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STUDIES ON CELLULOLYTIC ENZYMES

II. MULTIPLICITY OF THE CELLULOLYTIC ENZYMES OF *POLYPORUS VERSICOLOR*

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SUMMARY

1. Four cellulolytic components have been isolated from culture filtrate of *Polyporus versicolor*.

2. The molecular weight of one of the components has been determined to be 51 000.

3. A comparison has been made of the amino acid compositions of the 51 000-component and the previously isolated 11 000-component.

4. An explanation for the occurrence of multiple enzymes of varying cellulolytic activities has been proposed.

INTRODUCTION

The cellulolytic enzymes of a large number of fungi have been purified using block electrophoresis on potato starch as described by MANDELS *et al.*[1]. These studies have shown that most of the fungi studied to date appear to produce several cellulolytic components. The existence of multiple components also has been demonstrated by electrophoresis of other enzyme systems. Various explanations have been proposed to account for this heterogeneity[2]. For example, MILLER *et al.*[3] recently have shown that electrophoretically separated components differ in specific activity on carboxymethyl cellulose and cellopentaose. It therefore appears likely that some of the cellulolytic components may play different roles in the degradation of cellulose.

The present work was undertaken to elucidate the multiple-component cellulase system of *Polyporus versicolor*. It is a continuation of the investigation reported in the preceding article[4].

METHODS AND RESULTS

Source of the enzyme

The material for the present experiments was obtained as filtrates from cultures of the wood-destroying basidiomycete, *Polyporus versicolor*, grown in a mineral salts medium with cellulose as sole source of carbon. The filtrate was concentrated and fractionated on Sephadex as described earlier[4], giving 3 fractions, designated B, C and D.

Enzyme assays

The cellulase activity of all fractions was determined as the amount of soluble reducing substances formed when the enzyme was incubated with carboxymethyl cellulose or cellulose sol. Details of the analysis procedures were given previously[4].

Zone electrophoresis

Purification of Fraction B (small scale)

Zone electrophoresis was performed in a vertical bed[6] of Pevikon powder (Pevikon C 870 obtained from Fosfatbolaget, Stockholm (Sweden)). The material was purified by extensive washing with ethanol, then with 4 *M* hydrochloric acid and finally with distilled water. The powder was then suspended in 0.05 *M* pyridine–acetate buffer (pH 4.5). The electrophoresis column, a 1 × 35 cm glass tube, was closed at the bottom with a plate of porous plastic (Vyon available from Porous Plastic Ltd. Dagenham Docks, Essex (England)). The column was provided with a horizontal capillary side tube (1 mm in diameter) 2 cm from the bottom. Tubing of polyvinylchloride 60 cm in length and 1 mm inside diameter was connected to the capillary side tube. The column was packed with glass beads (Ballotini No. 12) to a height of 2 mm above the capillary side arm. Pevikon paste freed from air by evacuation was transferred to the column through a packing tube to form a 30-cm high column. The filtering properties of the bed were tested by passing a narrow zone of dinitrophenylethanolamine through the column.

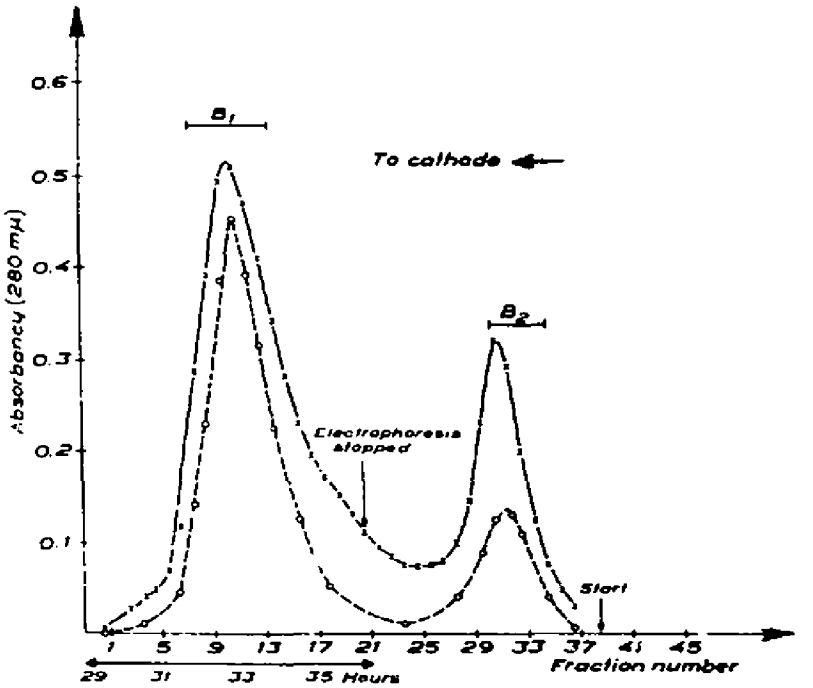


Fig. 1. Zone electropherogram of Fraction B. Bed dimensions 1 × 28 cm; buffer 0.05 *M* pyridine–acetate (pH 4.5); current, 9 mA; voltage, 700 V. Fractions 7–14 (B_1) and 30–36 (B_2) were pooled. ×- - ×, absorbancy at 280 mμ; ○– ○, cellulase activity against carboxymethyl cellulose (arbitrary units).

20 mg of Fraction B were dissolved in 0.4 ml 0.05 *M* pyridine–acetate buffer (pH 4.5) and placed on top of the column. The zone was allowed to filter down 1 cm and the column then arranged as described by PORATH *et al.*[6]. Each electrode vessel contained 4 l of buffer (0.05 *M* pyridine–acetate (pH 4.5)). The polyvinylchloride-tubing was connected with a peristaltic pump via 0.2 × 10-cm latex tubing. The buffer in the electrode vessels was mixed twice at suitable time intervals to keep the pH constant. After electrophoresis for 20 h at 700 V and 9 mA, continuous elution was begun at a constant flow rate of 4.5 ml/h. After a second period of 20 h, electrophoresis was stopped and the material on the column displaced with fresh buffer at a flow rate of 1.5 ml/h. Fractions containing 0.5 ml were collected. After dilution to 1.5 ml the absorbancy at 280 mμ was measured in a Beckman DU spectrophotometer.

The results are shown in Fig. 1 and reveal two active fractions: B_1 and B_2*. The distribution of cellulase activity and absorbancy was found to coincide over the greater part of the B_1 zone. Within experimental error, the curves for activity on cellulose sol and carboxymethyl cellulose were coincident. The fractions were pooled as indicated in Fig. 1 and then freeze-dried.

Purification of Fraction B (large scale)

Larger quantities of Fraction B (50 mg/ml buffer) were fractionated by a similar procedure. A 2 × 30-cm column was used. It was provided with a cooling jacket of methylpolymethacrylate (Perspex) but was not equipped for continuous elution. The electrophoresis time required at 400 V and 22 mA was calculated from the results of earlier experiments. Thus it was possible to stop the electrophoresis when the fast-moving component was near the bottom of the column. After elution, the analysis of the fractions collected revealed that the material distribution was comparable with the earlier findings as shown in Fig. 1.

Purification of Fraction C

Fraction C also was fractionated on the same column and in exactly the same manner as Fraction B (small scale). The plots of activity and absorbancy of the various fractions are very similar to those for Fraction B, but the activity and absorbancy curves are not coincident. The fractions were pooled and freeze-dried as indicated in Fig. 2.

Carbohydrate analysis

The hexose content of component B_1 was estimated to be 1% (w/w) by the orcinol method as described by VASSEUR[7]. The hexose content of component D previously had been determined to be 33% (w/w)[4].

Amino acid analysis

The amino acid composition of oxidized samples of components B_1 and D were determined using a Beckman-Spinco automatic amino acid analyzer and the proce-

* The enzymes present in the fractions obtained from gel filtration or electrophoresis will be referred to using the same labels as the fractions B_1, B_2 etc. whether they are pure or not.

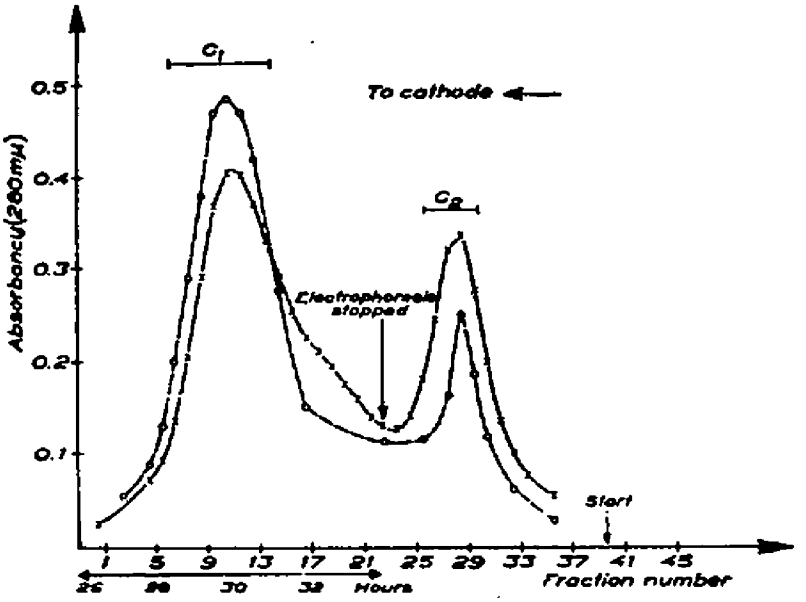


Fig. 2. Zone electropherogram of Fraction C. Bed dimensions, buffer, current and voltage as in Fig. 1. Fractions 6–14 (C_1) and 26–31 (C_2) were pooled. ×—×, absorbancy at 280 mμ; ○– –○, cellulase activity against carboxymethyl cellulose (arbitrary units).

TABLE I

PRELIMINARY AMINO ACID COMPOSITION OF COMPONENTS B_1 AND D

Amino acid	*Sample B_1 (μmoles)*	*Sample D (μmoles)*	*Ratio of amino acid content (B_1/D)*
Lysine	0.257	0.029	9.0
Histidine	0.079	0.015	5.3
Ammonia	0.944	0.395	2.4
Arginine	0.150	0.030	5.0
Cysteic acid	0.030	0.017	1.8
Aspartic acid	0.832	0.1605	5.2
Methionine sulfone	0.098	0.008	12
Threonine	0.493	0.1051	4.7
Serine	0.516	0.1041	5.0
Glutamic acid	0.490	0.1010	4.9
Proline	0.210	0.0779	2.7
Glycine	0.609	0.0683	8.9
Alanine	0.415	0.0703	5.9
Valine	0.265	0.0744	3.6
Isoleucine	0.193	0.0405	4.8
Leucine	0.341	0.0726	4.7
Tyrosine	0.120	Destroyed*	—
Phenylalanine	0.223	0.0288	7.7

* The oxidizing agent was destroyed using hydrobromic acid[9].

dure of SPACKMAN *et al.*[8]. Because of the small amount of material available, only a preliminary determination of amino acid content could be made. The samples consisted of freeze-dried material—2.60 mg of component B_1 and 1.20 mg of component D. The hydrolysis time was 24 h. One third of the material was placed on each column of the analyzer. The amino acid compositions of the samples are shown in Table I.

Sedimentation studies

Sedimentation analysis was performed with a Spinco model E ultracentrifuge provided with a model D analytical rotor and a synthetic boundary cell. The experiments were carried out at an average speed of 59 780 rev./min and at a temperature of 20°. 1 ml of a 1% (w/v) solution in 0.1 *M* pyridine acetate buffer (pH 5.0) was used. Component B_1 gave a single sedimentating boundary. The sedimentation coefficient $s_{20,w}$ was found to be 4.21 S. Component B_2 when examined under the same conditions also appeared to be homogeneous and $s_{20,w}$ equal to 4.26 S was obtained. Lack of material made it necessary to run component C_1 and C_2 at a low concentration namely 0.3% (w/v). Under these suboptimal conditions it was difficult to discover any polydispersity. $s_{20,w}$ was found to be 2.0 S for C_1 and 1.9 S for C_2.

Determination of molecular weight

The molecular weight of component B_1 was determined by the method of ARCHIBALD[12]. The centrifuge cell was filled to a height of 10 mm with a 1% (w/v) solution of the protein in 0.1 *M* pyridine acetate (pH 5.0). The average speed of rotation was 11 573 rev./min at 20°. The molecular weight estimates in Table II were determined

TABLE II

MOLECULAR WEIGHT OF CELLULASE COMPONENT B_1 ESTIMATED BY SEDIMENTATION ANALYSIS

The variation among the values is probably due to uncertainty in extrapolation of increments in refractive indices at the boundaries rather than polydispersity

Time after start (min)	*Molecular weight*	
	Meniscus (g/mole)	*Bottom (g/mole)*
8		—
16	55 400	48 500
24	53 200	46 000
32		51 200
40	51 900	48 700
48	51 000	51 600
56	48 900	52 600
64	—	48 900
72	53 800	54 200
80	52 400	51 300
88	49 300	52 200
96	47 100	52 900
Average	51 600	50 800

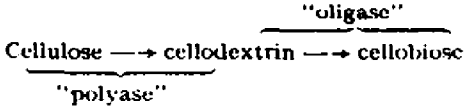
from extrapolated meniscus and bottom values, assuming $1 - \bar{v}\rho = 0.29$. The value 0.71 for $\bar{v}$ was calculated from the amino acid composition according to McMEEKIN AND MARSHALL[11].

DISCUSSION

The results of this investigation clearly demonstrate the occurrence of multiple cellulolytic enzymes in *Polyporus versicolor*. The enzyme components differ in molecular size and differences in substrate specificity have been observed. In addition to β-glucosidase, at least 5 cellulolytic components seem to be present in the culture filtrate. Additional components are indicated by the pattern of distribution of activity in the various fractions (Figs. 1, 2). We have recognized two pairs of components moving with slightly different relative velocities on Sephadex G 75 (see ref. 4) and with sedimentation coefficients of 4.2 and 2.0 S respectively. In a preceding paper[4] a fifth component, designated D, has been described. Lack of material has prevented examination of the possible significance of the occurrence of paired enzymes. Only components B_1 and D have been obtained in quantities sufficient for semiquantitative amino acid analysis and molecular weight determinations.

Despite the uncertainty of amino acid analysis, several points of evidence in the results appear worthy of mention. Components B_1 and D contained conspicuous amounts of acidic amino acids. The ammonia content in the hydrolysate was very high. Electrophoresis revealed that B_1 was only slightly positively charged at pH 4.5 while component D was immobile at this pH. These facts together strongly suggest that a large number of carboxamide side groups are present in both cellulase components. As shown in Table I, nine amino acids out of sixteen occur in about the same proportions. These findings suggest the existence of common polypeptide units.

A number of cellulolytic organisms produce multi-component cellulases[12]. It has been shown that these components occasionally differ in specificity on polysaccharides of various molecular sizes. Based on these observations STONE[13] proposed the following scheme for the degradation of cellulose.



It is evident from the present work that the basidiomycete *Polyporus versicolor* produced several cellulolytic components. The gel filtration method, described in the preceding article[4], gave a fractionation according to molecular size; the low-molecular-weight proteins being less mobile than those of high molecular weight. These earlier results therefore show, that the β-glucosidase in Fraction A, is of higher molecular weight than the enzymes present in Fractions B, C and D. The latter enzymes are active on cellulose and carboxymethyl cellulose. The activity curves for cellulose and carboxymethyl cellulose are displaced in relation to one another in a manner that suggests that the enzymes most active on cellulose are of larger molecular size than those active on carboxymethyl cellulose. Lack of coincidence between these two kinds of activity has been demonstrated even more convincingly for cellulolytic enzymes of some other organisms (to be published later). These circumstances indicate

that an inverse relationship may exist between the molecular size of the enzyme components and that of the substrates: Low-molecular-weight cellulases showing highest activity on glucosidic bonds in polysaccharide chains while the high-molecular-weight enzymes, with the β-glucosidase as the most extreme case, preferentially catalyze the hydrolysis of oligosaccharides. Such a molecular size relationship should facilitate the degradation of cellulose by a series of enzymes acting in a sequential manner. To test the validity of this hypothesis, these investigations will be extended to include cellulases from other fungi, together with a kinetic characterization of the components isolated.

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