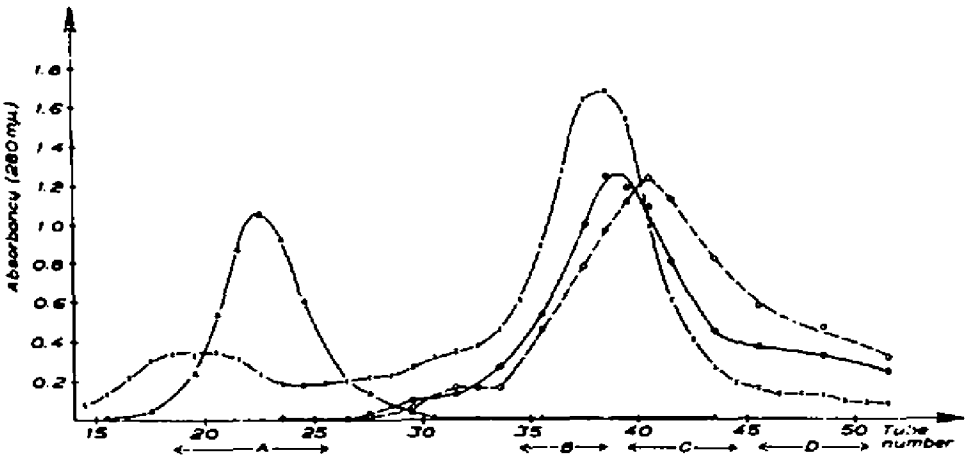

Fig. 2. Distribution of protein, cellulase, and β-glucosidase activity after gel filtration of a concentrated culture filtrate of *Polyporus versicolor* on Sephadex G 75 (column I). × — ×, absorbancy at 280 mμ; △—△, β-glucosidase activity measured against *p*-nitrophenyl-β-D-glucoside; ○—○, cellulase activity measured against carboxymethyl cellulose; ● ●, cellulase activity measured against cellulose sol.

After dissolving 18 mg of component D in 0.5 ml of 0.1 *M* pyridine–acetate buffer (pH 5.0) and refiltering with this same buffer on column II at an elution rate of 6 ml/h, coincident and symmetrical single peaks for protein and cellulase activity were obtained as shown in Fig. 3. The central fractions in component D were pooled as shown and again freeze dried in preparation for sedimentation studies.

From the original 85 l of culture filtrate the yield of each major component after freeze drying was: 200 mg of component A (with a very high carbohydrate content), 175 mg of B, 110 mg of C, and 45 mg of component D.

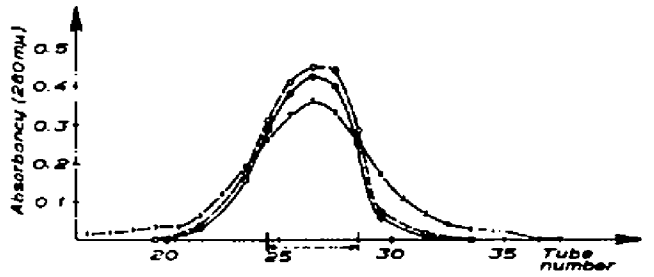

Fig. 3. Distribution of protein and cellulase activity of component D after refiltration on Sephadex G 75 (column II) with 0.1 *M* pyridine acetate (pH 5.0). ×—×, absorbancy at 280 mμ; ○···○, cellulase activity measured against carboxymethyl cellulose; ● ●, cellulase activity measured against cellulose sol.

Sedimentation studies

Sedimentation analyses were performed on components B, C and D in a Spinco Analytical Centrifuge model E with an analytical D-rotor and a synthetic boundary cell. The freeze dried components were dissolved in 0.1 *M* pyridine–acetate buffer (pH 5.0) to give 1% (w/v) solutions of components B and C and a 0.6% (w/v) solution of component D. The average speed of rotation was 59 780 rev./min and the temperature during the experiment varied between 19.5 and 20.0°. Component B gave a single symmetrical peak with a sedimentation constant ($s_{20,w}$) of 3.75. Component C gave 2 peaks with an $s_{20,w}$ of 3.75 for the rapid component and 1.55 for the slow one. After filtration on column II, component D gave a single symmetrical peak with an $s_{20,w}$ of 1.53.

Determination of molecular weight

A tentative estimate of the molecular weight of fraction D was determined after filtration on column II by the method of ARCHIBALD[22]. The centrifuge cell was filled to a height of 8 mm with a 1% (w/v) solution of the enzyme in 0.1 *M*

TABLE II

MOLECULAR WEIGHT OF CELLULASE COMPONENT D ESTIMATED BY SEDIMENTATION ANALYSIS

Time after start (min)	Molecular weight	
	Meniscus (g/mole)	Bottom (g/mole)
8	11 700	—
34	9 800	10 700
42	10 800	12 600
50	11 100	11 500
58	9 200	12 400
66	9 800	12 700
Average	10 700	12 000
Average for both estimates	11 400	

pyridine–acetate buffer (pH 5.0). During centrifugation at an average of 12 590 rev./min at 21°, the molecular weight estimates shown in Table II were determined from the meniscus and bottom readings assuming 1 $-\bar{v}\rho = 0.29$.

The value 0.71 for $\bar{v}$ has been estimated from the amino acid composition[23]. If $\bar{v}$ for the carbohydrate moiety is considered to be 0.60, the mean molecular weight will be reduced to 10 000.

The variation among these molecular-weight estimates probably was due to uncertainty in the determination of increments in refractive index at the boundaries rather than to polydispersity. This uncertainty resulted in part from the necessity of using a lower than optimal protein concentration in the cell.

Electrophoretic analysis

It soon became evident that no suitable electrophoretic method was available for the characterization of component D. The substance remained at the starting point after electrophoresis in starch gel and polyacrylamide gel. This could be due to the fact that the substance was close to its isoelectric point at the actual pH 4.5 or it could depend on inability of the enzyme to penetrate the gel. To eliminate the latter possibility a density gradient composed of water and heavy water was used as medium for electrophoresis (the method will be described in a later publication). The material did not migrate neither could any heterogeneity be observed. Because of lack of material further experiments could not be performed.

Carbohydrate analysis

The hexose content of fraction D after refiltration was determined with the orcinol method of VASSEUR[24] to 33% (w/w).

DISCUSSION

The results of these experiments clearly demonstrate that dextran gels can be used to concentrate, desalt and provide a preliminary fractionation of the cellulolytic enzymes of *Polyporus versicolor*. Extensive losses in cellulase activity usually associated with precipitation and evaporation methods of concentration, precipitation methods of fractionation, and with desalting by dialysis in cellulose membranes have been avoided entirely.

The filtration procedure gave very reproducible fractionations when performed on the same gel column. Each of 3 buffer systems tested gave identical patterns of distribution for protein and enzyme activities within the limits of errors of analysis. The patterns achieved were consistent with the principle of fractionation according to molecular size.

β-Glucosidase has been separated completely from cellulase and component D has been separated from at least 2 other cellulolytic components by filtration on 2 dextran gel columns. The curves (Fig. 2) for activity of components B and C against carboxymethyl cellulose and cellulose sol do not coincide. This may be explained by the presence of several cellulolytic components of different substrate specificities. This explanation also is supported by the polydispersity observed during sedimentation analysis of these components.

Component D was monodisperse both in filtration on gel column II and in the ultracentrifuge. Its activity was about 5 times as high as the original culture filtrate when calculated on the basis of protein content (280 mμ absorbancy). The extent of purification calculated on solids content was very much higher. The molecular weight 11 400 calculated for component D places this enzyme among the smallest known enzymically active native proteins and it is very much lower than the 63 000 reported by WHITAKER[25] for a purified cellulase from *Myrothecium verrucaria*.

Further purification of the impure components here obtained will be reported later. Kinetic studies of component D are in progress and studies of the comparative activity of the various components are planned.

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