SYNERGISTIC HYDROLYSIS OF CELLULOSE BY COMPONENTS OF THE EXTRACELLULAR CELLULASE SYSTEM OF *TALAROMYCES EMERSONII*

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1. Introduction

Forecasts of shortages in food and fossil fuel have stimulated an increased interest in cellulose as a potential alternative [1–5]. To this end the enzymic degradation of cellulase is being investigated. Many organisms possess enzymes that can hydrolyze soluble celluloses. By contrast, few species produce the 'complete' cellulase complex capable of catalying the extensive degradation of the crystalline substrates found in nature [6–8].

Talaromyces emersonii, a thermophyllic fungus, when grown on cellulose-containing media, produces an extracellular enzyme system that catalyzes the degradation of various insoluble crystalline celluloses [9,10]. The observed extent of hydrolysis of these substrates implied that the cellulase system produced by this organism is a 'complete' complex. Here we provide direct evidence that such is indeed the case.

2. Materials and methods

Talaromyces emersonii CBS 814.70 was obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. The 'white colony'-producing form (Folan and M.P.C., in preparation) of this organism was grown as in [9] except that the medium used contained 2% (w/v) BW.40 (purified ball-milled spruce cellulose) instead of crystalline cellulose and 0.5% (w/v) corn steep liquor plus 1% (w/v) ammonium nitrate instead of peptone. The pH of the medium which started at 5 and reached 6 at 29 h had fallen to 3–3.5 at 56 h. The culture medium was usually collected at 48–56 h and filtered through celite.

Enzyme activities, unless stated otherwise, were determined by measuring the release of reducing equivalents by the dinitrosalicylate method [11] following incubation of enzyme at 50°C with the appropriate substrate in 0.1 M sodium acetate buffer (pH 5.0) (final vol. 2.0 ml). Activities and substrates were as follows: total cellulase activity, 60 mg filter paper (Whatman no. 1) or of microcrystalline cellulose (Sigma type 38); CM-cellulase (endocellulase or C_x) activity, 6% (w/v) carboxymethyl cellulose (low viscosity material from Sigma). At 33°C this activity was linear up to 0.66 mg glucose reducing equivalents; exocellulase (C_1) activity, H_3PO_4-swollen cellulose. This substrate was prepared by treating filter paper with 85% (w/v) H_3PO_4 for 1 h at 4°C following by washing in turn with distilled water, 1% (w/v) $NaHCO_3$ and distilled water; laminarinase, 2 mg laminarin (from *Laminaria hypertorea;* Koch-Light); dextranase, 20 mg Sephadex G-75. β-Glucosidase (cellobiase) activity was measured as in [12] but using *p*-nitrophenyl-β-D-glucoside as substrate. Amylase activity was assayed by monitoring the loss of colour of the starch–iodine complex [13] using a kit supplied by Roche. Protease activity was demonstrated qualitatively by the clarification of casein–agar gels (2% casein in 1.5% agar). Protein concentration was determined from A_{280} and by the method in [14] using bovine serum albumin as standard.

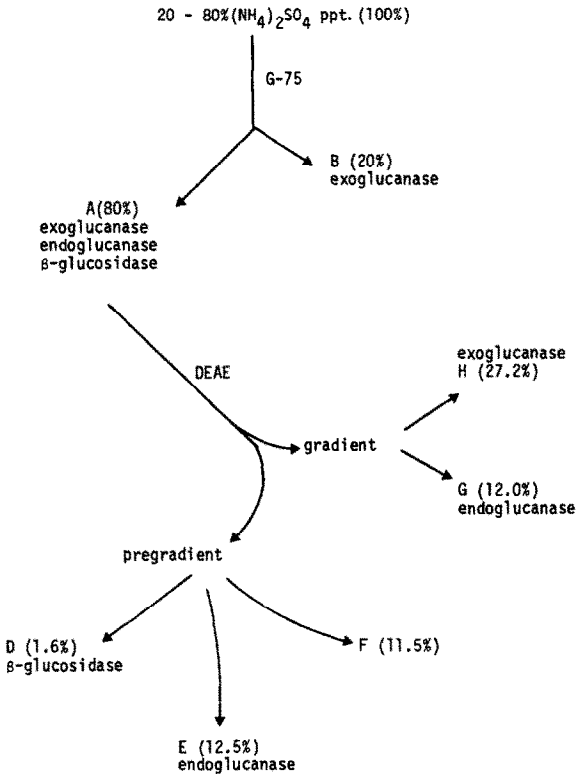
The products of the action of total cellulase, endocellulase (C_x) and exocellulase (C_1) on H_3PO_4-swollen cellulose were identified by paper and thin-layer chromatography as in [15] except that the thin-layer chromatograms were sprayed with alkaline potassium permaganate [16] instead of anisaldehyde/H_2SO_4.

3. Results

3.1. *Fractionation of the* T. emersonii *cellulase complex*

The fraction of the culture filtrate that precipitates at 4°C and 20–80% saturation with ammonium sulphate was collected by centrifugation, redissolved in distilled water and freeze-dried. The freeze-dried material was redissolved in 0.1 M potassium acetate buffer (pH 3.5) and subjected to gel filtration on a column (4.5 X 72 cm) of Sephadex G-75 equilibrated with the same buffer. Two major peaks, A and B, of protein were obtained. Peak A containind β-glucosidase (cellobiase), endocellulase (C_x or CM-cellulase), exocellulase (C_1), laminarinase and a trace of protease activity and accounted for 80% of the total protein of the ammonium sulphate precipitate. Peak B, which accounted for the remaining 20% of the protein, contained exocellulase (C_1), some laminarinase and at the trailing edge, dextranase. Peak A was concentrated ~10-fold using an Amicon and PM-10 filter and desalted by passage of several column volumes of distilled water through the apparatus. The desalted mixture was then freeze-dried for storage. No loss of activity occurred over a period of at least 1 month. Aliquots of this material were redissolved in 50 mM sodium acetate buffer (pH 5.0) containing 0.15 M NaCl and chromatographed on a column (1.7 X 26 cm) of DEAE–Sephadex A-50. Protein was eluted by irrigation with ~4 column vol. of the same buffer. The eluted proteins, fractions D–F, included β-glucosidase, traces of endocellulase and laminarinase. Further protein was eluted on application of a 500 ml exponential gradient, 0.15–0.35 M NaCl in 50 mM sodium acetate buffer (pH 5.0). Two peaks, G and H, of protein, one of which contained endocellulase (C_x) and the other exocellulase (C_1) activity, were obtained. The purification procedure and the relative proportions of the various components of the culture filtrate are summarized in scheme 1. The purification procedure used was designed primarily to show that a 'complete' cellulase system was present in the culture filtrate. However, by judicious choice of the fractions to be pooled, β-glucosidase was purified over 200-fold and endocellulase (C_x) and exocellulase (C_1) activities were completely separated from one another.

Scheme 1
Fractionation of the components of the *T. emersonii* cellulase complex



The scheme illustrates the procedures used to isolate the various fractions, their activities and their protein contents relative to that of the ammonium sulphate precipitate. The fractions containing exocellulase (the so-called C_1 or cellobiohydrolase) activity were identified by their ability to hydrolyze H_3PO_4-swollen cellulose almost exclusively to cellobiose while being unable to degrade CM-cellulose. Endocellulase (the so-called C_x or CM-cellulase) activity was identified by ability to hydrolyze CM-cellulose. The end-products of the action of this enzyme on H_3PO_4-swollen cellulose were mainly cellobiose with lesser amounts of glucose and traces of higher oligomers

3.2. *Synergism in the hydrolysis of crystalline cellulose*

The ability of the various components, individually and in combination with one or more others, to degrade filter paper and microcrystalline cellulose was investigated. Table 1 shows that none of the individual components could effect significant hydrolysis of either substrate under the conditions of the experiment. However, a mixture of endocellulase (C_x) and

Table 1
Hydrolysis of cellulose by the purified components of the *T. emersonii* cellulase system

	Sample	Microcrystalline cellulose (mg reducing equiv. /ml enzyme)	(%)	Filter paper (mg reducing equiv. /ml enzyme)	(%)
1.	Full complex	2.32	100	6.5	100
2.	Full complex	2.44	100	7.0	100
3.	G	0	0	0	0
4.	G + H	0.4	17	1.2	18
5.	G + B	0.12	5	0.3	4.6
6.	G + F	0	0	0	0
7.	G + E	0	0	0	0
8.	G + E + H + B	1	41	2.7	39
9.	G + E + H + B + D	1.4	57	4.2	60
10.	G + E + H + B + D + F	1.44	59	4.3	61

Enzyme activity was determined by monitoring the release of reducing equivalents following incubation with 60 mg substrate (microcrystalline cellulose or filter paper) for 36 h at 50°C in 0.1 M sodium acetate buffer (pH 5.0) as in section 2. Samples B–H are as in scheme 1. The various samples were recombined with one or more others in the ratios, as judged by their relative protein concentrations, in which they occurred in the full complex (i.e., the 20–80% satd. ammonium sulphate precipitate). The full complex was then diluted to give the same endocellulase (C_x) activity as that displayed by the reconstituted samples. The β-glucosidase activity of the diluted full complex was the same as that of the recombined sample, however the latter was deficient in exocellulase (C_1) activity. Samples 1 and 2 are the controls for samples 3–6 and 7–10, respectively. The protein concentrations of samples 2 and 10 were 130 μg/ml and 90 μg/ml, respectively

exocellulase (C_1) did allow an extensive hydrolysis thereby demonstrating the marked synergism in the actions of these two components. The addition of β-glucosidase to those two resulted in a further increase in activity to 60% of that of the full unfractionated complex. This value is an underestimate since no allowance was made for the fact that the protein concentration of the reconstituted mixture (no. 10, table 1) was 30% less than that of the full complex. In fact one may calculate from the data in table 1 that under the experimental conditions the full complex effected 29.8% and 81.6% solubilization of microcrystalline cellulose and filter paper, respectively, per mg protein. The corresponding values for the reconstituted mixture were 24% and 72.6%, respectively. Thus, at equivalent protein concentrations the reconstituted mixture displayed up to 90% of the activity of the unfractionated complex. The synergistic action of the endo- and exocellulases in hydrolyzing cellulose and the stimulation of this process by β-glucosidase is even more evident if one monitors the time-course of the conversion of substrate as in fig.1.

4. Discussion

In the introduction it was stated that while many microorganisms can degrade soluble or amorphous celluloses few produce the enzymes necessary for the hydrolysis of crystalline material. The C_1–C_x theory was proposed to explain these observations [6]. Thus, organisms of the first class produce only the C_x enzyme, an endocellulase now officially known as 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4). Organisms in the latter group were considered to synthesize in addition a non-hydrolytic C_1 factor which functioned in some unknown way to prepare the substrate for attack by C_x. This postulate was necessarily refined when it was shown that the supposed C_1 factor was in fact an exocellulase (1,4-β-glucan cellobiohydrolase; EC 3.2.1.91) [17]. Thus, it is now concluded that cellulase breakdown results from the combined and concomitant actions of these two hydrolytic enzymes [15,18]. Conversion of the resulting cellobiose to glucose is catalyzed by β-glucosidase (cellobiase; EC 3.2.1.21). By coincidence, this also relieves the inhibition of cellulase action by

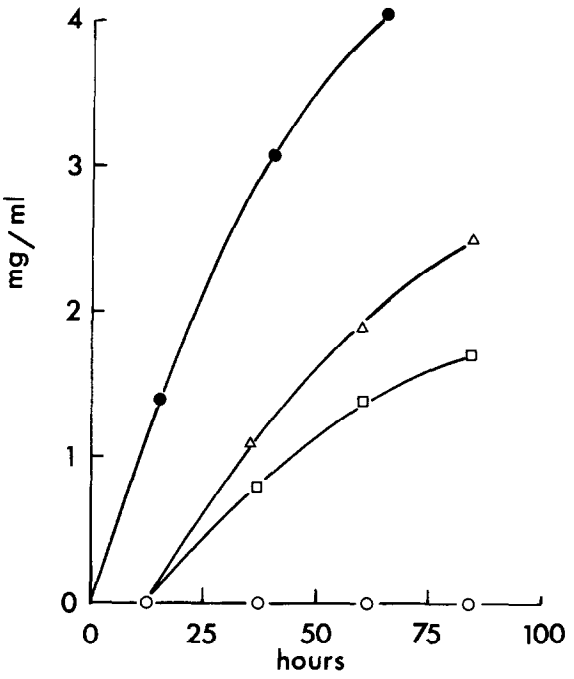


Fig.1. Time course of hydrolysis of cellulose. Aliquots of endocellulase were incubated at 40°C without shaking in sealed tubes containing 60 mg microcrystalline cellulose in 0.1 M sodium acetate buffer (pH 5). To some such reaction mixtures an aliquot of exocellulase was added at 12 h while to other exocellulase and β-glucosidase were added. The release of reducing equivalents at various times was measured as in section 2. (•) Full complex; (○) endocellulase alone; (□) endocellulase with exocellulase added at 12 h; (△) endocellulase with exocellulase plus β-glucosidase added at 12 h. Components were mixed in the appropriate ratios as described in table 1.

cellobiose [10,19] and explains, at least in part, the stimulation of the rate of cellulose hydrolysis on addition of β-glucosidase to the reaction mixtures of table 1 and fig.1.

The white-rot fungus, *Sporotrichum pulverulentum*, produces, in addition the usual hydrolytic enzymes, two oxidative enzymes involved in cellulose degradation. These are cellobiose oxidase and cellobiose : quinone oxidoreductase [20]. However, we have found that the extent of cellulose hydrolysis by the culture filtrate of *Talaromyces emersonii* is the same whether the reaction is carried out under a nitrogen or an oxygen atmosphere. Similar results have been found with *Trichoderma reesei* [21]. Accordingly, in neither of these cases would an oxidative mechanism appear to operate. We may conclude, therefore, that the hydrolysis of crystalline cellulose by *Talaromyces emersonii* results from the synergistic actions of the hydrolytic enzymes, endocellulase, exocellulase and β-glucosidase.

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