Cellulolytic Enzymes in Culture Filtrates of *Aspergillus clavatus*

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Aspergillus clavatus grew in synthetic liquid media with soluble or insoluble cellulose as sole carbon source, releasing cellulolytic enzymes into the media. These enzymes were separated by molecular exclusion and ion exchange chromatography into four components. When assayed for hydrolysis of insoluble cellulose, the combined fractions released more glucose than when each component was tested alone.

INTRODUCTION

Aspergillus clavatus Desm. is a phytopathogenic fungus associated with internal mouldiness of cocoa beans in Nigeria (Broadbent, 1968; Oyeniran, 1970). Many plant pathogens produce a complex of extracellular cellulases which hydrolyse the cellulosic components of host cell wall. Such a complex of cellulases has been isolated from some species of *Aspergillus* and other fungi (Olutiola & Cole, 1976; Youatt, 1958; Coutts & Smith, 1976; Almin & Eriksson, 1968).

Production of fungal cellulases is greatly influenced by cultural conditions. For example, maximum production of cellulase from *Aspergillus tamarii* was at pH 4.5 (Olutiola & Cole, 1976) but that of *Aspergillus luchuensis* was at pH 6.5 to 7.0 (Reese & Levinson, 1952).

This paper examines the ability of *A. clavatus* to produce cellulolytic enzymes *in vitro*. The composition and activity of these enzymes are also examined.

METHODS

Organism and culture conditions. The isolate (NSPRI109) of *Aspergillus clavatus* used was from the culture collection of the Nigerian Stored Products Research Institute. It was isolated from mouldy cocoa beans. The organism was routinely grown and maintained on 1 % (w/v) malt yeast extract agar. Each flask was inoculated with 1 ml of an aqueous spore suspension containing approximately 4×10^6 spores ml⁻¹. Cultures were grown in a defined liquid medium (Olutiola, 1976) containing 1 % (w/v) carboxymethylcellulose (CM-cellulose). Other carbon sources, cellulose film (Lansil Ltd., Lancaster), cellobiose (BDH), cellulose powder (Difco), Whatman no. 1 filter paper and viscose cellulose (Gallenkamp) were each used separately to give 1 % (w/v) carbon source. The CM-cellulose and cellobiose were dissolved by mechanical agitation. Discs (2 cm diam.) of Whatman no. 1 filter paper were used. Cellulose powder was ball-milled for 72 h. The cellulose film and viscose cellulose were each boiled to remove plasticizers. Each 150 ml Erlenmeyer flask contained 50 ml of medium and was incubated without shaking at 30 °C. On each carbon source, growth was judged to occur when there was a noticeable increase in the fungal mats produced.

Preparation of enzyme solution. After 9 days of incubation, unless otherwise stated, the contents of each flask were filtered through a glass-fibre filter (Whatman GF/A). The reducing sugars and protein content of the filtrates were measured using the methods of Miller (1959) and Lowry *et al.* (1951), respectively.

One litre of the filtrate was precipitated with (NH₄)₂SO₄ (analytical grade, BDH) at 40 to 95 % (w/v) saturation (Wood, 1968; Whitaker, Hanson & Datta, 1963; Dixon & Webb, 1971). The precipitate was redissolved in phosphate buffer, pH 6.5 (containing 6.7 mM-NaH₂PO₄, 100 mM-NaCl and 3.3 mM-Na₂HPO₄) to give a 20-fold concentration of the original culture filtrate. The filtrate was then dialysed for 24 h at 4 °C using acetylated cellophane tubing prepared from Visking dialysis tube (Gallenkamp) as previously described (Whitaker *et al.*, 1963). The filtrate was sterilized by membrane filtration (pore size 0.45 µm, Oxoid) before

separation and assay procedures. The dialysed filtrate, when diluted 20-fold, possessed all the activity of the original crude culture filtrate towards cellulosic substrates.

Enzyme separation. A column (2.5×70 cm) of Sephadex G-75, surrounded by a water jacket maintained at 20°C , was prepared and calibrated as previously described (Olutiola, 1976). Ten ml of the enzyme concentrate were applied to the column and eluted with phosphate buffer, pH 6.5, containing (mm): NaN_3 , 5; NaCl , 100; NaH_2PO_4 , 6.7; and Na_2HPO_4 , 3.3. The protein content of the eluted fractions was recorded by continuous measurement of the extinction at 280 nm and fractions (4.5 ml per tube) were collected in an LKB automated fraction collector (7000 A Ultra Rac).

Fractions (16 to 23) which showed appreciable cellulase activity after gel filtration, were combined and low molecular weight substances were removed by mixing with dry, coarse Sephadex G-25 for 10 min (Flodin, Gelotte & Porath, 1960). This process also helped to concentrate the enzyme fraction. The enzyme concentrate was made up to 15 ml in succinate buffer, pH 5.2 (containing 5 mM- NaN_3 , 0.2 M- $(\text{CH}_3\text{COOH})_2$ and 0.2 M- NaOH), and applied to a column (2.5×40 cm) of CM-Sephadex C-50 surrounded by a water jacket at 20°C and equilibrated with succinate buffer (pH 5.2). Fractions (3.5 ml per tube) were collected and measured as described above.

Assay methods. C_x cellulase and cellobiase activities were obtained by measuring the reducing sugars released in reaction mixtures. Each reaction mixture contained 0.4 ml of the enzyme preparation and 1 ml of CM-cellulose (5 mg ml^{-1}) or cellobiose (0.7 mg ml^{-1}) in 0.1 M-citrate/phosphate buffer (pH 4.5), and was incubated at 30°C for 1 h. The reducing sugars released were measured by the method 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.

C_x cellulase activity was also assayed viscometrically in an Ostwald viscometer (British Standard Units, size C, Gallenkamp) containing 4 ml enzyme solution and 6 ml CM-cellulose (10 mg ml^{-1}) in 0.1 M-citrate/phosphate buffer (pH 4.5) at 30°C for 30 min. The activity of the enzyme was expressed as the percentage loss in viscosity of the CM-cellulose (Norkrans & Hammarström, 1963).

The activity of the protein fractions, singly and in combination, towards insoluble cellulose was measured by incubating 5 mg cellulose powder, 1 ml 0.1 M-citrate/phosphate buffer (pH 4.5), 0.4 ml enzyme solution and 5 mM-sodium azide (BDH) for 72 h at 30°C . The unhydrolysed cellulose left after incubation was separated from the reaction mixture by centrifuging at 10000 g for 10 min at 4°C . The supernatant was carefully removed with a pasteur pipette and analysed for glucose by a modified technique of De Verdier & Hjelm (1962) in which the galactose oxidase was replaced by glucose oxidase. The unit of activity (u.) is defined as the amount of enzyme in 1 ml of reaction mixture that liberates 10 μg glucose.

RESULTS AND DISCUSSION

Growth on cellulosic substrates and enzymic activity of culture filtrates

Aspergillus clavatus was able to grow on both CM-cellulose and cellobiose and insoluble forms of cellulose (Whatman no. 1 filter paper, viscose cellulose, cellulose film and cellulose powder) indicating production of C_1 cellulase (Selby & Maitland, 1967; Whitney, Chapman & Heale, 1969). Hydrolysis of CM-cellulose was indicated by a noticeable decrease in the viscosity of the CM-cellulose medium and by an increase in cell mass which corresponded to the protein concentration and reducing sugars liberated into the medium.

Culture filtrates liberated reducing sugars from CM-cellulose and cellobiose over a wide range of pH values with an optimum at pH 4.5 (Table 1). Ability of the filtrate to hydrolyse CM-cellulose and cellobiose is indicative of C_x cellulase and cellobiase activity.

Fractionation of cellulase complex

Fractionation of the enzyme concentrate (10 ml) on Sephadex G-75 produced a single peak designated A (Fig. 1). The molecular weight of this component estimated from the elution volume was approximately 105900. The component possessed C_x cellulase and cellobiase activity, indicating that both types of enzymes were eluted together. Previous workers (Selby & Maitland, 1967; Wood, 1968; Olutiola & Ayres, 1973) were also unable to separate C_x cellulases from cellobiases by passage through Sephadex G-75.

Fractionation of component A on Sephadex C-50 gave four new absorption peaks designated Aa, Ab, Ac and Ad respectively (Fig. 2). When these components were mixed

Table 1. Effect of pH on the enzymic activity of culture filtrates of *Aspergillus clavatus* incubated with CM-cellulose (0.5 %, w/v) or cellobiose (0.7 mg ml⁻¹) for 90 min

Each value represents enzyme activity (u. ml⁻¹) and is the mean of three replicates.

	pH							
Substrate	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5
CM-cellulose	1.9	5.8	8.1	6.1	4.9	1.8	1.2	0.9
Cellobiose	2.7	4.9	17.7	14.7	7.6	3.2	2.4	1.5

Fig. 1

Fig. 2

Fig. 1. Separation by gel filtration of proteins in concentrated culture filtrates (40 to 95 % saturated $(\text{NH}_4)_2\text{SO}_4$ fraction redissolved to give a 20-fold concentration) of *A. clavatus*, and enzymic activity of the fractions towards CM-cellulose and cellobiose. $\circ$ — $\circ$, Protein (E_{280}); ---, C_x cellulase activity; ..., cellobiase activity.

Fig. 2. Separation by ion exchange chromatography of proteins (fractions 16 to 23) previously separated from culture filtrates of *A. clavatus* by gel filtration (Fig. 1), and enzymic activity of the fractions towards CM-cellulose and cellobiose. $\circ$ — $\circ$, Protein (E_{280}); ---, C_x cellulase activity; ..., cellobiase activity.

and rechromatographed on Sephadex G-75 column, they all produced a single absorption peak, and were collected in exactly the same fraction tubes as component A, indicating that these components (Aa, Ab, Ac and Ad) are of similar molecular weight.

The enzymic activity of each component was examined (Fig. 2). Components Aa and Ab were able to hydrolyse CM-cellulose and cellobiose and thus still possess C_x cellulase and cellobiase activities. Component Ac hydrolysed CM-cellulose and failed to act on cellobiose. This component therefore appears to be a cellobiase-free form of C_x cellulase. Component Ad degraded cellobiose but not CM-cellulose, indicating a separated form of cellobiase, completely free from C_x cellulase activity. Thus, there were differences in substrate specificity between the components separated on the CM-50 column. Other workers (Selby & Maitland, 1967; Streamer, Eriksson & Petterson, 1975) have also demonstrated differences in the type of cellulosic substrates degraded by various components of cellulolytic enzyme systems.

The activity of the components towards insoluble cellulose powder was also examined. Components were examined singly and in various combinations such that the original cellulase complex was reconstituted in a stepwise manner (Table 2). Release of glucose from the cellulose was indicative of enzyme activity. Each component hydrolysed insoluble cellulose to release glucose as an end-product. When the components were combined, more glucose was released from insoluble cellulose than when each component was tested alone. Whenever the component under test contained the component Ac, there was a synergistic increase in the hydrolysis of cellulose, since under such conditions more glucose was released than could

Table 2. *Enzymic activity (towards insoluble cellulose powder) of cellulase components separated from culture filtrates of Aspergillus clavatus*

Reaction mixtures were incubated for 72 h.		
Means of separation	Components	Enzyme activity (u. ml ⁻¹)
None	Culture filtrate	3.8
None	40 to 95 % saturated (NH ₄) ₂ SO ₄ concentrate (20-fold)	14.9
None	40 to 95 % saturated (NH ₄) ₂ SO ₄ concentrate (20-fold), diluted × 20	4.5
Sephadex G-75	A	13.6
Sephadex C-50 of component A	Aa	3.5
	Ab	2.0
	Ac	2.8
	Ad	3.2
	Aa + Ab	3.9
	Aa + Ac	9.6
	Aa + Ad	4.3
	Ab + Ac	7.4
	Ab + Ad	3.5
	Ac + Ad	8.4
	Aa + Ab + Ac + Ad	13.0

be predicted from an examination of the components alone. Thus, component Ac has the character of a C₁ cellulase (Selby & Maitland, 1967; Eriksson & Rzedowski, 1969; Shikata & Nisizawa, 1975). It resembles the C₁ component of Li, Flora & King (1965) and Wood (1968), but differs from the C₁ component of Selby & Maitland (1967), Olutiola & Ayres (1973) and Olutiola (1976), because of its ability to hydrolyse soluble cellulose. Such synergism between separated components of cellulases has been reported for a number of phytopathogenic fungi (Umeruzike, 1970; Olutiola & Cole, 1976; Olutiola 1976; Streamer *et al.*, 1975).

The cellulolytic enzyme system of *A. clavatus* consists of components which exhibit substrate specificity. Production of a complex of cellulase enzymes is believed to be of advantage to a phytopathogen, since it can then attack celluloses at different stages of degradation, from the highly complex and insoluble forms to the simpler and soluble ones which can be absorbed and utilized by the organism (Whitney *et al.*, 1969; Olutiola, 1972; Shikata & Nisizawa, 1975).

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