Purification and Some Properties of the Extracellular β -D-Glucosidase of the Cellulolytic Fungus *Trichoderma koningii*

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The β -D-glucosidase which was associated with the cellobiohydrolase and endo-(1 $\rightarrow$ 4)- β -D-glucanase activities in the cellulase of the fungus *Trichoderma koningii* was purified by gel filtration on a column of Ultrogel AcA 44, ion-exchange chromatography on DEAE-Sephadex and sulphoethyl-Sephadex (SE-Sephadex), and finally by isoelectric focusing in a pH gradient supported in a sucrose density gradient. The separation of β -glucosidase and endo-(1 $\rightarrow$ 4)- β -glucanase, which was effected with some difficulty on SE-Sephadex, could be achieved readily by chromatography on a column of concanavalin A-Sephadex. Isoelectric focusing yielded two β -glucosidase components (pI 5.53 and 5.85) of identical molecular weight (39800), but with different affinities for *o*-nitrophenyl- β -glucoside (K_m 0.37 ± 0.04 and 0.85 ± 0.03 mM), cellobiose (K_m 1.18 ± 0.09 and 0.86 ± 0.02 mM), cellotriose (K_m 5.10 ± 0.22 and 7.10 ± 0.33 mM), cellotetraose (K_m 2.38 ± 0.14 and 4.0 ± 0.4 mM) and cellopentaose (K_m 1.51 ± 0.04 and 2.19 ± 0.06 mM). One of the β -glucosidases was devoid of carbohydrate, the other contained approximately 2% carbohydrate. Gluconolactone was a powerful inhibitor of the action of both enzymes on *o*-nitrophenyl- β -glucoside (K_i 1.8 ± 0.08 and 1.17 ± 0.05 μ M); glucose was less effective (K_i 1.05 ± 0.07 and 0.66 ± 0.04 mM). Inhibition was of the competitive type in each case. Both β -glucosidases showed the same capacity for acting in synergism with a mixture of the endo-(1 $\rightarrow$ 4)- β -glucanase and cellobiohydrolase in solubilizing the cellulose in cotton fibre. They differed markedly, however, in the degree of synergism they showed when acting in concert with the cellobiohydrolase on partially degraded H_3PO_4 -swollen cellulose.

INTRODUCTION

The cellulases synthesized by the cellulolytic fungi *Trichoderma koningii* (Wood, 1972), *Trichoderma reesei* (Berghem *et al.*, 1975; Gong *et al.*, 1979), *Penicillium funiculosum* (Wood & McCrae, 1977*a*) and *Fusarium solani* (Wood & McCrae, 1977*a, b*) consist of three principal types of enzyme, namely, exo-(1 $\rightarrow$ 4)- β -glucanase (cellobiohydrolase), endo-(1 $\rightarrow$ 4)- β -glucanase and β -glucosidase. Detailed studies on the purified enzymes have established that when acting separately these enzymes have little or no action on highly ordered cellulose, but when together act synergistically to extensively solubilize the most refractory cellulosic materials. Two types of synergism seem to operate during the enzymic degradation. In the first stage of the reaction an enzyme A (endo-(1 $\rightarrow$ 4)- β -glucanase) produces a reactive product for enzyme B (cellobiohydrolase) while in the second stage an enzyme C (β -glucosidase) removes the inhibitory product (cellobiose) of the action of the other enzymes. The β -glucosidase enzyme is obviously an important component of the cellulase system and it has attracted much interest in recent years.

Trichoderma species seem to be the best sources of extracellular cellulases that can solubilize highly ordered cellulose, and they have been studied widely because of their potential importance in the industrial saccharification of cellulose. Unfortunately *Trichoderma* species, particularly *T. reesei*, are relatively poor synthesizers of β -glucosidase, and this has resulted in

attempts by some to improve the performance of the cellulase by supplementation with β -glucosidase from other sources (Sternberg *et al.*, 1977) or by the isolation of mutants that can produce higher yields of the enzyme (Beja da Costa & van Uden, 1980).

In view of the commercial importance of β -glucosidase a better understanding of the enzyme is required. In this report we describe the purification and some properties of the β -glucosidase of *Trichoderma koningii*.

METHODS

Materials. The sources of the materials were as follows: *O*-carboxymethylcellulose sodium salt CellofasB (CM-cellulose), ICI, Nobel Division; Texas-cotton fibre, Shirley Institute, Manchester, U.K.; Sephadex G-25, DEAE-Sephadex and sulphoethyl-Sephadex (SE-Sephadex), Pharmacia; Ampholine carrier ampholyte and Ultrogel, LKB Instruments; D-glucose oxidase (type II), arbutin, phloridzin, salicin and amygdalin, Sigma; peroxidase, Boehringer; collodion tubes (Sartorius), V. A. Howe, London, U.K.; cytochrome *c* (type II), α -chymotrypsinogen (type II) and thyroglobulin (type II), Sigma; bovine α -globulin, Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; and *o*-nitrophenyl- β -glucoside, BDH. Ovalbumin was a gift from Dr J. Conchie, Rowett Research Institute. All other reagents were of analytical reagent grade.

Preparation of *T. koningii* cellulase. Cultures and cell-free enzyme preparations were prepared from *T. koningii* IMI 73022 by batch culture as already described (Wood, 1968). The crude cell-free culture filtrate contained 41.2 units β -glucosidase activity ml⁻¹. Concentrated (100-fold) and partially purified [20–80% saturated (NH₄)₂SO₄ fraction] cell-free culture filtrate contained 4100 units β -glucosidase ml⁻¹. A unit of activity is defined as the amount of enzyme needed to liberate 25 μ g *o*-nitrophenol under the conditions of the assay described by Wood (1968).

Preparation of cello-oligosaccharides. Cello-oligosaccharides were prepared by the acetolysis (Miller *et al.*, 1960) of cellulose powder (Whatman CC41), and separated by gradient elution (0 to 35%, v/v ethanol) from a column (90 $\times$ 3.5 cm) of charcoal (BDH; acid-washed)/Celite (1:1, w/w).

Isoelectric focusing. Separations were performed in an LKB electrofocusing column (110 ml). After focusing at 5 °C, the column was emptied at a rate of 120 ml h⁻¹. Fractions (2 ml) were collected, and the pH of each was measured at 5 °C with a Corning-Eel pH meter fitted with a combination electrode.

Enzyme assays. **CM-cellulase activity.** CM-cellulase activity was measured by the reducing-sugar method using modified Park-Johnson reagent, described previously (Wood & McCrae, 1972).

Activity against cotton. Dewaxed cotton (2 or 20 mg) was incubated for 7 d (2 mg cotton) or 18 h (20 mg) and the activity calculated as previously described (Wood, 1969). A 1 ml volume of the 20–80% saturated (NH₄)₂SO₄ fraction, diluted 50-fold, solubilized 1.4 mg of the 2 mg sample of cotton under these conditions.

Protease. Proteolytic activity was assayed with casein as substrate (Kunitz, 1947).

β -Glucosidase. β -Glucosidase was measured with *o*-nitrophenyl- β -glucopyranoside as substrate by the method already described (Wood, 1968).

Other assays. Reducing sugars were measured by the Nelson–Somogyi method (Nelson, 1952), D-glucose by a modified D-glucose oxidase method (Lloyd & Whelan, 1969) and total sugar by the phenol-sulphuric acid method (Dubois *et al.*, 1956). In each case the reagents were calibrated against D-glucose. Protein was determined by the Lowry method with crystalline bovine serum albumin used as the standard. In some of the fractionations the protein content was estimated from the A_{280} .

TLC. Thin-layer plates (Kieselgel G) were developed (two ascents) with ethyl acetate/propan-2-ol/water (18:13:9, by vol.) and sprayed with silver nitrate or anisaldehyde/sulphuric acid (Stahl & Kaltenbach, 1961).

RESULTS

Fractionation of the cellulase complex. The β -glucosidase activity in a 5 ml sample of 100-fold concentrated partially purified [20–80% saturated (NH₄)₂SO₄ fraction] cell-free culture filtrate was separated from a low molecular weight endo- β -(1 $\rightarrow$ 4)-glucanase component by gel filtration on a column (2.5 $\times$ 87.5 cm) of Ultrogel AcA 44, and from cellobiohydrolase activity by ion-exchange chromatography on DEAE-Sephadex equilibrated with 0.1 M-NaOH/acetic acid buffer, pH 5.0 (Wood & McCrae, 1978). The β -glucosidase and high molecular weight endo-(1 $\rightarrow$ 4)- β -glucanase components, which were not adsorbed on the ion-exchange medium under such starting conditions, were precipitated with (NH₄)₂SO₄, desalted on a column of Sephadex G-25 equilibrated with 0.01 M-ammonium acetate and freeze-dried. A column (27 $\times$ 1.5 cm) of SE-Sephadex equilibrated with 0.01 M-sodium succinate buffer, pH 4.5, was used to separate β -glucosidase from the bulk of the endo-(1 $\rightarrow$ 4)- β -glucanase as previously described (Wood, 1968;

Fig. 1. Purification of β -glucosidase on a column of concanavalin A-Sepharose. The column (1.5×27 cm) was equilibrated with 0.1 M-acetate buffer, pH 6.0 which contained 1 M-NaCl, 1 mM-MnCl₂, 1 mM-CaCl₂ and 0.003 mM-NaN₃. The enzyme (2 ml), which had been equilibrated by dialysis (collodion tube) against the same equilibrating buffer, was applied to the column in the cold room and then washed with the same buffer solution at 20 ml h⁻¹. Fractions (5 ml) were collected and assayed for β -glucosidase (●) and CM-cellulase (▲) activities. After tube 85 the column was eluted with a linear gradient of 0 to 0.5% (w/v) α -methyl-D-mannoside in the above buffer. The gradient was formed by an LKB Ultrograd.

Wood & McCrae, 1978). Fractions rich in β -glucosidase were pooled, diluted with 2% (v/v) carrier ampholyte, pH range 4–6, and subjected to isoelectric focusing.

A more convenient method of separating β -glucosidase from the high molecular weight endo-(1→4)- β -glucanase activity was by using a column of concanavalin A-Sepharose (Fig. 1). Under the conditions given in the legend to Fig. 1, β -glucosidase passed straight through the column along with only 3% of the total enzyme activity of endo-(1→4)- β -glucanase added; the bulk of the endo-(1→4)- β -glucanase was finally eluted with α -D-mannoside. Recovery of β -glucosidase from the concanavalin A-Sepharose column was normally in excess of 90%.

Separation and purification of β -glucosidase components. A typical distribution of β -glucosidase, CM-cellulase and protein from an electrofocusing experiment using carrier ampholyte, pH range 4–6, is shown in Fig. 2. β -Glucosidase₁ (which had a pI of 5.53) was virtually free from CM-cellulase activity. β -Glucosidase₂ (pI 5.85) differed from β -glucosidase₁ in this latter respect, but it could be purified further by the isoelectric focusing technique (Fig. 3), when a small CM-cellulase component (pI 6.28) was separated using carrier ampholyte covering a higher pH range (5–7). Both β -glucosidase₁ and β -glucosidase₂ were purified by re-runs in ampholyte pH 4–6 (Fig. 4) and pH 5–7 (data not shown), respectively.

Purified in this way, β -glucosidase₁ was free from CM-cellulase activity. β -Glucosidase₂ was, however, still able to attack [0.1% total increase in reducing power (glucose equivalent) in 1 h] CM-cellulose with a degree of substitution of 0.5, but a more highly substituted sample of CM-cellulose (degree of substitution 0.7) was not a substrate.

Purified β -glucosidase₁ and β -glucosidase₂ amounted to 0.14% (w/w) and 0.39% (w/w), respectively, of the total protein found in the crude cell-free culture filtrate, as determined by the Lowry method. Many purification runs were necessary to prepare significant amounts of purified enzyme as it was found impossible to scale-up the purification and still achieve the same degree of purity.

Molecular weight of the enzymes. The molecular weight of the purified enzymes was estimated by gel filtration through a column of Ultrogel AcA 44 calibrated with the standard proteins cytochrome *c* (mol. wt 12400), chymotrypsinogen (25800), ovalbumin (45000), bovine serum albumin (67000) and bovine γ -globulin (160000). A linear relationship was obtained when the elution volumes of the standard proteins were plotted against the log of the molecular weight. The molecular weights of both enzymes were estimated from this graph to be 39800.

Fig. 2. Separation of β -glucosidase₁ and β -glucosidase₂ by isoelectric focusing. The β -glucosidase-rich component (20 ml) isolated from *T. koningii* cellulase by chromatography on Ultrogel AcA 44, DEAE-Sephadex and SE-Sephadex as described in the text, was mixed with 10 ml carrier ampholyte (pH 4–6) and added to an isoelectric focusing column of 110 ml capacity. After focusing for 70 h (600 V, 3 mA at the start; 700 V, 1 mA at the end of the run) the column was emptied and the fractions (2 ml) assayed: ●, β -glucosidase activity; ▲, CM-cellulase activity; ----, A_{280} (protein content); ○, pH.

Carbohydrate content of the enzymes. Using the phenol-sulphuric acid method (Dubois *et al.*, 1956) for measuring carbohydrate and the Lowry method for estimating protein, β -glucosidase₂, which had been purified by isoelectric focusing, was found to be associated with approximately 2% carbohydrate (glucose equivalent). However in view of the small amounts of protein obtained from the isoelectric focusing, this value must be regarded as very approximate. β -Glucosidase₁ purified by isoelectric focusing appeared to be free from carbohydrate.

When β -glucosidase activity from another cell-free cellulase preparation of *T. koningii* (cultured as above) was separated from the endo- and exo-(1→4)- β -glucanase activities using a column of concanavalin A-Sepharose (Fig. 1) and subsequently fractionated into β -glucosidase₁ and β -glucosidase₂ on an isoelectric focusing column, neither component appeared to be associated with carbohydrate.

Transferase activity of the enzymes. With high concentrations of cellobiose (5%, w/v), large amounts of a component with the same mobility as authentic cellotriose were detected by TLC on silica gel plates; traces of a slower moving component travelling at the same speed as authentic cellotetraose appeared on prolonged (5 h) incubation.

Specificity of β -glucosidase components. Both β -glucosidases acted rapidly on both *o*- and *p*-nitrophenyl- β -glucosides and more slowly on the aryl- β -glucosides salicin and arbutin (Table 1). The only alkyl- β -glucoside tested, methyl- β -glucoside, was not a substrate for either component. Both β -glucosidases acted on all the various β -glucosyl glucoses, although at markedly different rates (Table 2). The activity on sophorose (β -1→2-diglucose) or laminaribiose (β -1→3-diglucose), for example, was approximately four times that shown on gentiobiose (β -1→6-diglucose). Cellobiose was attacked relatively slowly by both components.

Effect of heat. The effect of heat on β -glucosidase₁ and β -glucosidase₂ was almost identical under the conditions tested. When the enzymes were heated in the absence of substrate for

Fig. 3. Purification of β -glucosidase₂ by isoelectric focusing. Fractions 42–50 (Fig. 2) were pooled, concentrated to 10 ml on an Amicon cell using a PM-10 membrane, diluted with 10 ml 2% (v/v) carrier ampholyte solution (pH 5–7) and re-focused using the same column and conditions listed previously (Fig. 2). Fractions (2 ml) were assayed: ●, β -glucosidase activity; ▲, CM-cellulase activity; ----, A_{280} (protein content); ○, pH.

Fig. 4. Purification of β -glucosidase₁ by isoelectric focusing. Fractions 30–35 (Fig. 2) were pooled (54 ml), concentrated in an Amicon cell using a PM-10 membrane, diluted with 10 ml of 2% (v/v) carrier ampholyte solution (pH 4–6) and added to the 100 ml LKB electrofocusing column. The voltage at the end of the run (40 h) was 810 V and current 0.5 mA. The contents of the column were collected (2 ml) and assayed: ●, β -glucosidase activity; ----, A_{280} (protein content); ○, pH.

Table 1. *Hydrolysis of different glucosides by the β -glucosidase components*

The reaction mixture contained 0.5 ml of a solution of the substrate (5 mM), 0.5 ml 0.2 M-sodium acetate buffer (pH 5.0), 1.0 ml enzyme, and water to give a total volume of 2 ml. Incubation was for 1 h at 37 °C, after which the glucose released was estimated by the method of Nelson (1952).

Glucoside	Glucose released (μ g)	
	β -Glucosidase ₁	β -Glucosidase ₂
<i>o</i> -Nitrophenyl- β -glucoside (aryl- β -glucoside)	80	70
<i>p</i> -Nitrophenyl- β -glucoside (aryl- β -glucoside)	213	193
Methyl- β -glucoside (alkyl- β -glucoside)	0	0
Phloridzin (phloritin- β -glucoside) (aryl- β -glucoside)	0	0
Amygdalin (aryl- β -gentiobioside)	12	NT
Salicin (2-hydroxymethylphenyl- β -glucopyranoside) (aryl- β -glucoside)	27	25
Arbutin (hydroquinone- β -glucopyranoside) (aryl- β -glucoside)	36	30

NT, Not tested.

Table 2. *Relative activities of β -glucosidase₁ and β -glucosidase₂ on β -linked diglucoses*

The enzymes used were those separated by isoelectric focusing. The assay consisted of approximately 1 mM-substrate, 0.5 ml 0.1 M-sodium acetate buffer pH 5.0, and enzyme plus water to give a total volume of 1 ml. After 30 min incubation at 37 °C the glucose released was measured by the glucose oxidase method (see Methods); 5 units of *o*-nitrophenyl- β -glucosidase were used in each assay.

Substrate	Enzyme activity (μ g glucose equivalent)	
	β -Glucosidase ₁	β -Glucosidase ₂
Sophorose (1 $\rightarrow$ 2- β)	62	68
Laminaribiose (1 $\rightarrow$ 3- β)	60	72
Cellobiose (1 $\rightarrow$ 4- β)	33	47
Gentiobiose (1 $\rightarrow$ 6- β)	15	17

Table 3. *Michaelis constants of the β -glucosidase components*

Substrate	K_m (mM)*	
	β -Glucosidase ₁	β -Glucosidase ₂
<i>o</i> -Nitrophenyl- β -glucoside	0.37 $\pm$ 0.04	0.85 $\pm$ 0.03
Cellobiose	1.18 $\pm$ 0.09	0.86 $\pm$ 0.02
Cellotriose	5.1 $\pm$ 0.22	7.1 $\pm$ 0.33
Cellotetraose	2.38 $\pm$ 0.44	4.0 $\pm$ 0.4
Cellopentaose	1.51 $\pm$ 0.04	2.19 $\pm$ 0.06

* Values shown are $\pm$ the S.D.

30 min at 45 °C, at pH values between 4 and 6, no loss of activity was observed when the pH was below 4.5; at pH 6.0 approximately 60% of the original activity was lost. At pH 5.0 the enzymes were stable for at least 30 min at temperatures less than 50 °C; the stability fell dramatically at 65 °C and at 70 °C the enzymes were completely inactivated.

K_m and K_i values. The two enzymes purified by isoelectric focusing were used for the calculation of K_m and K_i values using Lineweaver-Burk plots; K_m values for the aryl- β -glucoside, cellobiose and the soluble, short-chain cello-oligosaccharides, cellotriose, cello-tetraose and cellopentaose are listed in Table 3. The affinity of β -glucosidase₁ for the aryl- β -glucoside was much higher than that of β -glucosidase₂.

The effect of substrate concentration on the kinetics of hydrolysis of the soluble trisaccharides, tetrasaccharides and pentasaccharides by β -glucosidase₁ and β -glucosidase₂ was tested using concentrations up to 2.4 mM. The two enzymes were affected in a similar fashion. Substrate inhibition occurred with relatively low concentrations of cellotriose (0.6 mM), cellotetraose (0.2 mM) or cellopentaose (0.4 mM). However, with cellobiose as substrate, concentrations of approximately 10 mM were required before significant inhibition was observed. *o*-Nitrophenyl- β -glucoside was inhibitory to both β -glucosidases at relatively low concentrations (0.8 mM). The inhibition constant (K_i , $\pm$ the S.D.) for gluconolactone with *o*-nitrophenyl- β -glucoside as substrate was 1.8 ± 0.08 μ M in the case of β -glucosidase₁ and 1.17 ± 0.05 μ M in the case of β -glucosidase₂; glucose was less inhibitory, values of K_i being 1.05 ± 0.07 mM and 0.66 ± 0.04 mM, respectively. Inhibition was of the competitive type in all cases.

Synergism between the β -glucosidase components and the other enzymes of the cellulase complex in solubilizing various celluloses. Synergism was apparent between the β -glucosidases and either the endo-(1 $\rightarrow$ 4)- β -glucanase or the cellobiohydrolase. However, the degree of co-operation shown depended on the substrate as well as the particular β -glucosidase used. With cotton fibre as substrate both β -glucosidases co-operated with the cellobiohydrolase but not with the endo-(1 $\rightarrow$ 4)- β -glucanase (Table 4). With H₃PO₄-swollen cellulose, synergism was apparent with all combinations of enzyme tested, although the degree to which the enzymes co-operated varied considerably. When endo-(1 $\rightarrow$ 4)- β -glucanase was acting in admixture with either of the β -glucosidases, essentially the same amount of hydrolysis was effected. However, mixtures of cellobiohydrolase (200 μ g protein) and β -glucosidase (10 units) acted differently. In 24 h, β -glucosidase₁ (37 °C, 5 ml reaction mixture) produced a considerably greater breakdown of 10 mg H₃PO₄-swollen cellulose (approximately 30% by wt) when acting in conjunction with cellobiohydrolase than did β -glucosidase₂ (approximately 20% by wt).

Both β -glucosidases co-operated to approximately the same extent with a mixture of cellobiohydrolase and endoglucanase to solubilize cotton fibre (Table 4).

Table 4. *Synergism between the β -glucosidases of *T. koningii* and the other hydrolytic enzymes of the cellulase system in solubilizing the cellulose in cotton fibre*

β -Glucosidase₁ and β -glucosidase₂ were purified as described in the text, after separation from cellobiohydrolase and endo-(1 $\rightarrow$ 4)- β -glucanase by chromatography on concanavalin A-Sepharose. Cellobiohydrolase and endo-(1 $\rightarrow$ 4)- β -glucanase were then separated by ion-exchange chromatography on DEAE-Sepharose. The assays contained cellobiohydrolase (200 μ g), endo-(1 $\rightarrow$ 4)- β -glucanase (100 μ g) and β -glucosidases (10 units of *o*-nitrophenyl- β -glucosidase). The activity against cotton (2 mg) was determined by estimating the residual cellulose left after 7 d incubation (see text); the weight of cellulose solubilized was calculated by difference and expressed as a percentage of the initial weight.

Enzyme	Solubilization (%)
β -Glucosidase ₁	0
β -Glucosidase ₂	0
Cellobiohydrolase	1
Endo-(1 $\rightarrow$ 4)- β -glucanase	6
Endo-(1 $\rightarrow$ 4)- β -glucanase + cellobiohydrolase	53
β -Glucosidase ₁ + cellobiohydrolase	20
β -Glucosidase ₂ + cellobiohydrolase	19
β -Glucosidase ₁ + endoglucanase	6
β -Glucosidase ₂ + endoglucanase	5
β -Glucosidase ₁ + cellobiohydrolase + endoglucanase	68
β -Glucosidase ₂ + cellobiohydrolase + endoglucanase	66

DISCUSSION

β -Glucosidases appear to exist in multiple forms in many cellulase systems. In the case of *Stachybotrys atra* β -glucosidase (Jermyn, 1962) the multiplicity has been ascribed to the formation of a number of complexes between a single enzyme and a number of polysaccharides, but modification of the enzymes by proteolysis has been tentatively suggested to be the cause of the apparent heterogeneity of the β -glucosidase of *Sporotrichum pulverulentum* (Deshpande *et al.*, 1978). Neither of these explanations would seem to describe satisfactorily the reason for the apparent multiple nature of the β -glucosidase of *T. koningii*, since (a) the crude cell-free culture filtrate seemed to be free from proteolytic activity under the assay conditions used, and (b) after thorough purification the two β -glucosidase components did not seem to be associated with significant amounts of carbohydrate. However, it is still possible that one or both of the β -glucosidases are artefacts reflecting small structural changes in the protein during culture, isolation or purification. The existence of an association-dissociation phenomenon similar to that found for the β -glucosidase of *Botryodiplodia theobromae* (Umezurike, 1975), is another possibility that cannot be ruled out; in these circumstances the β -glucosidases would in effect be subunits of a large molecule.

The molecular weights of the β -glucosidases of *T. koningii* (39800) compare with values of 76000 and 47000 reported for *Trichoderma viride* β -glucosidases (Li *et al.*, 1965; Berghem & Pettersson, 1974), but are much smaller than those of *Fusarium solani* (400000; Wood, 1971), *Aspergillus wentii* (170000; Legler, 1967) and *Sporotrichum pulverulentum* (165000–182000; Deshpande *et al.*, 1978). Umezurike (1975) contends that there is a similarity in the β -glucosidases from various sources in terms of molecular weight and concludes that all β -glucosidases are aggregates of a monomer with a molecular weight of approximately 40000. Clearly, this is highly speculative, but it is noteworthy that the β -glucosidases of *T. koningii* have a molecular weight similar to the basic unit envisaged.

The whole question of the number of enzyme components synthesized by the cellulolytic fungus for efficient hydrolysis of native cellulose is currently the subject of some debate in the literature. This applies as much to the β -glucosidases as it does to the enzymes with exo-(1 $\rightarrow$ 4)- β -glucanase and endo-(1 $\rightarrow$ 4)- β -glucanase activities which are required for the conversion of insoluble cellulose to soluble sugars. Under the conditions used in this study, *T. koningii* β -glucosidase₁ and β -glucosidase₂ would appear to be more or less identical in the extent to which they co-operate with the exo-(1 $\rightarrow$ 4)- β -glucanase (cellobiohydrolase) and/or endo-(1 $\rightarrow$ 4)- β -glucanase of the same cellulase system in hydrolysing native cellulose (e.g. cotton cellulose). This has led us in the past to conclude tentatively that the β -glucosidases were isoenzymes. However, a more detailed examination of the properties of the β -glucosidases now shows that they do differ somewhat in terms of (a) their capacities for acting co-operatively with the cellobiohydrolase to solubilize H₃PO₄-swollen cellulose, and (b) their affinities for cellobiose, cellotriose, cellotetraose and cellopentaose. Clearly, since cellobiose, in particular, is a potent inhibitor of cellulase action (Halliwell & Griffin, 1973; Wood & McCrae, 1975), it is possible that under some test conditions the variation in affinity of the β -glucosidase for the dimer would be manifested in different rates of hydrolysis of cellulose when the β -glucosidase was acting in admixture with the other members of the cellulase system.

The affinities of the two β -glucosidases of *T. koningii* for cellobiose (K_m values 1.18 mM and 0.86 mM) were very similar to that displayed by the β -glucosidase of *Trichoderma viride* isolated by Berghem & Pettersson (1974) (K_m 1.5 mM) and Emert *et al.* (1974) (K_m 1.8 mM) and the β -glucosidase of *Aspergillus phoenicis* (K_m 0.76 mM) studied by Sternberg *et al.* (1977). However, *T. koningii* β -glucosidase does differ from *T. viride* β -glucosidase in that the K_m values for longer-chain oligosaccharides, such as the trimer and the tetramer, are higher than that for the dimer; this would indicate that *T. koningii* β -glucosidase has a lower affinity for these oligomers than *T. viride* β -glucosidase.

The *T. koningii* β -glucosidases are similar to the β -glucosidases of *Sporotrichum pulverulentum* (Deshpande *et al.*, 1978), *Aspergillus phoenicis* (Sternberg *et al.*, 1977), *Trichoderma viride* (Berghem & Pettersson, 1974) and *Trichoderma reesei* (Sternberg *et al.*, 1977) in that they can hydrolyse both aryl- β -glucosides and cellobiose. However, while such substrate specificity is

common, it is not universal. Thus, the aryl- β -glucosidases from *Stachybotrys atra* (Jermyn, 1962) cannot attack cellobiose. A broad substrate specificity is characteristic of β -glucosidases, however, and this property has been used to distinguish the β -glucosidases from the exo-glucanases (Reese *et al.*, 1968). Typically, the β -glucosidases of *T. koningii* have the ability to hydrolyse other β -linked dimers of glucose, albeit at widely varying rates. Sophorose and laminaribiose are better substrates than cellobiose or gentiobiose, and in this respect the β -glucosidases are similar to the β -glucosidase of *Aspergillus fumigatus* (Rudick & Elbein, 1973). The β -glucosidases of *Aspergillus phoenicis* (Sternberg *et al.*, 1977) and *Aspergillus niger* (Reese *et al.*, 1968) differ, however, in that activity on cellobiose is greatest.

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