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THE CELLULOLYTIC SYSTEM OF *TALAROMYCES EMERSONII*

PURIFICATION AND CHARACTERIZATION OF THE EXTRACELLULAR AND INTRACELLULAR β-GLUCOSIDASES

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The thermophyllic fungus *Talaromyces emersonii* produces three extracellular and one intracellular enzymes exhibiting β-glucosidase (1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.21) activity. Two of the extracellular forms β-glucosidase I and β-glucosidase III have been purified as has the intracellular, β-glucosidase IV. The pH and temperature optima, stability, kinetic parameters and substrate specificity of each has been determined. We conclude that β-glucosidase I and β-glucosidase IV are true cellobiases while β-glucosidase III is an exo-β-1,4-glucose hydrolase.

Introduction

Talaromyces emersonii produces a 'complete' cellulase system when grown on cellulose-containing media [1–3]. Conversion of the crystalline substrate to glucose is accomplished by the concerted action of endocellulases, exocellulases and β-glucosidases [4,5]. The latter by cleaving cellobiose not only produce the glucose but also diminish the inhibition of cellulase activity [2,6–8]. However, as in the case of *Trichoderma sp.* [9], the level of β-glucosidase activity in the culture filtrate of *T. emersonii* is low [2,4]. Such activity must be optimized in order to maximize saccharification of cellulosic substrates [10]. But, is each of the four forms of this enzyme [11] equally important in the hydrolytic process? It was hoped that examination of the properties of the various forms, the subject matter of this paper, would answer this question and provide clues to their individual roles in vivo.

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Materials

Chemicals were obtained from the following suppliers: glucose oxidase (Type II), peroxidase (Type II), Triton X-100, *p*-chloromercuribenzoate, cellobiose and all monosaccharides from Sigma (London) Chemical Co.; 2,2′-azino-di(3-ethylbenzenethiazoline-sulphonic acid) from Boehringer, Mannheim, F.R.G.; dinitrosalicylate and *N,N,N′*,N-tetramethylethylenediamine (TEMED) from BDH: NP-40 from Shell Chemicals (Republic of Ireland) Ltd., Dublin; mercaptoethanol and Biogel P-2 (minus 400 mesh) from Bio-Rad Laboratories, U.K. Solka Floc (BW.40; purified ball-milled spruce cellulose) was from Brown & Co., Berlin, NH, U.S.A. Cello-oligosaccharides were prepared as described below.

Methods

Organism. T. emersonii CBS 814.70 was routinely grown (10 l batches) on cellulose/NH_4NO_3/corn steep liquor medium as described in the accompanying paper [11]. Culture filtrate and mycelia were harvested

at the times appropriate to the isolation of the various β-glucosidases. Three extracellular forms of this enzyme, termed β-glucosidase I, II and III in order of decreasing molecular size, and one intracellular form β-glucosidase IV have been identified [11].

Enzyme assay. β-Glucosidase activity was measured as follows: Reaction mixtures containing *p*-nitrophenyl-β-D-glucoside (1.25 mM in the case of β-glucosidase I and IV, 5 mM in the case of β-glucosidase III). 50 mM sodium acetate buffer, pH 5.0, and an aliquot of enzyme in a final volume of 4 ml were incubated at 37°C. Under these conditions activity was constant up to 1 h for β-glucosidase I and III but for only 10 min in the case of β-glucosidase IV (see below). Reactions were stopped by the addition of 4 ml 0.4 M glycine/NaOH buffer, pH 10.8 and the absorbance at 430 nm was read. Activity is expressed as μmol *p*-nitrophenol released/min per ml enzyme. With cellobiose as substrate conditions were as described above except that the volume of the mixture was 1 ml and the reaction was stopped by boiling in sealed tubes for 3 min. On cooling, the amount of glucose released was measured using the glucose oxidase method of Messer and Dahlqvist [12] except that 2,2′-azino-di(3-ethylbenzenethiazolinesulphonic acid) was used instead of *O*-dianisidine. Protein concentration was determined as described by Hartree [13].

Enzyme purification. The culture filtrate was harvested at the appropriate time and fractionated essentially as described earlier [4]. β-Glucosidase I and III, present in fraction D of Ref. 4, were separated and purified to apparent homogeneity by gradient gel electrophoresis [14]. Each form of the enzyme was located by staining a representative portion of gel for activity [11] and was eluted by homogenization of the appropriate section of gel in 0.2 M sodium acetate buffer, pH 5.0. The suspension was centrifuged to remove gel particles and the supernatant was dialyzed against distilled water and freeze-dried for concentration and storage. β-Glucosidase III was purified to apparent homogeneity by harvesting filtrate at approx. 36 h, fractionation with $(NH_4)_2SO_4$ (20–80% cut), gel filtration on Sephadex G-75 (fraction B of Ref. 4), and ion-exchange chromatography on DEAE-Sephadex A-50 (equilibrated with 50 mM sodium acetate buffer, pH 5.0/0.25 M NaCl) and eluting with a linear gradient of 0.25–0.55 M NaCl in the above buffer. The β-glucosidase III isolated in this way migrated as a single band on gradient gel electrophoresis. β-Glucosidase IV, and intracellular form of the enzyme, was purified as follows: mycelia were washed successively with 10 vol. distilled water/0.5 M sodium acetate buffer, pH 5.0/distilled water/0.2 M sodium acetate buffer, pH 5.0, and then suspended in the latter. The suspension was homogenized in the presence of glass beads (0.5–0.75 mm) in a Waring blendor for 3 × 1-min intervals. Following centrifugation at 100 000 × *g* for 1 h to remove all particulate matter the supernatant was dialyzed against distilled water and subjected to gradient gel electrophoresis and elution as above.

Preparation of cello-oligosaccharides. These substrates were prepared by acetolysis/deacetylation of Solka Floc according to the method of Miller et al. [15] followed by fractionation of the products by gel filtration on Biogel P-2. Oligosaccharides eluting from the column were detected by the dinitrosalicylate method [16] and their identities established by TLC as in [17] except that chromatograms were sprayed with alkaline potassium permanganate [18] instead of anisaldehyde/H_2SO_4. The various frac-

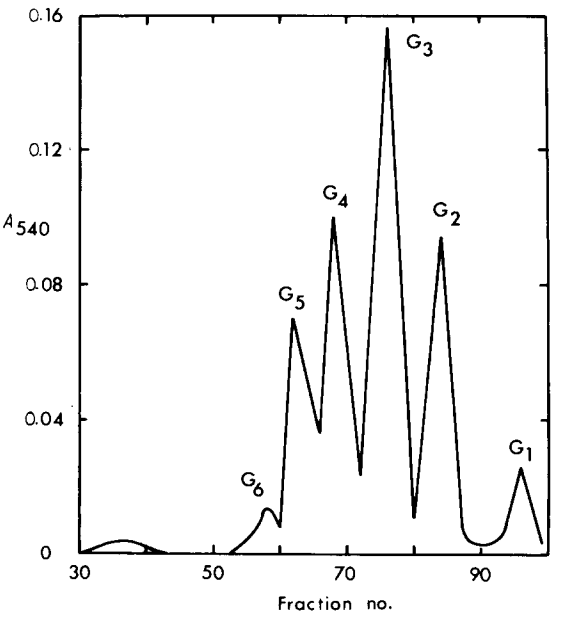


Fig. 1. Preparation of cello-oligosaccharides. Aliquots of each fraction were reacted with the dinitrosalicylate reagent [16] and the A_{540} determined. G_1, G_2, G_3...G_6 represent glucose, cellobiose, cellotriose . . . cellohexaose.

tions were judiciously pooled (see Fig. 1 for an example of a typical run) and freeze-dried.

Results and Discussion

In the accompanying paper [11] we reported that *T. emersonii* produces three extracellular enzymes exhibiting β-glucosidase activity. These were called, in terms of decreasing molecular weight, β-glucosidase I, II and III. An intracellular form of this enzyme, β-glucosidase IV, was also identified [11]. While sufficient β-glucosidase II was not available for characterization the other three enzymes were purified to apparent homogeneity as described in Methods.

pH optima. The pH activity profiles for β-glucosidase I and β-glucosidase IV are relatively sharp, the optima being at 4.1 and 5.7, respectively (Fig. 2). By contrast, that for β-glucosidase III is broad with 50% of maximal activity at almost 2.5 pH units on either side of the optimum, i.e., at pH 5.1 (Fig. 2). These pH optima are similar to the values at which the activities of β-glucosidases from other fungi are maximal. Thus, for example the extracellular enzymes from *Pyricularia oryzae* are most active at pH 5.0–5.5 [20,21], that from *Schizophyllum commune* at 30°C is most active at pH 5.3 [22] while at 50°C the *Aspergillus phoenicis* enzyme has a broad pH optimum centred at 4.3 [23]. Fig. 3 shows that the activity of each of the β-glucosidases from *T. emersonii* decreases with increasing ionic strength. In this respect β-glucosidase I and IV are more sensitive than the β-glucosidase III. It should be mentioned however that the ionic strength of the culture medium remained fairly constant during the growth of this organism. Therefore, one cannot invoke variation in ionic strength to explain the observed variation in β-glucosidase activity in the culture filtrate during fermentation [11].

Temperature optima. At pH 5 β-glucosidase I and

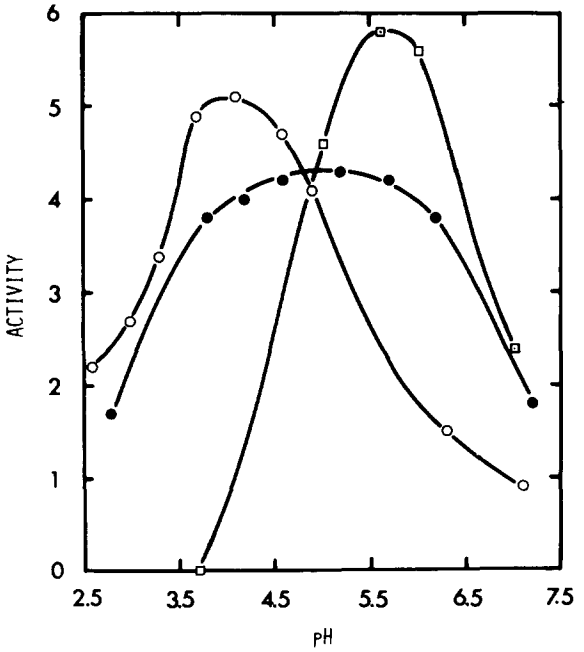


Fig. 2. Effect of pH on β-glucosidase activity. Enzyme activity with *p*-nitrophenyl-β-D-glucoside as substrate was determined at 37°C in 125 mM citrate-phosphate buffer [19] at the indicated pH values. Ionic strength was standardized at each pH value by the addition of appropriate amounts of KCl. Protein concentrations (μg/4 ml) were: β-glucosidase I (○), 0.11 μg; β-glucosidase III (●), 0.58 μg; β-glucosidase IV (□), 1.46 μg.

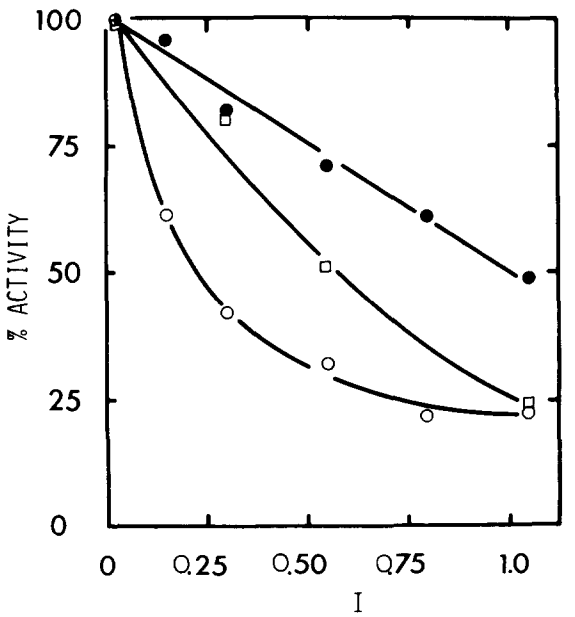


Fig. 3. Effects of ionic strength on β-glucosidase activity Reactions were carried out at 37°C in 50 mM sodium acetate buffer (pH 5.0) with NaCl added to give the indicated ionic strength values. Activities with *p*-nitrophenyl-β-D-glucoside as substrate are in each case expressed as a percentage of the control reaction. β-glucosidase I (○), β-glucosidase III (●) β-glucosidase IV (□).

III are most active at 70°C. Activity drops very sharply on either side of this temperature. These high temperature optima, to be expected of extracellular enzymes produced by a thermophyllic organism, may accord the *Talaromyces* system an advantage over those of mesophiles in the practical saccharification of cellulosic substrates. By contrast with the extracellular forms the intracellular β-glucosidase IV is most active at 35°C, though again activity drops sharply on either side of this value. Arrhenius plots for the hydrolysis of *p*-nitrophenyl-β-D-glucoside by each enzyme are shown in Fig. 4. In the case of β-glucosidase I and III plots were linear between 15 and 45°C and energy of activation values (Kcal/mol) over this temperature range were calculated to be β-glucosidase I, 14.1; β-glucosidase III, 17.3. The plot for β-glucosidase IV was linear only between 15 and 20°C even though substrate was saturating. The energy of activation value for β-glucosidase IV was calculated to be 37.8 kcal/mol. This unusually high value suggests that the enzyme may have to undergo some association or dissociation (deaggregation) before becoming active or it may be that the reaction measured is not the only reaction catalyzed by the enzyme under the experimental conditons used. Answers to these questions must await further investigation. The above values may be compared with the value of 15.0 kcal/mol reported for the β-glucosidase from sweet almonds [24].

Stability. Once again as expected of enzymes produced by a thermophyle the extracellular β-glucosidases exhibit marked stability at high temperatures (Fig. 5 and Table I). Thus, β-glucosidase I and III have half-lives at 70°C and pH 5 of 6 h and 3 h, respectively, whereas the intracellular β-glucosidase IV is very much less stable under these conditions. For comparative purposes we may note that the β-glucosidase from *Aspergillus phoenicis,* one of the best sources of this enzyme, is only 0.5 h at 70°C [23]. Thermal stability is also a function of pH (Table I). At lower pH values both β-glucosidase I and β-glucosidase III are much more labile. For example, at 45°C and pH 3, conditions that develop during the growth of *T. emersonii* on cellulose/corn steep liquor/NH_4NO_3 medium [11], the half-life of β-glucosidase III is less than 2 min. This would explain the loss of β-glucosidase III from the culture filtrate at about 45 h during growth on this medium [11]. It would seem

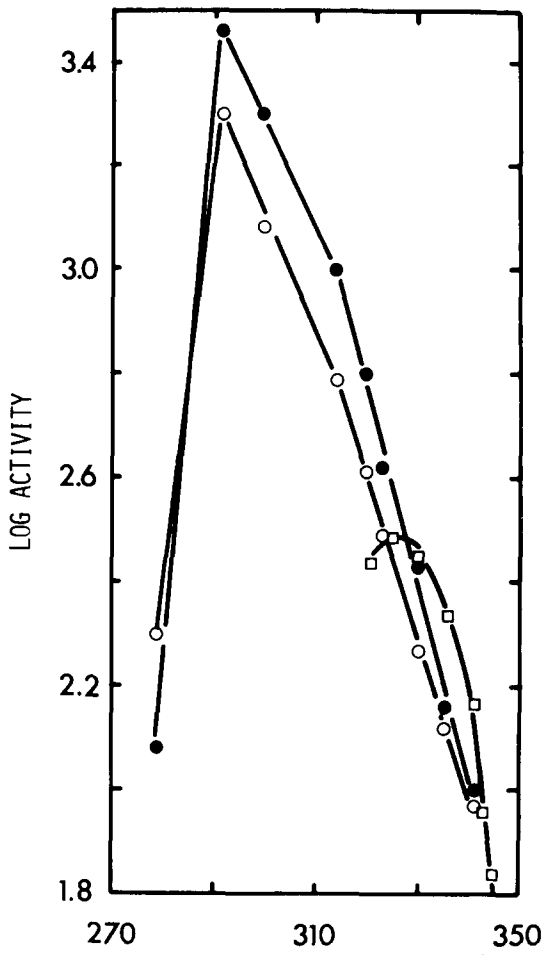


Fig. 4. Arrhenius plots of the hydrolysis of *p*-nitrophenyl-β-D-glucoside. The extent of hydrolysis of substrate (μmol/min per ml enzyme on incubation of enzyme with *p*-nitrophenyl-β-D-glucoside for 30 min at the indicated temperature was determined. Enzyme concentrations (μg/4 ml assay mixture) were: β-glucosidase I (○) 0.11; β-glucosidase III (●), 0.58; β-glucosidase IV (□), 0.29.

that the β-glucosidases from most if not all fungal species are unstable below pH 3 and above pH 8. This is certainly the case with the enzymes from *Trichoderma viride* [25], *Aspergillus phoenicis* [23] and *Schizophyllum commune* [22].

Substrate specificity. Gong andTsao [26] in their review state that there are two types of β-glucosidase, one being aryl-β-glucosidase and the other β-glucosidase. The former hydrolyzes aryl-β-glucosidases but is unable to cleave cellobiose while the latter is active against both types of substrate and in the context of

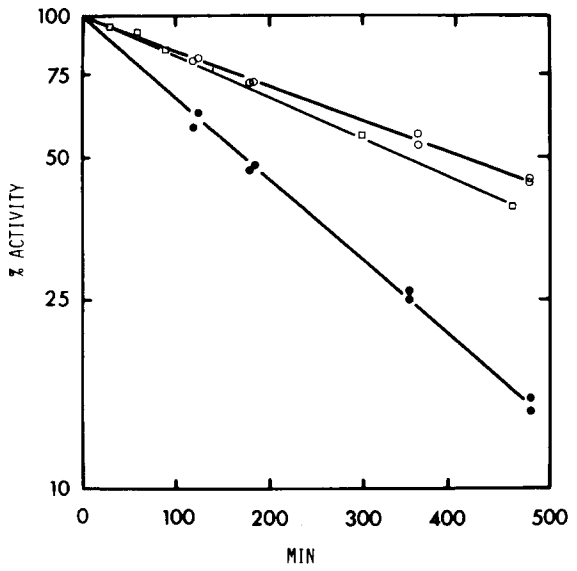


Fig. 5. Thermal stability of the β-glucosidases from *T. emersonii*. Samples of enzyme in 50 mM sodium acetate buffer pH 5 were incubated in sealed tubes at 70°C (β-glucosidase I and III) or at 37°C (β-glucosidase IV). Activity at various times is expressed as a percentage of the appropriate zero time control. Enzyme concentrations were: β-glucosidase I (○), 1.1 μg/ml; β-glucosidase III (●), 5.8 μg/ml; β-glucosidase IV (□), 14.6 μg/ml.

cellulose saccharification has been termed 'cellobiase'. The enzymes from many fungal and bacterial sources fall into this category [26] as indeed do the β-glucosidases from *T. emersonii* (see below).

The enzymes from *Pyricularia oryzae* [20 and *Aspergillus phoenicis* [23] cleave not only cellobiose but also various other β-linked dimers and higher polymers of glucose. However, in order to examine the possible roles of the *Talaromyces* enzymes in cellulolysis we investigated only their ability to degrade the β-1,4-linked polymers of glucose, i.e., the cello-oligosaccharides. Table II shows that whereas the relative rates of hydrolysis of these substrates by β-glucosidase I and IV decrease in going from cellobiose (G_2) to cellohexaose (G_6) the rate of hydrolysis by β-glucosidase III increases dramatically at least up to G_6.

Cellobiose can be cleaved in one way only to give glucose. In contrast, the higher polymers can in theory be hydrolyzed in a number of different ways, internally and externally, ultimately to produce glucose. Accordingly, the products formed from cellotetraose during the course of reaction with β-glucosidase I, III and IV were identified by TLC (Fig. 6). The presence of large quantities of cellotriose in the early stages of the reaction and of cellobiose at later stages clearly shows that each of these enzymes acts by removing glucose units one at a time from the end of the substrate, presumably the non-reducing end [20].

Examination of the kinetic properties of the *T. emersonii* enzymes (Table III) shows that β-glucosidase I and IV are quite similar. Both enzymes are subject to inhibition by excess of substrate. In the

TABLE I

pH AND TEMPERATURE OPTIMA FOR *p*-NITROPHENYL-β-GLUCOSIDE HYDROLYSIS BY β-GLUCOSIDASES

βGI, β-Glucosidase I, βGIII, β-glucosidase III and βGIV, β-glucosidase IV.

Property	βGI	βGIII	βGIV
Optimum pH at 37°C	4.1	5.1	5.7
Optimum temp. (°C) at pH 5	70	70	35
E_a (kcal/mol)	14.1	17.3	37.8
$T_{1/2}$ (min) at 70°C, pH 5	410	175	2
$T_{1/2}$ (min) at 37°C, pH 5	–	–	350
$T_{1/2}$ (min) at 45°C, pH 3	157	1.9	–

TABLE II

RELATIVE RATES OF HYDROLYSIS OF CELLO-OLIGOSACCHARIDES BY β-GLUCOSIDASES

Activities were determined by measuring the release of glucose using the glucose oxidase method. In each case activity with cellobiose as substrate was arbitrarily put at 100. No correction was made for the fact that cleavage of one bond in cellobiose releases two molecules of glucose, whereas initially at any rate only one molecule of glucose is released per bond hydrolyzed in the other substrates. See legend to Table I for abbreviations.

Substrate (1 mM)	βGI	βGIII	βGIV
Cellobiose	100	100	100
Cellotriose	76	912	43
Cellotetraose	74	2089	30
Cellopentaose	66	2647	27
Cellohexaose	60	3029	23

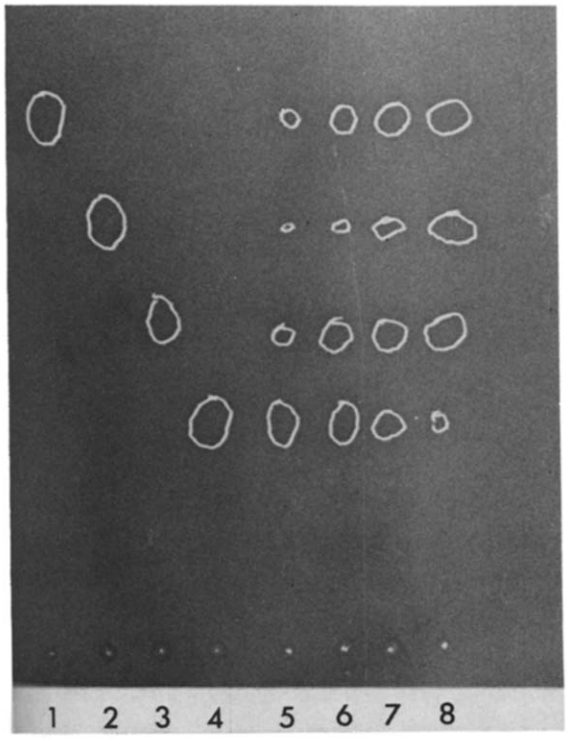


Fig. 6. Products of hydrolysis of cellotetraose by β-glucosidase III. Cellotetraose (2 mM) was incubated in 50 mM sodium acetate buffer, pH 5.0. Samples of the reaction mixture obtained at the indicated times were boiled for 2 min and then subjected to TLC. 1, 2, 3 and 4 are the glucose, cellobiose, cellotriose and cellotetraose standards, respectively. 5, 6, 7 and 8 are samples of reaction mixtures taken at 0.5, 1.0, 2.0 and 4.0 h after the start of the reaction. Qualitatively similar results were obtained with β-glucosidase I and β-glucosidase IV.

case of cellobiose this occurs at concentrations over 2.5 mM. In contrast, no such inhibition of β-glucosidase III was observed even at concentrations up to 80 mM. Table IV shows that cellobiose competitively inhibits the hydrolysis of *p*-nitrophenyl-β-D-glucoside by all three enzymes. One may note the very good agreement between the K_i values and the corresponding apparent K_m values listed in Table III. This indicates that the latter do indeed represent the affinity of each enzyme for cellobiose. It may also be noted that in the case of β-glucosidase III, the K_i value for cellotetraose (Table IV) is very close to the apparent K_m value for this substrate.

While each of these enzymes catalyzes hydrolysis of cello-oligosaccharides (Table II) examination of the apparent K_m value (Table III) and K_i values (Table IV) provides an interesting insight into their individual roles in vivo. Thus, β-glucosidase I and IV exhibit a 2-fold greater affinity for cellotetraose than for cellobiose. However, the relative V values decrease by 2-fold and 30-fold, respectively. In this context the most notable feature about β-glucosidase III is the fact that it displays a 25-fold greater affinity for cellotetraose than for cellobiose. These results coupled with those of Table II and Fig. 6 suggest to us that while β-glucosidase I and IV may be regarded as true 'cellobiases' β-glucosidase III should more properly be considered as a β-1,4-glucose hydrolase (glucohydrolase). The fact that β-glucosidase III but not β-glucosidase I is induced cotemporaneously with the cellulases [11] is consistent with this interpretation.

For comparative purposes we may note that the enzymes from *Trichoderma viride* [25,27,28], *Pyricularia oryzae* (GB-1 enzyme, Ref. 20) and *Aspergil-*

TABLE III

MICHAELIS-MENTEN CONSTANTS FOR THE β-GLUCOSIDASES OF *TALAROMYCES EMERSONII*

Kinetic parameters were estimated by fitting the Michaelis-Menten equation to the data using MLAB (a software package supplied by DCRT, NIH, Bethesda, Md., U.S.A.) running on a DEC 2040 computer. K_m was given in mM and V as μmol/min per mg enzyme. For abbreviations see Table I.

Substrate	βGI		βGIII		βGIV	
	K_m	V	K_m	V	K_m	V
p-Nitrophenyl-β-glucoside	0.14	35.4	1.03	9.7	0.81	19.9
Cellobiose	0.58	96.2	23.70	2.6	1.47	79.8
Cellotetraose	0.22	43.0	0.90	1.6	0.67	2.8

TABLE IV

COMPETITIVE INHIBITION OF *p*-NITROPHENYL-β-GLUCOSIDE HYDROLYSIS

For abbreviations see Table I.

Inhibitor	K_i (mM)		
	βGI	βGIII	βGIV
Cellobiose	0.57	34.50	1.08
Cellotetraose	–	1.60	–
Glucose	0.71	none	52.0
Galactose	–	none	45.0

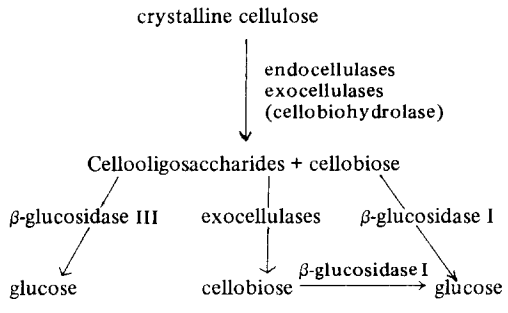
lus phoenicis [23] all behave like β-glucosidase I in exhibiting a higher affinity for but lower V with cellotetraose than with cellobiose. Unlike these enzymes a second β-glucosidase from *Pyricularia oryzae* called GB-2 [30] is said by the authors to be an allosteric protein, with two binding sites for substrate, displaying negative cooperativity with cello-oligosaccharides as substrate. In this case affinity for substrate decreases with increasing chain length [30]. Sternberg [25] stated that, as judged from kinetic patterns, culture filtrates of *Trichoderma viride* Qm9414 showed two types of β-glucosidase activity. One such β-glucosidase enzyme had high affinity for cellobiose (K_m 1.5 mM), was subject to substrate inhibition above 7.5 mM and was acid-labile. The other enzyme had low affinity for cellobiose ($K_m >$ 50 mM), was not inhibited by excess of substrate and was more acid-stable. Apart from reversal of the acid stability properties this situation is reminiscent of β-glucosidase I and III here. Investigation of the time course of production of the low affinity *T. viride* enzyme should prove interesting as would its isolation and characterization.

Table IV also shows that glucose, an effective inhibitor of β-glucosidase I, is without effect on β-glucosidase III and almost so against β-glucosidase IV. Indeed at 50 mM, glucose inhibited the β-glucosidase IV-catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucoside by only 29%. Inhibition of this enzyme by galactose and fructose, both at 50 mM, was 41 and 16%, respectively. Neither of the latter sugars inhibited β-glucosidase I and III, while mannose, arabinose and xylose had no effect on substrate hydrolysis by any of the enzymes. For comparative purposes we note that glucose is a competitive inhibitor of the GB-1 enzyme from *Pyricularia oryzae* [30] and of the β-glucosidase from *Botryodiploidia theobromae* [29] while at concentrations between 4 to 90 mM the enzyme from *Trichoderma viride* is apparently stimulated [31].

The inclusion in reaction mixtures of the ionic detergent SDS at a concentration of 0.25% (v/v) inhibited β-glucosidase I, III and IV by 26, 7 and 88%, respectively. On the other hand, the non-ionic detergents Triton X-100, which is frequently used to control foaming during fermentation, and NP-40 at the same concentration were with effect on any of the enzymes. Also without effect were EDTA (1.25 mM), mercaptoethanol (1 mM) and ethanol (1.25%). The latter finding augurs well for the practical application of the *Talaromyces* enzymes in the production of alcohol from cellulosic wastes. Tris is known to inhibit disaccharidases and indeed is used in glucose estimation by glucose oxidase so that it can inhibit the disaccharidase contaminating these reagents [12]. At 0.25 mM Tris inhibited β-glucosidase I by 12% and β-glucosidase IV 19% but did not inhibit β-glucosidase III. Although not dramatic these findings are also consistent with the interpretation that β-glucosidase III is not a true cellobiase (see earlier discussion). Finally, we note that the inclusion of *p*-chloromercuribenzoate (1 mM) in reaction mixtures completely inactivated β-glucosidase IV, inhibited β-glucosidase I by 30% but had no effect on β-glucosidase III. Further work will be required to determine whether in β-glucosidase I and β-glucosidase IV this inactivation results from modification of sulphydryl groups.

Conclusion

The results in this and the accompanying paper indicate that β-glucosidase I and β-glucosidase IV are true cellobiase, whereas β-glucosidase III is a glucohydrolase. Cellulose hydrolysis by the extracellular system of *Talaromyces emersonii* may therefore be envisaged as follows:



Thus, β-glucosidase I is the major enzyme involved in cleaving the cellobiase resulting from the action of the endo- and exocellulases. β-Glucosidase III meanwhile removes glucose residues from the oligosaccharides arising during the saccharification process. However, it may also come into its own as a cellobiase if because of glucose accumulation β-glucosidase I action is inhibited. β-Glucosidase IV the intracellular enzyme is clearly involved in cleaving any cellobiose taken into the cell. As such it may also have a role in regulating the synthesis of the various components of the extracellular cellulase system in the manner postulated by Gong and Tsao [26].

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