Carboxymethylcellulase Production by *Verticillium albo-atrum*

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SUMMARY

A strain of *Verticillium albo-atrum* produced CM cellulase in liquid culture containing either cellulose powder or carboxymethyl-cellulose (CM cellulose) as sole carbon source, and when the fungus was growing in its natural host (lucerne). The CM cellulase liberated when the fungus was grown in liquid culture was fractionated on Sephadex G75, giving three major peaks and two minor peaks of cellulase activity. The materials of the three major peaks differed from each other in molecular weight, Michaelis constant and pH/activity curve, but were the same whether induced by cellulose powder or CM cellulose. Incubation of the materials of the three major peaks with CM cellulose resulted in the production of only medium-length polymers and cellobiose. No cellobiase was detected in liquid culture. CM cellulase activity was found to be only slight in the living infected lucerne tissue but was considerable in the dead infected tissue.

INTRODUCTION

The components involved in the enzymic breakdown of cellulose (cotton) by *Trichoderma viride* were separated by Selby & Maitland (1967) into a carboxymethylcellulase (CM cellulase), a cellobiase and a component (C₁) which had no action alone but operated synergistically with the other two components to convert cotton completely into water-soluble products. Previous investigations with *Myrothecium verrucaria* (Selby & Maitland, 1965) had shown that this fungus produces three major cellulolytic components, one having 90% of the total CM cellulase activity, the other two being mainly responsible for the activity of the filtrates towards cotton. Recently cellulases have been separated from the culture medium of *Penicillium notatum*. The larger of the two peaks obtained on DEA Sephadex A25 was further resolved into two components by fractionation on Sephadex G-75 (Pettesson, 1968).

The active role of cellulases during the penetration of plant pathogenic fungi through the cellulose walls of host tissues is in doubt. Talboys (1958) clearly demonstrated cellulase activity in cultures of *Verticillium albo-atrum* causing a vascular wilt disease in hops. Enzyme activity in cultures grown on filter-paper pulp led to the production of a reducing sugar, tentatively identified as cellobiose. Starch, sucrose, glucose and lactose were found to inhibit methylcellulase activity in the presence of cellulose, and from the likely presence of these substances in the host cells Talboys assumed that the passage of the very fine penetration hyphae through cell walls was accomplished by mechanical rather than by chemical means. He suggested that the cellulase system of *V. albo-atrum* involved both an initial cellulase (C₁) and a CM

cellulase (Cx), although no attempt at separation was made. Stoddart & Carr (1966) found that the major wilting activity in *V. albo-atrum* filtrates could be ascribed to a high molecular weight fraction of protein and polysaccharide components, and since Selby & Maitland (1967) found that the C1 component in *Trichoderma* cultures was a glycoprotein, it seems that protein-carbohydrate complexes are of considerable importance in the extra-cellular products of these fungi. In the present paper we report on the results of an investigation into CM cellulase produced by a strain of *V. albo-atrum* which attacks lucerne.

METHODS

Cultures. A strain of *Verticillium albo-atrum* isolated from diseased lucerne (from Norfolk in 1964) was cultured in 16 oz. medical flats containing 100 ml. of medium at 25° in the dark for 20 days. The medium contained either cellulose powder (Whatman no. 1) 5.0 g./l., or carboxymethylcellulose (CM cellulose) (Na⁺ salt, D.S. 0.45 to 0.55, British Drug Houses), 5.0 g./l., and the following mineral salts: KCl, 0.5 g./l.; MgSO₄·7H₂O, 0.5 g./l.; FeSO₄·7H₂O, 0.01 g./l.; in 0.05 M-sodium phosphate buffer (pH 6.0). The fungus was removed by centrifugation at 3000 g for 15 min. in a swing bucket centrifuge. The supernatant fluid was reduced to about 5% of its original volume in a rotary evaporator at 45° and the concentrate sterilized by membrane filtration (Oxoid 'standard' grade) before separation and assay procedures.

Enzyme separation. Approximately 25 ml. of sterile concentrate of culture fluid were applied to a column (95 cm. × 2.5 cm.) of Sephadex G-75 (dry sieved particle size < 75 μ) contained in a water jacket at 25°. The column was eluted with a solution containing NaCl 100 mM, Na₂HPO₄ 3.3 mM, NaH₂PO₄ 6.7 mM (pH 6.5) (Selby & Maitland, 1965), at a rate of 12 ml./hr., and the eluate collected in 5 ml. fractions. The Sephadex column was calibrated by using blue dextran (MW 2,000,000), bovine serum albumin (MW 64,000), peroxidase (MW 45,000), cytochrome c (MW 12,000) and sucrose (MW 342). The blue dextran, bovine serum albumin and cytochrome c were assayed by measuring the extinction at 625, 230 and 412 mμ, respectively, the peroxidase by the hydrogen peroxide/pyrogallol method (Polis & Shmukler, 1953) and the sucrose estimated by total fructose (Bacon & Tell, 1948).

Assay methods. Eluted fractions of the culture medium were assayed viscometrically in an Ostwald type viscometer (BS/1P/U size D) with 1 ml. samples and 25 ml. 1% carboxymethylcellulose (CM cellulose) (Na⁺ salt, D.S. 0.45 to 0.55, B.D.H.; prepared by mechanical stirring at room temperature) in 6 mM-acetate buffer (pH 5.0). Reaction mixtures were incubated at 25° for 30 min.

Michaelis constants of the materials of the three main enzyme peaks, separated by the above method from cultures grown in media containing either cellulose powder or CM cellulose as the sole carbon source, were calculated from results of viscometric assays. The disappearance of substrate is expressed in CM cellulose equivalents, i.e. the concentration of CM cellulose that would have the same viscosity. The molecular weight of the CM cellulose used for Km determinations was calculated from osmotic pressure measurements with a high speed membrane osmometer (Mechrolab model 502) at 25° with concentrations ranging from 0.01 to 0.07%.

The effect of pH value on the activity of the three main enzyme components was investigated viscometrically by using 2 ml. of 0.2 M-citrate phosphate buffer, 0.3 ml.

enzyme solution and 25 ml. 1% CM cellulose. Results are expressed as a percentage of the maximum activity.

The long-term breakdown of CM cellulose by the three main components separated from cultures grown in a medium containing cellulose powder was followed viscometrically over a period of 120 hr (chloroform was added to maintain sterility). At the end of these incubations 5 ml. of each mixture was made up to 70% (v/v) ethanol in water (to precipitate long-chain polymers) and then de-salted on a bio-deminrolit column (12 cm. $\times$ 2 cm.) (B.D.H. 1965). The eluate was concentrated under infra-red lamps before paper chromatography. Chromatograms (Whatman no. 1 paper) were run for 20 to 24 hr with solvent *n*-propanol + ethyl acetate + water (13 + 2 + 5, by vol.). Carbohydrates were detected by spraying with trichloroacetic acid + benzidine (2 g. trichloroacetic acid + 0.24 g. benzidine + 5 ml. water made up to 100 ml. with ethanol (Bacon & Edelman, 1951).

Fig. 1. Elution pattern of standards from Sephadex G-75 column (showing volume of buffer required to elute standards of known molecular weight). Δ , Blue dextran (M.W. 2×10^6); $\circ$, serum albumin (M.W. 6.4×10^4); $\blacksquare$, peroxidase (M.W. 4.5×10^4); $\square$, cytochrome C (M.W. 1.2×10^4); $\bullet$, sucrose (M.W. 342).

Cellobiase activity was estimated (Nelson, 1944; Somogyi, 1952) by measuring the increase in reducing sugar after incubation of cellobiose solution with both the culture filtrate and the three separated CM cellulase peak components.

To investigate the production of CM cellulase in the host, tissue from various parts of healthy and diseased lucerne plants (Du Puit strain) was ground in 0.1 M-phosphate buffer (pH 5.0; 0.5 g. tissue/ml. buffer). The slurry was centrifuged at 3000g for 10 min. and 1 ml. of the supernatant fluid assayed viscometrically for CM cellulase activity. The assay was done twice with different samples of lucerne material.

RESULTS

In the Sephadex column calibration (Fig. 1) the blue dextran indicates the void volume since it is totally excluded from the gel because of its high molecular weight (2×10^6). The sucrose was below the lower limit of separation obtainable with G-75, but the other three substances were clearly resolved. The molecular weight estimations based on the physical characteristics of the column (Whitaker, 1963) showed good correlation with the known weights of the standards (Fig. 2).

Fig. 2. Estimation of molecular weight by calibration of G-75 column. —, From standards; ---, theoretical; ●, serum albumin; ▽, peroxidase; ○, cytochrome C.

The separation of CM cellulases from cultures grown in CM cellulose or cellulose powder medium resulted in three main peaks designated A, B and C and two smaller peaks D and E (Fig. 3). The approximate molecular weights of these components estimated from the standard curve (Fig. 2) are A 75,000, B 32,000, C 16,000, D 6500 and E 2200.

K_m determinations of components A, B and C gave values of 1.33×10^{-5} , 2.86×10^{-5} and 3.33×10^{-5} at pH 5.0, respectively, i.e. A had the highest affinity and C the lowest affinity for its substrate (Fig. 4a, b, c). High concentrations of substrate inhibited the reaction. The molecular weight of CM cellulose used for these determinations was calculated as 2500 (Fig. 5). These three components all showed activity

over a wide pH range (Fig. 6*a, b, c*); A and B had two maxima, A at pH 4.5 and 7.5 and B at pH 5.0 and above 8.0, whereas C had a single maximum at pH 8.0. There was no difference in the viscosity of the CM cellulose controls at any pH value used, either initially or after incubation.

Incubation of A, B and C materials (derived from cultures grown in cellulose medium) separately with CM cellulose over an extended period showed that the viscosity of all the mixtures was tending towards the same value (Fig. 7*a*). Component B caused the most rapid breakdown of substrate (Fig. 7*b*). Since its K_m was intermediate between that of components A and C this indicated a greater proportion of the B component in these cultures. At the end of this incubation, paper chromatograms

Fig. 3. Elution pattern of CM cellulases from culture filtrates. ●, Cultures grown on cellulose powder; O, cultures grown on CM cellulose.

of the mixtures were run. These showed only the presence of cellobiose (authenticated by comparison with standard) and medium-length polymers (still at the origin). The ratio of cellobiose to medium-length polymers was much higher in the mixtures resulting from incubation with C than in those with either A or B. There was no indication of short-chain polymers other than cellobiose. None of the three main peak components showed any cellobiase activity. The only glucose detected was a contaminant of the CM cellulose; this was shown by the chromatographic detection of glucose in CM cellulose in the same small quantities before enzymic hydrolysis as were found after reaction with the enzyme components.

There was a greatly increased (approximately 20-fold) degree of CM cellulase activity in the dead infected lucerne stem as compared with the healthy stem (Table 1). The living tissue of infected plants had only a slightly (approximately three-fold)

increased CM cellulase activity as compared with those of healthy plants. The dead root material was at this time extensively invaded by numerous saprophytic soil organisms and so a cellulase assay was not attempted.

Fig. 4. Lineweaver-Burk reciprocal plots for K_m determinations of enzyme peaks A (a), B (b) and C (c).

$$\frac{I}{S} = \frac{I}{\text{Substrate concentration (mM)}}$$

$$\frac{1}{V} = \frac{1}{\text{initial reaction velocity (decrease in CM cellulose equivs (g./100 ml./sec.))}}$$

(i) Cultures grown on cellulose powder; (ii) cultures grown on CM cellulose.

Fig. 5. Calculation of molecular weight of CM cellulose from osmotic pressure measurements. When the concentration tends to zero, $\pi/C = 0.01025$.

$$\text{Average molecular weight} = \frac{RT}{\pi/C} = \frac{0.082 \times 307}{0.01025} = 2456.$$

Table 1. *Production of CM cellulase by Verticillium albo-atrum in lucerne tissue*

	Decrease in CM cellulose equivalents. Assayed viscometri- cally
Living stem	
Uninfected	0.010
Lightly infected young stem	0.030
Heavily infected old stem	0.025
Dead stem	
Uninfected	0.000
Infected	0.230
Living root	
Uninfected	0.010
Heavily infected old root	0.030

DISCUSSION

These experiments indicate that several distinct CM cellulases are produced extracellularly by *Verticillium albo-atrum*. The data also show that the components possess the same characteristics (molecular weight, Michaelis constant, activity/pH curve) whether induced by CM cellulose or cellulose powder as sole carbon source in the medium. The K_m determinations were not ideal since the calculations assumed the breakdown of a highly viscous substrate to a product of viscosity similar to water though it is obvious that the medium-length polymers would have appreciable viscosity. However, since all the incubation mixtures progressed towards a similar viscosity, a valid comparison could be made between them and with other K_m determinations similarly calculated (Mandels & Reese, 1963). Although inhibition at high substrate concentrations is commonly encountered in hydrolytic enzyme systems it is doubtful whether a similar restricting mechanism was operating here, since we used a relatively low maximum substrate concentration. Any such

inhibition might have been due to an increase in the viscosity of the reaction mixture decreasing molecular movement and hence availability of substrate to enzyme. The double pH optima obtained with components A and B were unexpected, since accord-

Fig. 6. pH/activity range of CM cellulase peaks A (a), B (b) and C (c). ●, Culture grown on cellulose powder; ○, culture grown on CM cellulose.

ing to Mandels & Reese (1965) most cellulases have a single optimum between pH 4.5 and 5.5. The double optima may indicate that the enzyme enters two active states, or alternatively that the presence of two enzymes of similar molecular weight which we had failed to separate.

Of the five enzyme components separated, only B (MW 32,000) and D (MW 6500) can be correlated with any of those found by Selby & Maitland (1965) in *Myrothecium verrucaria* cultures. The high molecular weights of components C, B and particularly of A suggest that their action is largely restricted to exposed surfaces and loose chain ends, since molecules of this size would have difficulty in penetrating naturally occurring cellulose in the plant cell wall. Because of the difficulties inherent in investigating the initial steps of cellulose degradation no attempt was made to

Fig. 7. Viscosity of CM cellulose plotted against time during incubation with enzyme peaks A, B and C.

(a) On a linear scale for about 1 day, O, Enzyme peak A; ▲, enzyme peak B; △, enzyme peak C.

(b) On a logarithmic scale for about 7 days.

establish whether a CI component was produced by *Verticillium albo-atrum*. However, Selby & Maitland (1967) showed that CM cellulases have no detectable effect alone on natural forms of cellulose so the fact that *V. albo-atrum* can utilize these as sole carbon source is circumstantial evidence for the presence of a CI component. No cellobiase activity was associated with any of the peak components and cellobiose was identified in the CM cellulose incubation mixtures and in harvested culture fluids containing cellulose powder as sole carbon source. This strongly suggests that cellobiose is the end product of cellulose breakdown by *V. albo-atrum* and is presumably taken up in this form by the hyphae.

The simplest hypothesis to explain the different ratio of CM cellulose products shown in the three main peak components is that component C removes a cellobiose unit from the chain when it cleaves β 1-4 linkages, whereas components A and B hydrolyse the linkage without removing a cellobiose unit (unless it is at the end of a chain). The degree of substitution controls the hydrolysis of cellulose by cellulases, the enzyme not attacking substituted glucose units (Mandels & Reese, 1965). In the present case the CM cellulose was substituted at 0.45 to 0.55 carboxymethyl groups per glucose unit (i.e. an average of one substituent group per two glucose units). It is likely that in some parts of the chain there would be two adjacent substituted glucose units and in other parts one substituent group per three or four glucose units. This would explain why all the CM cellulose was not hydrolysed to cellobiose and yet some cellobiose was produced.

The high values for CM cellulase in the dead infected stem, compared to living infected stem and healthy stem, indicate that the enzyme was only produced in large quantities after the death of the host tissue. This almost complete repression throughout the parasitic phase can be explained by an inhibition of enzyme production by sugars such as glucose and sucrose, as shown in *in vitro* studies by Talboys (1958) and by D. P. Gupta (personal communication). We cannot at this stage rule out the possibility that CM cellulases might be produced in minute amounts by fine penetration hyphae before and during passage through the host cell walls, although we have not yet successfully designed a technique to detect this. The activity detected in dead and dying tissue is of obvious importance to the pathogen. It enables an otherwise poorly competitive saprophyte (Heale & Isaac, 1963) to utilize at least part of the cellulose fraction of the host (after the more easily assimilable sugars are exhausted) before general saprophytic invasion occurs. The darkly pigmented resting mycelium characteristic of *Verticillium albo-atrum* is formed during this latter stage of the disease and is of some importance in the survival of the fungus in the soil.

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