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Affinity Chromatography of the Cellulase System of *Trichoderma koningii*

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A procedure involving affinity chromatography on cellulose was developed for separating enzymic components of the cellulase complex. Cellobiase, carboxymethylcellulase, component C_2 and cellobiohydrolase are adsorbed with increasing tenacity, and released, as highly purified components, as the ionic strength of the eluent is decreased.

Isolation of the cellulolytic components cellobiase, CM-cellulase (carboxymethylcellulase), cellobiohydrolase (component C_1) and component C_2 from culture filtrates requires complex and lengthy chromatography on ionic as well as non-ionic gel-filtration media, followed by recycling on similar materials for minimal purification (for review see Bailey *et al.*, 1975). An alternative approach based on the ability of cellulase to bind to its substrate (Halliwell, 1961) would be useful, particularly for isolation of cellobiohydrolase and component C_2, both of which occur in small amounts and are unstable in the absence of substrate (cf. cellobiohydrolase; Halliwell & Griffin, 1973). Past attempts at cellulose chromatography of the cellulase complex have failed to give satisfactory resolution of the several components (Li *et al.*, 1965; Halliwell & Riaz, 1970; Berghem *et al.*, 1976). The present report describes the isolation by affinity chromatography on cellulose of four highly purified cellulolytic components, cellobiase, CM-cellulase, component C_2 and cellobiohydrolase, from culture filtrates of *Trichoderma koningii*.

Materials and Methods

Culture filtrates were prepared and concentrated 10-fold for chromatography as previously described (Halliwell & Riaz, 1970). Enzyme activities were measured as follows. Cellobiase was assayed at 37°C and pH 5.5 for 1h, with cellobiose as substrate (Halliwell & Griffin, 1973). CM-cellulase was assayed at 37°C for 1h in 44mM-sodium phosphate buffer, pH 6.5, for individual eluate fractions (or 22mM-sodium acetate buffer, pH 4.8, for pooled fractions) by the modified ferricyanide procedure (Halliwell & Riaz, 1970; Halliwell, 1961). These references and Halliwell (1965) also give procedures for assaying component C_2 and cellobiohydrolase activities on 2mg of dewaxed Texas cotton fibres, pH 4.8; for component C_2, assay mixtures were shaken at 25°C for 20h (in the presence of added pure CM-cellulase if necessary; see below) and examined for short-fibre formation; for solubilizing activity (cellobiohydrolase or otherwise) assay mixtures were left unshaken at 37°C for 7 days. Total soluble carbohydrates produced by cellulolysis were measured with the phenol reagent (Halliwell & Riaz, 1971).

Chromatography was accomplished in long (15mm × 900mm) and short (15mm × 300mm) columns of cellulose, Cellex MX (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and Sigmacell, types 38 and 100 (Sigma, Kingston upon Thames, Surrey, U.K.). Cellulose was stirred gently in 20mM-NaH_2PO_4/Na_2HPO_4, pH 6.5 (30ml/g), for 30min, and allowed to settle for 30min; the aqueous phase was removed and replaced with fresh buffer. After overnight equilibration, liquid was again discarded (if necessary replaced and discarded repeatedly to remove 'fines'), and the wet settled cellulose added to half its volume of fresh buffer for pouring into columns. Buffers, sterilized by filtration, contained a final concentration of 0.05% chlorbutol (BDH Chemicals, Poole, Dorset, U.K.) and were run through columns overnight to ensure pH stability and column packing at 9–12ml/h before samples were applied. Sephadex G-75 (bead size 40–120 μm) in 25mm × 450mm columns was eluted with 0.1M-sodium acetate buffer, pH 5.0, at 15ml/h, and Bio-Gel P-2 (200–400 mesh) in 15mm × 900mm columns was eluted with water at 5ml/h.

Results and Discussion

Affinity chromatography of culture filtrates of *T. koningii* on cellulose was done at 4°C (Fig. 1). Cellobiase was quantitatively eluted with 20mM-sodium phosphate buffer, with nearly all the pigment (characteristic of culture filtrates) in fractions 10–24 and most of the CM-cellulase activity appearing in fractions 25–60. Neither pooled fractions 10–24 (cellobiase) nor 25–60 (CM-cellulase) attacked cotton to form short fibres (20h) or sugars (7 days). Pooled fractions 72–90 and 108–118 each solubilized cotton, but only the former produced short

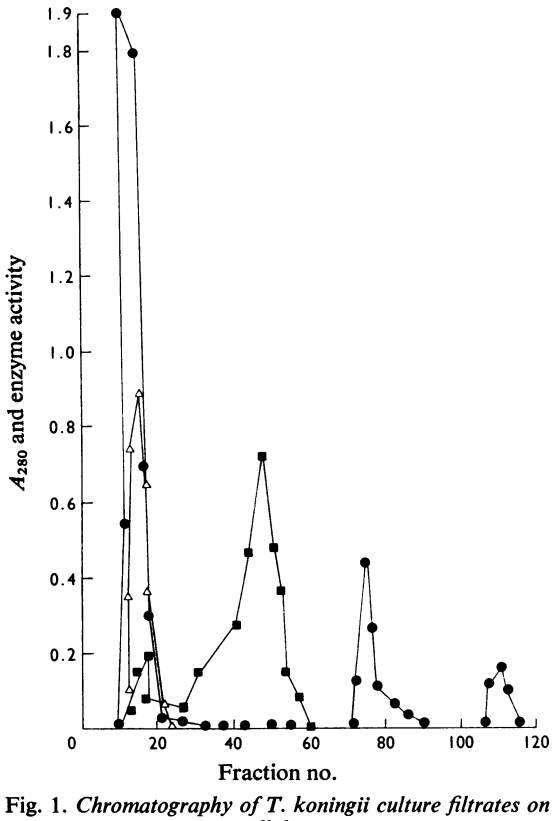

Fig. 1. *Chromatography of T. koningii culture filtrates on cellulose*

Filtrate (5ml of 10-fold concentrated) was allowed to drain into Cellex (15mm×900mm) before being eluted with 20mM-phosphate buffer, pH6.5, up to fraction 60, then with 0.5mM-phosphate buffer up to fraction 92 and then by water. Continued elution with each eluent for 40 fractions beyond those stated produced no further protein or activity. Fraction size was 5ml. ●, Protein (A_{280}); △, cellobiase; ■, CM-cellulase. Enzyme activity is expressed as A_{436} for cellobiase and A_{420} for CM-cellulase.

fibres (component C_2 activity) on addition of the CM-cellulase. The main CM-cellulase component and pooled fractions 72–90 (in the absence of added CM-cellulase) both formed short fibres when incubated longer (60h), indicating the presence of residual component C_2 activity. Hence in stage 2 the four components from stage 1 (Fig. 1), pooled fractions 10–24 (component 1), 25–60 (component 2), 72–90 (component 3) and 108–118 (component 4), were concentrated 10-fold (relative to culture filtrates) and each was rechromatographed separately with the same eluents and techniques used in Fig. 1 but on short cellulose columns. This separated residual minor activities from the four main (purified) enzymes (shown here by their fraction number from short columns): component 1 gave cellobiase (fractions 4–9) plus CM-cellulase; component 2 gave CM-cellulase (fractions 10–30) plus component 3P; component 3 gave component 3P (fractions 44–56) plus CM-cellulase; component 4 gave component 4P (fractions 63–77) plus CM-cellulase. The minor component 3P and CM-cellulase activities released from components 2 and 3 respectively amounted to 25% of the main component-3P protein and to 10% of the main CM-cellulase activity respectively and were discarded. The main 3P and CM-cellulase components from stage 2 were compensated for their loss by a proportionate increase in the amount taken for subsequent assays. These two activities, unlike their parent fractions (components 3 and 2 above), no longer formed short fibres on shaking (60h) unless mixed with one another.

Purified components from stage 2 could be treated further if required. Thus cellobiase and CM-cellulase components from stage 2 were chromatographed on Sephadex G-75 to remove pigment associated mainly with cellobiase, which was eluted with the solvent front, immediately ahead of the pigment. The cellobiase and CM-cellulase and component 4P (stage 2) were then dialysed. Component 3P (stage 2) lost activity on dialysis and was passed through Bio-Gel P-2, arriving with the solvent front. Of the four final purified components, cellobiase, CM-cellulase, 3P and 4P, only the first two hydrolysed cellobiose (pH5.5) and CM-cellulose (pH4.8) respectively in 1h and lacked activity on cotton in 7 days. Component 3P acting on dewaxed fibres, either alone or supplemented with component 4P, formed no short fibres even in 5 days, but did so readily when combined with the purified CM-cellulase, and is therefore identifiable as component C_2. Likewise purified component 4P has the properties of cellobiohydrolase prepared by conventional gel-filtration procedures (see the introduction). Its recovery was estimated to be 95% from the solubilization of cotton in the presence of other components relative to whole-culture filtrates. Recoveries of the other final purified components, including the CM-cellulase and 3P discarded in stage 2, were, for cellobiase 100%, for CM-cellulase 99%, and for component 3P (component C_2) 95% (measured as short fibres produced in the presence of added final purified CM-cellulase). Component C_2 and the cellobiohydrolase were equally and weakly effective in solubilizing the substrate (14%) when assayed separately, but when combined were inhibitory (Table 1). The only two-component system that increased solubilization appreciably over that achieved by the sum of the individual components was that containing cellobiase and cellobiohydrolase, in which solubilization was doubled. This combination in turn formed the basis of the most effective ternary mixture when supplemented with component C_2, where

Table 1. *Cellulolysis of native cellulose by purified components from T. koningii*

Dewaxed Texas cotton fibres (2mg) were incubated for 7 days at 37°C with each final purified component (stage 3) equivalent to 1ml of culture filtrate. CM-cellulase and component C_2 (3P) were compensated for activities discarded during purification (see the text). Solubilization was measured with the phenol reagent. (In the range examined here solubilization is virtually linearly related to cellulolytic activity of culture filtrates.)

Component	Solubilization of substrate (%)
Cellobiase	0
CM-cellulase	0
Component C_2	14
Cellobiohydrolase	14
Cellobiase+CM-cellulase	0
Cellobiase+component C_2	16
Cellobiase+cellobiohydrolase	27
CM-cellulase+component C_2	16
CM-cellulase+cellobiohydrolase	14
Component C_2+cellobiohydrolase	23
Cellobiase+CM-cellulase+component C_2	16
Cellobiase+CM-cellulase+cellobiohydrolase	27
Cellobiase+component C_2+cellobiohydrolase	41
CM-cellulase+component C_2+cellobiohydrolase	32
Cellobiase+CM-cellulase+component C_2+cellobiohydrolase	41
Culture filtrate	43

cellulolysis attained a value equal to that when all four components were acting together.

In conclusion, certain points in the application of affinity chromatography to the cellulase system deserve emphasis. The best celluloses were microcrystalline varieties (see the Materials and Methods section for the types used), since others apparently adsorbed enzymes permanently or were attacked. Reduction of the preferred celluloses with borohydride (Wolfrom *et al.*, 1966) was unhelpful. Long cellulose columns (Fig. 1) were more effective in separating components directly from culture filtrates than were short columns. These also separated filtrates into four main components, but all contained considerably more CM-cellulase; the main CM-cellulase component also hydrolysed cotton and reprecipitated cellulose; the 3P component attacked cotton to give short fibres and reducing sugars. Shorter cellulose columns (7cm) emphasized this multiplicity of enzyme activities in each component while providing good recoveries. Elution at pH6–6.5 was preferred to that at pH6.9, where adsorption of components was weaker, resolution less effective and instability of some components, isolated by conventional procedures, was considerable (Halliwell & Griffin, 1973); at pH5, stability was greatest, but adsorption of some components was too strong. Columns were eluted at 10ml/h, since higher rates, (25ml/h) favoured transfer of CM-cellulase to the cellobiase fractions, and cellobiase to the normally faster-running pigment. Under the recommended conditions, affinity chromatography on cellulose has been successfully applied to culture filtrates containing cellulolytic components. Their differential binding to the substrate permits an effective separation of purified forms of cellobiase, CM-cellulase, component C_2 and cellobiohydrolase.

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