Cellulolytic Enzymes in Culture Filtrates of *Rhizoctonia lamellifera*

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SUMMARY

During growth in a liquid culture containing a single soluble or an insoluble cellulosic carbon source, *Rhizoctonia lamellifera* released cellulolytic enzymes into the medium. These enzymes were separated by gel filtration and ion-exchange chromatography into seven components, three of high and four of low molecular weight. One of the components had the character of a C_1 cellulase. When the components were combined they released more reducing sugars from cellulosic substrates than when used singly.

INTRODUCTION

Rhizoctonia lamellifera penetrates healthy roots, causing a rapid degeneration of both cortical and vascular tissues (Small, 1928), and is associated with the blast disease of the oil palm (*Elaeis guineensis*) in Nigeria (Robertson, 1959). Many plant pathogens produce a distinct C_1 cellulase which hydrolyses crystalline forms of cellulose to soluble polyanhydroglucose chains which can be degraded by other cellulolytic enzymes – C_x cellulase and cellobiase. Such a complex of cellulase enzymes has been isolated from other fungi (Wood, 1968; Whitney, Chapman & Heale, 1969; Olutiola & Ayres, 1973). This paper describes the production of cellulolytic enzymes by laboratory cultures of *R. lamellifera*, the composition and activity of this enzyme system and the growth of the fungus on celluloses.

METHODS

Organism and culture conditions. *Rhizoctonia lamellifera* (NIFOR-24.9.74) was obtained from the culture collection of the Nigerian Institute for Oil Palm Research, Benin, Nigeria. Stock cultures were maintained on 1 % (w/v) malt extract agar slants. Cultures were grown in a basal medium containing 29.4 mM- NaNO_3 , 7.3 mM- KH_2PO_4 , 1 mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 29 μM - $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 18 μM - $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 15.7 μM - $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 20 μM - $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$, 3.5 μM - $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 μM -thiamin and 0.04 μM -biotin: all chemicals were analytical reagent grade. The carbon source was usually 1 % (w/v) carboxymethylcellulose (CM-cellulose). Other carbon sources used were cellulose powder (Difco), Whatman no. 1 filter paper and viscose cellulose (Gallenkamp). Cultures were grown in 250 ml flasks containing 100 ml liquid medium. Each flask was inoculated with 1 ml of an aqueous spore suspension prepared from a 6-day-old agar slant culture, and incubated at 30 °C without shaking.

Preparation of enzyme solution. After incubation for 7 days (unless otherwise stated) stationary phase cultures were filtered through a glass-fibre filter (Whatman GF/A), and the reducing sugars and protein content of the filtrate were measured by the techniques of Miller (1959) and Lowry *et al.* (1951) respectively. The filtrate was then concentrated to about 12 % of its original volume in a rotary vacuum evaporator at 37 °C (Selby & Maitland, 1967;

Whitney *et al.*, 1969) and low molecular weight substances were removed by mixing with dry Sephadex G-25 for 10 min (Flodin, Gelotte & Porath, 1960). The concentrate was sterilized by membrane filtration (Oxoid) before fractionation and assay. The mycelium, retained on the filter, was rinsed with distilled water to free it from medium components, dried in an oven at 80 °C for 12 h and weighed.

Fractionation on Sephadex G-75. A column (700 × 25 mm) of Sephadex G-75 was prepared according to Whitaker (1963) and Andrews (1964) and was surrounded by a water jacket at 20 °C. Concentrated culture filtrate (10 ml) was applied to the column and eluted with a phosphate buffer (pH 6.5) containing 6.7 mM- NaH_2PO_4 , 100 mM- NaCl , 3.3 mM- Na_2HPO_4 and 5 mM- NaN_3 (Selby & Maitland, 1965; Olutiola & Ayres, 1973). The protein content of the eluted fractions (2.6 ml) was recorded by continuous measurement of the extinction at 280 nm and appropriate fractions were collected automatically. The fractions were also examined for activity towards CM-cellulose, cellobiose and insoluble cellulose powder. The Sephadex column was calibrated using ferritin (mol. wt 480000), calf catalase (mol. wt 240000), aldolase (mol. wt 147000), bovine serum albumin (mol. wt 67000), egg albumin (mol. wt 45000), myoglobin (mol. wt 17800), cytochrome *c* (mol. wt 12400), and DNP-L-alanine (mol. wt 255.2) which were obtained from Serva-Feinbiochemica (D-69 Heidelberg 1, Germany).

Fractionation by ion-exchange chromatography. After gel filtration, each series of fractions corresponding to an absorption peak at 280 nm was combined and concentrated to about 4 ml in a rotary evaporator, and made up to 10 ml in 0.09 M-sodium succinate buffer (pH 5.2) containing 5 mM- NaN_3 (Olutiola & Ayres, 1973). Components of high molecular weight (peak A fractions) were applied to a column of CM Sephadex C-50; those of lower molecular weight (peak B fractions) were applied to a column of CM Sephadex C-25. Each column (400 × 30 mm) was surrounded by a water jacket at 20 °C and eluted with sodium succinate buffer (pH 5.2). Fractions (2.6 ml) were collected and assayed as described for Sephadex G-75 columns.

Enzyme assays. Cellulase and cellobiase activities were determined by measuring the reducing sugars released in reaction mixtures. The reaction mixtures, containing 0.5 ml enzyme preparation and 0.5 ml CM-cellulose (1 %, w/v) or cellobiose (0.7 mg ml⁻¹) in citrate/phosphate buffer (pH 5.5), were incubated at 30 °C for 2 h. The reducing sugars released were measured by the technique of Miller (1959). The unit of activity (u.) is defined as the amount of enzyme in 1 ml of reaction mixture that liberates reducing sugar equivalent to 10 µg glucose.

Cellulase activity was also monitored in an Ostwald viscometer (British Standard Units, size C) containing 5 ml enzyme solution and 5 ml 1 % CM-cellulose in citrate/phosphate buffer (pH 5.5) at 30 °C for 1 h.

The activity of the protein fractions towards insoluble cellulose powder (Difco) was measured, singly and in combination, by incubating 5 mg cellulose, 1 ml citrate/phosphate buffer (pH 5.5), 0.5 ml enzyme solution and 5 mM- NaN_3 for 7 days at 35 °C (Olutiola & Ayres, 1973). The unhydrolysed cellulose was then removed by membrane filtration and the filtrate was analysed for glucose using the *o*-toluidine reagent of Wootton (1964).

RESULTS

In blast disease of the oil palm, *R. lamellifera* is associated with roots showing cortical decay. Extracts obtained by grinding such infected tissues in 0.1 M-phosphate buffer (pH 5.5), and clarified by centrifuging at 10000 g for 15 min at 4 °C, showed only very slight activity

Fig. 1

Fig. 2

Fig. 1. Separation of proteins in concentrated culture filtrates of *R. lamellifera* by gel filtration, and enzymic activity of the fractions towards CM-cellulose and cellobiose. —, Protein (E_{280}); ---, CM-cellulase activity; ···, cellobiase activity.

Fig. 2. Separation by ion-exchange chromatography of high molecular weight proteins (fractions 30 to 68 separated from culture filtrates of *R. lamellifera* by gel filtration; Fig. 1), and enzymic activity of the fractions towards CM-cellulose and cellobiose. —, Protein (E_{280}); ---, CM-cellulase activity; ···, cellobiase activity.

Fig. 3. Separation by ion-exchange chromatography of low molecular weight proteins (fractions 128 to 166 separated from culture filtrates of *R. lamellifera* by gel filtration; Fig. 1), and enzymic activity of the fractions towards CM-cellulose and cellobiose. —, Protein (E_{280}); ---, CM-cellulase activity; ···, cellobiase activity.

Table 1. *Enzymic activity towards insoluble cellulose of components separated from culture filtrates of Rhizoctonia lamellifera by gel filtration and ion-exchange chromatography*

Enzyme activity is expressed as the glucose equivalent of reducing sugars ($\mu\text{g ml}^{-1}$) released from cellulose powder.

Means of separation	Components	Glucose equivalent of reducing sugars ($\mu\text{g ml}^{-1}$)
None	Concentrated culture filtrate	71
Sephadex G-75	A	32
	B	25
	A + B	69
Sephadex C-50 of component A	Aa	5.6
	Ab	7.1
	Ac	8
	Aa + Ab	12
	Aa + Ac	20
	Ab + Ac	23
	Aa + Ab + Ac	31
Sephadex C-25 of component B	Ba	11
	Bb	6.5
	Bc	8
	Bd	5
	Ba + Bb	16
	Ba + Bc	20
	Ba + Bd	15
	Bb + Bc	17
	Bb + Bd	10
	Bc + Bd	14
	Ba + Bb + Bc + Bd	23

in reducing the viscosity of buffered 1 % (w/v) CM-cellulose; thus it was difficult to extract adequate quantities of active enzyme for laboratory work. However the organism grew rapidly in artificial media (producing white mycelia which finally turned black as abundant sclerotia were produced) and so laboratory cultures were used in the present work.

Growth on cellulosic substrates and enzymic activity of culture filtrates. *Rhizoctonia lamellifera* grew on CM-cellulose, cellulose powder, Whatman no. 1 filter paper and viscose cellulose when each was used as the sole carbon source. The ability of the organism to hydrolyse CM-cellulose was shown by a noticeable decrease in the viscosity of the medium and by an increase in cell mass which corresponded to the measured increase in the concentration of reducing sugars liberated into the medium.

Culture filtrates reduced the viscosity of buffered CM-cellulose solution, with an accompanying release of reducing sugars, over a wide range of pH values with an optimum at pH 5.5. They also showed cellobiase activity, liberating glucose from cellobiose (Fig. 1).

Fractionation on Sephadex G-75. Fractionation of the concentrated culture filtrate on Sephadex G-75 led to two absorption peaks designated A and B (Fig. 1). The molecular weights of the components of these peaks, estimated from their elution volumes, were approximately 89 100 and 9600 respectively.

Fractionation on CM Sephadex C-50 and C-25. Fractionation of component A on Sephadex C-50 gave three absorption peaks designated Aa, Ab and Ac (Fig. 2). Fractionation of component B on Sephadex C-25 gave four peaks, Ba, Bb, Bc and Bd (Fig. 3).

Components Aa, Ab and Bb showed C_x cellulase and cellobiase activity in that they released glucose from CM-cellulose and cellobiose. Component Ba showed only cellobiase

activity, and components Bc and Bd only C_x cellulase activity. Component Ac had neither C_x cellulase nor cellobiase activity.

The activity of the various components was also examined using insoluble cellulose powder. Components were examined singly and in various combinations such that the original cellulase complex was reconstituted in a stepwise manner (Table 1). Release of glucose from the cellulose was indicative of enzyme activity. All the components released glucose from insoluble cellulose, although to varying degrees. When the components were combined more glucose was released than when each was used alone.

DISCUSSION

Rhizoctonia lamellifera produced cellulolytic enzymes which enabled it to grow on both soluble and insoluble forms of cellulose, releasing glucose as an end-product. Culture filtrates exhibited both C_x cellulase and cellobiase activities. The ability of the organism to use insoluble cellulosic substrates as sole carbon source is indicative of C_1 cellulase production (Selby & Maitland, 1967; Whitney *et al.*, 1969).

After separation on Sephadex G-75, the two components, A and B, still exhibited both C_x cellulase and cellobiase activities. Thus although chromatography on Sephadex G-75 separated the high molecular weight component (A) from the low molecular weight one (B), it failed to separate components with cellobiase activity from those with C_x cellulase activity. Ion-exchange chromatography (Olutiola & Ayres, 1973) was used to separate cellobiases from C_x cellulases.

Components Aa, Ab and Bb degraded CM-cellulose and cellobiose, and thus showed C_x cellulase and cellobiase activities. Component Ba only degraded cellobiose and had no activity on CM-cellulose. Thus, this component seems to be a separated form of cellobiase, completely free from C_x cellulase activity. Components Bc and Bd degraded CM-cellulose but not cellobiose, and therefore represent a cellobiase-free form of C_x cellulase.

Component Ac has a distinct characteristic in that, although it could not break down soluble celluloses, it hydrolysed insoluble cellulose releasing glucose. This component therefore resembles the Aa component of Olutiola & Ayres (1973) and the C_1 component of Selby & Maitland (1967), but differs from the C_1 of Li, Flora & King (1965) and Wood (1968) because of its inability to degrade soluble cellulosic substrates.

When some of the components were combined, they showed synergistic effects (particularly when Ac was present). Synergism between separated components has been reported for a number of fungi (Selby & Maitland, 1967; Umeruzike, 1970; Olutiola & Ayres, 1973).

Culture filtrates of *R. lamellifera* contain high and low molecular weight proteins which exhibit differences in substrate specificity. The ability of *R. lamellifera* to produce such a system of cellulolytic enzymes means that the organism is equipped with enzymes to attack cellulose at different stages in its degradation from the crystalline form to the simpler, soluble forms which can be absorbed and utilized by the organism.

The disintegration of the cortical cells of infected tissues (Small, 1928; Robertson, 1959) strongly suggests that cellulase enzymes may play a role in pathogenicity. The importance of cellulolytic enzymes in pathogenicity has been indicated by other workers (Whitney *et al.*, 1969; Umeruzike, 1970; Wood, 1968; Olutiola, 1972).

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