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FRACTIONATION OF THE CELLULOLYTIC ENZYMES PRODUCED BY A SPECIES OF *Monilia*; PURIFICATION AND PROPERTIES OF AN EXTRACELLULAR β-D-GLUCOSIDASE

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ABSTRACT

Extracellular cellulolytic enzymes produced by a species of *Monilia* could be fractionated by chromatography on SP-Sephadex, Con A–Sepharose, and cellobiose–Sepharose. These methods did not separate the β-D-glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21) from the cellulases and xylanases within a single purification step. Fractionation by isoelectric focusing on a flat-bed granulated gel gave all of the β-D-glucosidase activity in a single zone isoelectric at pH 8–9. The β-D-glucosidase could be further purified to homogeneity by column isoelectric focusing at pH 8.0–10.5, and gel filtration on Biogel P-100. The purified β-D-glucosidase showed optimal activity at pH 4–5 and 50°, was isoelectric at pH 8.87, and had a molecular weight of 46,600. SDS–Polyacrylamide-gel electrophoresis demonstrated that the β-D-glucosidase was not dissociated into subunits and, hence, consisted of a single polypeptide chain. The enzyme is considered a glycoprotein, as it binds to Con A–Sepharose. The β-D-glucosidase hydrolyzed (1→2)-, (1→4)-, and (1→6)-β-D-glucosidic linkages but not cellulose. Nitrophenyl β-D-glucopyranosides and β-D-xylopyranosides were also degraded. The β-D-glucosidase was competitively inhibited by D-glucose (K_i 0.67mM).

INTRODUCTION

At least three classes of enzymes are involved in the hydrolysis of native cellulose. They include such exo-cellulases [(1→4)-β-D-glucanases] as cellobiohydrolase (EC 3.2.1.91) and glucohydrolase, several endo-cellulases (EC 3.2.1.4) of different specificities, and the cellobiases or β-D-glucosidases[1]. Together these enzymes constitute the cellulase complex and synenergistically degrade native cellulose to D-glucose[2]. The β-D-glucosidases (EC 3.2.1.21, β-D-glucoside glucohydrolases or cellobiases) specifically hydrolyze the β-linked D-glucosidic linkages of aryl and alkyl β-D-glucopyranosides, and *inter alia* such β-linked D-glucosaccharides as cellobiose and low-molecular-weight cello-oligosaccharides, but not

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cellulose[3]. The role of the β-D-glucosidases in cellulolysis is to hydrolyse cellobiose, produced by action of cellulases on cellulose, to D-glucose.

A species of the cellulolytic fungus *Monilia*, isolated from decomposing bagasse, produces[4] an inducible extracellular β-D-glucosidase and two intracellular β-D-glucosidases, one of which was shown[5] to be the nascent form of the extracellular enzyme prior to its secretion into the extracellular medium. The other intracellular β-D-glucosidase produced by *Monilia* sp. was constitutive. Highest activities of the inducible extracellular and intracellular β-D-glucosidase resulted when the fungus was grown on cellulosic substrates, such as Avicel, that were rather resistant to attack by cellulase. D-Glucose and cellobiose were also capable of inducing the formation of both extra- and intra-cellular enzymes, but only when these carbohydrates in the growth medium were nearly depleted. This finding suggested that the extracellular β-D-glucosidase of *Monilia* sp. was controlled by a repression–induction mechanism and that synthesis of enzyme became derepressed only when the level of D-glucose or cellobiose had fallen below a critical, albeit low, level.

We report here on the fractionation of the extracellular cellulases [exo- and endo-(1→4)-β-D-glucanases], (1→4)-β-D-xylanases, and β-D-glucosidases produced by *Monilia* sp. when grown on microcrystalline cellulose (Avicel), and describe the purification to homogeneity of an extracellular β-D-glucosidase and some of its properties.

EXPERIMENTAL

Materials. — A species of *Monilia* (Pers. ex Fries), tentatively identified as *Monilia sitophila* (Mont.) Sacc., was isolated from decomposing bagasse[6]. Avicel, a microcrystalline cellulose preparation, was obtained from FMC Corp., Philadelphia, U.S.A.; SP-Sephadex, Sepharose 4B, Sephadex G-100, Pharmalytes, Epoxy-activated Sepharose 6B and Con A–Sepharose 4B from Pharmacia Fine Chemicals AB, Sweden; Biogel P-100 from Bio-Rad Labs, U.S.A.; cellobiose, gentiobiose, salicin, *o*- and *p*-nitrophenyl β-D-glucopyranosides, methyl glucosides, *O*-(carboxymethyl)cellulose, larchwood xylan, and molecular-weight marker protein standards from Sigma Chemical Co., U.S.A.; sophorose monohydrate from Carl Roth, Karlsruhe, F.R.G.; Ampholine ampholytes and Ultradex gel from LKB Produkter AB, Sweden; Coomassie Brilliant Blue R-250 and molecular-weight marker protein standards for SDS–polyacrylamide gel electrophoresis from BDH Chemicals Ltd; and Tween-80 from Merck, Darmstadt, F.R.G.

Growth of fungus and preparation of culture filtrate. — The *Monilia* sp. was maintained on potato–dextrose–agar plates at 5°. Liquid cultures of the fungus were grown at 26° in a New Brunswick Magnaferm fermenter (10-L working volume) on a medium containing 1% (w/v) Avicel, 0.2% (w/v) Tween-80, and 0.075% (w/v) proteose peptone as described by Mandels and Reese[7] and Reese and Maguire[8]. The pH was not controlled during fermentation. The fermenter contents were inoculated with 1 L of 48 h-old cultures grown in shake flasks (2 L capacity

containing 750 mL of culture medium and inoculated with 7.5×10^7 spores) in a New Brunswick G-25 gyrotary shaker at 120 r.p.m. and 25°. After 8–10 days of growth, mycelia were removed by sequential filtration and the culture filtrate, or extracellular fluid, recovered. The mycelium was discarded.

Concentration of culture filtrate. — Extracellular fluid (10 L) was concentrated to ~120 mL by ultrafiltration employing a Millipore Pellicon Cassette system equipped with a single cassette filter (4,300 cm^2 filter area) having a nominal mol. wt. cut-off of 10,000. Column effluents were concentrated in an Amicon stirred ultrafiltration cell using a Diaflo PM-10 membrane.

Assay procedures. — β-D-Glucosidase activity was determined as previously described[4] by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenyl β-D-glucopyranoside when incubated at 45° for 10 min (pH 5.0). β-D-Glucosidase activity is expressed as μmol of *p*-nitrophenol released min^{-1} per mL enzyme under the conditions of assay.

O-(Carboxymethyl)cellulase (CM-cellulase) and (1→4)-β-D-xylanase activities were determined by measuring the increase in reducing end-groups arising from the hydrolysis of *O*-(carboxymethyl)cellulose (CM-cellulose) and larchwood xylan, respectively. A typical assay consisted of incubating substrate [0.5 mL of a 1% (w/v) solution in 50mM sodium acetate buffer, pH 4.5], enzyme (0.1–0.2 mL), and the same buffer in a final volume of 1.0 mL for 0.5 h at 45°. Aliquots (0.1–0.2 mL) were withdrawn and assayed for total reducing sugars by the method of Nelson[9]. D-Glucose and D-xylose were used as the respective standards. One unit of CM-cellulase and xylanase activity is defined as the amount of enzyme needed to liberate 1 μmol of reducing sugars per min.

Protein was determined by the method of Lowry *et al.*[10] as modified by Hartree[11]. Bovine serum albumin was used as the standard. In monitoring column chromatography effluents, protein was determined by measuring the absorbance at 280 nm.

D-Glucose was determined by the glucose oxidase method as described by Dekker and Richards[12].

Polyacrylamide-gel electrophoresis. — Analytical disc-gel electrophoresis was performed in a 7.5% (w/v) polyacrylamide gel according to the method of Reisfeld *et al.*[13], and Williams and Reisfeld[14]. Two different systems of polyacrylamide-gel electrophoresis were used: a cationic-buffer system (β-alanine–acetic acid, pH 4.6) to separate proteins isoelectric in the range 7–14, and an anionic system (Tris–glycine–HCl buffer, pH 8.3) for proteins isoelectric between pH 9–2. Electrophoresis was conducted at 5° with a constant current of 3.5 mA per tube. Protein was stained by Coomassie Brilliant Blue R250 and destained by diffusion.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber *et al.*[15]. The determination of mol. wt. by SDS–PAGE was computed from a calibration curve of $\log_{10}$ (mol. wt.) versus the relative electrophoretic mobility of marker standards whose mol. wts. ranged from 14,300 to 71,500.

Fractionation of the monilial cellulolytic enzymes. — (a) *Chromatography on SP-Sephadex.* Extracellular fluid (500 mL) was adjusted to pH 3.5 with acetic acid

and applied to a column of SP-Sephadex C-25 (3.5 × 8.0 cm) equilibrated with 50mM sodium acetate buffer (pH 3.5). Proteins adsorbed onto the column were displaced batchwise by elution with 50mM sodium acetate buffer (pH 6.5) containing M NaCl and the total effluent was collected.

(b) *Chromatography on Con A–Sepharose.* Extracellular fluid (0.8 mL) was applied to a column (0.6 × 5.5 cm) of Con A-Sepharose 4B equilibrated in Tris–HCl–NaCl buffer (20mM Tris, 0.5M NaCl, pH 7.0) containing mM $MgCl_2$ and mM $CaCl_2$ and washed with the same buffer to remove non-adsorbed proteins. The column was then eluted with a 0–0.5M linear gradient of methyl α-D-mannopyranoside (0.5M, 20 mL) in the same buffer to displace adsorbed proteins. Fractions of 1.33 mL were collected and assayed for CM-cellulase, xylanase, and β-D-glucosidase activity.

(c) *Affinity chromatography on cellobiose–Sepharose.* Cellobiose was coupled to epoxy-activated Sepharose 6B through an oxirane group according to the Pharmacia manual on "Affinity Chromatography". Extracellular fluid (3 mL) was adjusted to pH 4.5 with 50mM sodium acetate buffer and applied to a column (1.0 × 3.0 cm) of cellobiose–Sepharose which was equilibrated with 50mM sodium acetate buffer, pH 4.5. The column was next washed with the same buffer to remove non-adsorbed protein, followed by sequential elution with 50mM cellobiose in buffer (10 mL) and M NH_4Cl in buffer (10 mL) to displace proteins adsorbed onto the affinity matrix. Fractions of 1.2 mL were collected and assayed for CM-cellulase, xylanase, and β-D-glucosidase activities and protein (A_{280}).

Purification of β-D-glucosidase. — (a) *Preparative flat-bed and column isoelectric focusing.* Concentrated extracellular fluid (95 mL, containing 0.75–1.0 g protein) was subjected to preparative isoelectric focusing in a flat-bed granulated gel (4% w/v, Ultadex) using a Multiphor apparatus (LKB). Ampholytes in the pH range 3.5–10.0 (1%, w/v, final concentation in the gel) were used to generate the pH gradient as described in LKB Application Note 198. A constant power of 6W was applied for 16–18 h at 5°. β-D-Glucosidase activity was located in the gel after electrofocusing by means of a zymogram method employing *p*-nitrophenyl β-D-glucopyranoside as described by Deshpande *et al.*[16]. Enzyme activity was revealed by the presence of a yellow band (*p*-nitrophenol) on the zymogram indicating the location of β-D-glucosidase. Following electrofocusing, the zone on the gel was excised and eluted with water. The β-D-glucosidase fraction collected was referred to as EG-1.

Without any further treatment, EG-1 was then electrofocused within the pH range 8.0–10.5 in a preparative column (LKB 8101, 110 mL capacity) using D-glucitol as the density gradient. The final ampholyte concentration in the density gradient was 1% (w/v). Electrofocusing was carried out at a constant power of 15W (1.6 kV) for 18 h at 5°. The column was emptied from the bottom at the rate of 1 mL min^{-1} and fractions (2.5 mL) were collected. Each fraction was assayed for protein (A_{280}), β-D-glucosidase activity, and pH (measured at 5°).

(b) *Molecular exclusion chromatography.* An aliquot of a concentrated

solution of β-D-glucosidase fraction (5 mL, EG-1B from column electrofocusing) was applied to a column of Biogel P-100 (2.5 × 90.0 cm) equilibrated with 50mM potassium phosphate buffer (pH 6.6) and eluted with the same buffer at a flow rate of 1.9 $mL.h^{-1}$. Fractions (3.5 mL) were collected and assayed for protein (A_{280}) and β-D-glucosidase activity.

The Biogel P-100 column was calibrated for molecular-weight determination by the method of Fischer[17] using a mixture of mol. wt. calibration protein markers (bovine serum albumin, ovalbumin, pepsin, trypsinogen, and lyzozyme).

RESULTS

Fractionation of cellulase, (1→4)-β-D-xylanase, and β-D-glucosidase. — Enzymes constituting the cellulase complex of *Monilia* sp. were produced in relatively low concentrations in the extracellular fluid. We therefore investigated ways whereby these enzymes could be recovered from the extracellular fluid, subsequently fractionated by techniques that were relatively simple and fast. Enzyme concentration by ammonium sulphate precipitation followed by molecular-exclusion chromatography on Sephadex G-100 did not achieve any useful separation of the enzymes. Ion-exchange chromatography on SP-Sephadex at pH 3.5 caused all of the cellulolytic enzymes, and the (1→4)-β-D-xylanase, to bind to the matrix. This step separated out the non-cellulolytic enzymes, which did not bind, and also allowed concentration of the cellulolytic enzymes and xylanases by their adsorption onto the ion-exchange support.

The CM-cellulases and xylanases could readily be displaced from the SP-Sephadex matrix, with recoveries of >90% of that applied, by elution of the gel with 50mM sodium acetate buffer (pH 6.5) containing M NaCl. The β-D-glucosidases, however, were bound firmly to the cation exchanger and only 25% of that applied could be recovered by elution with the foregoing buffer. Elution of SP-Sephadex with 100mM sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5) followed by 100mM sodium tetraborate buffer (pH 11.0) containing 3M KCl (buffers used to regenerate this type of ion-exchanger) still left bound enzyme that showed enzyme activity towards *p*-nitrophenyl β-D-glucopyranoside. It therefore appears that the immobilized form of β-D-glucosidase was extremely stable to alkaline pH.

As monilial β-D-glucosidases were bound strongly to SP-Sephadex at pH 3.5, but only poorly at pH 4.0, SP-Sephadex could not be used for the succesful fractionation of β-D-glucosidase. Many cellulolytic enzymes are glycoproteins that bind specifically to the lectin concanavalin A if their carbohydrate component contains α-D-mannopyranosyl and α-D-glucopyranosyl residues. In this respect, Con A–Sepharose has been used in fractionation procedures for purifying cellulases produced by *Sporotrichum pulverulentum*[18]. CM-cellulases, xylanases, and β-D-glucosidases produced by *Monilia* sp. could also be fractionated on Con A–Sepharose. Two classes of CM-cellulases and xylanases appeared to be present in

the extracellular fluid. Those bound to the lectin are glycoproteins, whereas those not bound are considered to be nonglycosylated proteins, or glycoproteins containing carbohydrate moieties not recognized by the lectin. More than 90% of the β-D-glucosidase activity bound to Con A–Sepharose could be displaced by methyl α-D-mannopyranoside, indicating that the extracellular β-D-glucosidases produced by *Monilia* sp. were glycoproteins. Polyacrylamide-gel electrophoresis (anionic system) of the eluted β-D-glucosidase fraction showed the presence of at least 6 protein-staining bands. As no useful separation of the β-D-glucosidase component from CM-cellulase and xylanase occurred using Con A–Sepharose chromatography, we tried an alternative affinity-binding chromatography approach. The ligand, cellobiose in this case, was covalently coupled through a 1,4-bisoxirane group to Sepharose. The elution profile for the fractionation of CM-cellulase and β-D-glucosidase on the cellobiose-ligand matrix is shown in Fig. 1. Bound enzyme could not be displaced by cellobiose, but could be eluted from the column by M NH_4Cl. The activity of CM-cellulase and xylanase in the fraction not bound to the affinity support was increased relative to that in the sample applied. The increase in enzyme activity may have resulted from the removal of a dissociable inhibitor (e.g., a low mol. wt. cellooligosaccharide) which was displaced by the immobilized cellobiose on the affinity matrix. The cellobiose ligand, however, was susceptible to hydrolysis by the β-D-glucosidase, especially at high enzyme loadings, and this precluded its use for large-scale fractionation. Other ligands, such as D-glucose and salicin [2-(hydroxymethyl)phenyl β-D-glucoside] were also coupled to epoxy-activated Sepharose, but proved unsuccessful in that they showed no affinity for β-D-glucosidase or CM-cellulase.

We next tried preparative isoelectric focusing on a flat-bed granulated gel; relatively large sample volumes and high protein loadings may be applied in this type of fractionation procedure. A typical profile for the fractionation of monilial

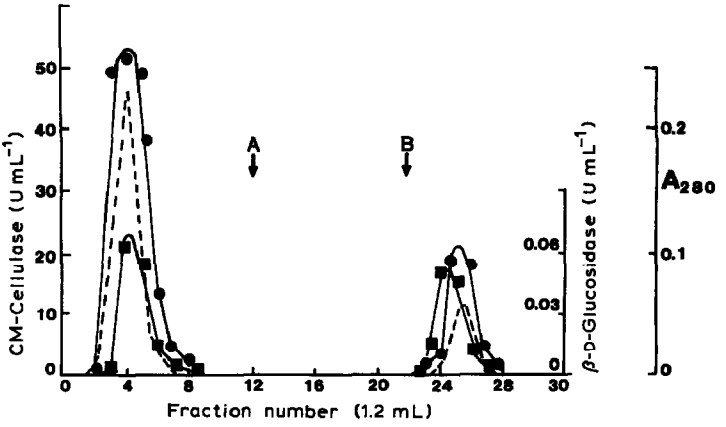

Fig. 1. Elution profile for the fractionation of CM-cellulases and β-D-glucosidases on cellobiose–Sepharose. The column was eluted with 50mM cellobiose (A), and M NH_4Cl (B). Fractions (1.2 mL) were collected and assayed for CM-cellulase activity (●), β-D-glucosidase activity (■), and A_{280} (——).

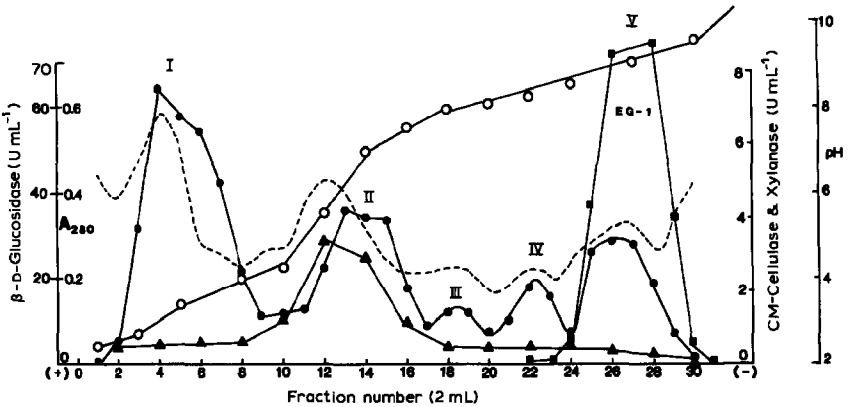

Fig. 2. Fractionation of *Monilia* sp. cellulolytic enzymes by preparative isoelectric focusing in a flat-bed granulated gel. Following isoelectric focusing of the extracellular fluid at pH 3.5–10.0, the flat-bed was divided into 1-cm wide segments and the gel excised and eluted with water (2 mL). Each fraction was assayed: ●, CM-cellulase activity; ■, β-D-glucosidase activity; ▲, (1→4)-β-D-xylanase activity; ---, A_{280} (protein content); and O, pH.

cellulolytic enzymes by flat-bed isoelectrofocusing within a pH 3.5–10.0 range is shown in Fig. 2. Five fractions showing CM-cellulase activity, one with xylanase activity, and one with β-D-glucosidase activity resulted. CM-cellulase fractions I, II, and V rapidly decreased the viscosity of a 1% (w/v) solution of *O*-(carboxymethyl)cellulose and are considered to be typical endo-cellulases, whereas fractions III and IV did not decrease the viscosity of *O*-(carboxymethyl)cellulose and appear to be cellulases of the exo-type. The apparent pI values of fractions I, II, III, IV, and V were 4.5, 6.25, 6.80, 7.75, and 9.0, respectively. All of the β-D-glucosidase activity applied resided in a single fraction, i.e. fraction V.

Preparative flat-bed isoelectric focusing routinely demonstrated that the bulk of the extracellular proteins secreted by *Monilia* sp. could be separated from β-D-glucosidase. This fractionation procedure was therefore selected as the first step in the purification of an extracellular β-D-glucosidase.

Purification of β-D-glucosidase. — The β-D-glucosidase fraction (EG-1) obtained upon electrofocusing in a flat-bed granulated gel at the pH 3.5–10.0, was further resolved into 2 fractions of β-D-glucosidase activity (EG-1A and EG-1B) by electrofocusing in a column within the pH range 8.0–10.5 (Fig. 3). Analysis of both fractions by polyacrylamide-gel electrophoresis (PAGE, cationic system) and SDS–PAGE revealed the presence of 4 and 5 protein-staining bands, respectively. The major β-D-glucosidase fraction (EG-1B) was fractionated further by molecular-exclusion chromatography on Biogel P-100. SDS–PAGE of the resultant β-D-glucosidase component (EG-1B-1) revealed the presence of a major and one minor protein-staining band. Rechromatography of EG-1B-1 on Biogel P-100, under identical conditions, yielded a single symmetrical peak of β-D-glucosidase activity. Both SDS- and analytical disc-polyacrylamide gel electrophoresis (cationic and anionic systems) of this component revealed the presence of a single protein band.

TABLE I

PURIFICATION OF β-D-GLUCOSIDASE FROM THE EXTRACELLULAR FLUID OF *Monilia* SP.

Purification step (fraction)	*Specific activity (IU/mg)*[a]	*Fold-purification*
Extracellular fluid	0.17	1.0
Preparative flat-bed gel isoelectric focusing (EG-1)	0.23	1.4
Column isoelectric focusing (EG-1B)	1.13	6.8
Chromatography on Biogel P-100 (EG-1B-1)	1.61	9.7
Re-chromatography on Biogel P-100 (Purified enzyme)	3.58	21.6

[a]Units of β-D-glucosidase/mg protein.

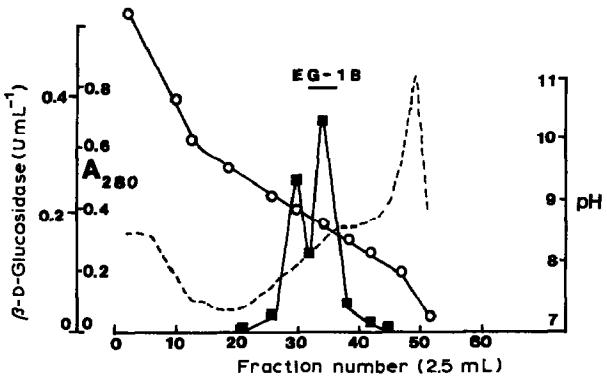

Fig. 3. Purification of β-D-glucosidase by column isoelectric focusing. β-D-glucosidase (Fraction V, Fig. 2) was electrofocused at pH 8.0–10.5 as described in the text, and fractions (2.5 mL) were collected and assayed: ■, β-D-glucosidase activity; ——, A_{280} (protein content); and O, pH.

This β-D-glucosidase component was thus considered to be homogeneous. Table I summarises the purification scheme, in which a net 21.6-fold purification was achieved.

Physicochemical properties. — The molecular weight of the purified enzyme as estimated by gel filtration (Biogel P-100) was computed from the expression: $\log_{10}$ (mol. wt.) = -1.0649 (K_{av}) + 4.805 (K_{av} was 0.1304), and was calculated to be 46,500. The relative mobility of β-D-glucosidase on SDS–PAGE indicated a mol. wt. of 46,700. As the mol. wt. of the purified enzyme determined by gel-filtation chromatography and SDS–PAGE were very similar, it was concluded that the *Monilia* sp. β-D-glucosidase consisted of a single polypeptide chain.

The dependence of β-D-glucosidase activity upon temperature and pH was found to be optimal at 50° and between pH 4–5, respectively. The pI of the purified β-D-glucosidase was 8.87.

The substrate specificity of β-D-glucosidase is shown in Table II. The enzyme

TABLE II

HYDROLYSIS OF DIFFERENT D-GLUCOSIDES BY THE β-D-GLUCOSIDASE PRODUCED BY *Monilia* SP.

Substrate [linkage]	*Specific activity (U/mg)*	*Relative activity (%)*
p-Nitrophenyl β-D-glucoside	3.58	100
o-Nitrophenyl β-D-xyloside	0.21	5.7
Cellobiose [β-(1→4)]	0.65	18.3
Sophorose [β-(1→2)]	0.47	13.2
Gentiobiose [β-(1→6)]	0.85	23.8
Maltose [α-(1→4)]	0	0
Methyl β-D-glucoside	0.78	21.9
Methyl α-D-glucoside	0	0
Salicin	0.03	0.9

The mixture contained substrate (0.9 mL of a 2mM solution of each glucoside made up in 50mM sodium acetate buffer, pH 5.0) and enzyme (0.1 mL) and was incubated for 10 min at 50°. The activity of enzyme against the nitrophenyl glucosides was determined by measuring the amount of nitrophenol liberated, whereas the activity towards the other glucosides was estimated by measuring the amount of D-glucose liberated by the glucose oxidase method.

was active towards only β-D-glucosidic bonds; CM-cellulose was not degraded. The purified enzyme preparation also showed some activity towards *o*-nitrophenyl β-D-xyloside, but this was <6% of that towards *p*-nitrophenyl β-D-glucopyranoside. The purified enzyme was not specific exclusively for (1→4)-β-D-glucosidic bonds, but could also hydrolyze (1→2)- and (1→6)-β-D-glucosidic linkages.

The K_m value of β-D-glucosidase was computed from the Lineweaver–Burk plot as 0.075mM and 5.7mM for *p*-nitrophenyl β-D-glucopyranoside and cellobiose, respectively. D-Glucose and D-glucono-1,5-lactone strongly inhibited monilial β-D-glucosidase. The inhibition by D-glucose using with *p*-nitrophenyl β-D-glucopyranoside as substrate was competitive and the K_i value calculated from a Dixon plot was 0.67mM.

DISCUSSION

Procedures for the fractionation of enzymes constituting the cellulase complex are rather complicated and involve many purification steps. It was advantageous therefore to employ fractionation methods which were relatively simple and fast, and which would allow the separation of enzyme components or their partial purification within a few steps. Affinity-binding chromatography has this potential and has decreased the number of fractionation steps required to achieve homogeneity. For example, cellulases have been purified on cross-linked cellulose[19], and also on an immunoadsorbent using an antiserum ligand prepared by immunization with a purified endo-cellulase[20]. β-D-Glucosidases have been separated from cellulases on Con A–Sepharose[2,21].

Employment of Con A–Sepharose affinity chromatography as the first step in

the separation of monilial cellulolytic enzymes did not achieve any real advantage, as the fungus produced enzymes that are both glycosylated and nonglycosylated. This fractionation step would be more useful in the later stages of a purification procedure (see, e.g. ref. 18). Likewise unsuccessful was the use of a cellobiose ligand for attempted affinity separation of the β-D-glucosidase (or an exo-cellulase) from the other enzymes of the cellulase complex. Furthermore, the ligand was susceptible to hydrolysis by the β-D-glucosidase itself, and hence the affinity matrix could not be employed more than once. A more-suitable ligand might be a thio-cellobiose, e.g., the substrate analogue, 4-*S*-(β-D-glucopyranosyl)-4-thio-D-gluco-pyranose, or the 1-thiocellobiosides and -glucosides that are known to be strong inhibitors of β-D-glucosidases[22]. In this respect, the 4-thiocellobioside could be a suitable ligand, as this compound is reported not to be hydrolyzed by a crude cellulolytic enzyme-preparation of *Schizophyllum commune*[23].

The merits of employing preparative flat-bed isoelectric focusing within a granulated gel have been already discussed. β-D-Glucosidases of *Monilia* sp. could be separated rather conveniently from the bulk of the CM-cellulases and xylanases by electrofocusing. As a consequence, all β-D-glucosidase activity (EG-1) resided in a single zone isoelectric at pH 8–9. Although some CM-cellulase was present in the β-D-glucosidase fraction, this could be removed relatively easily in a further purification step employing Biogel P-100.

Multiple forms of β-D-glucosidases commonly exist in many cellulolytic fungi. For example, *Sporotrichum pulverulentum,* produces up to five extracellular β-D-glucosidase components[18]; *Sclerotium rolfsii,* four[24]; *Trichoderma koningii,* two[2]; while *Sporotrichum thermophile* produced two intracellular β-D-glucosidases[25]. *Monilia* sp. was therefore no exception, and produced two extra- and one intra-cellular β-D-glucosidase.

The mol. wt. of the *Monilia* sp. β-D-glucosidase (46,600) was similar to that of the extracellular β-D-glucosidases[2] from *T. koningii* (MW 39,800) and[26] *Clostridium thermocellum* (MW 43,000), and the intracellular β-D-glucosidase[25] of *S. thermophile* (MW 40,000). SDS–polyacrylamide gel electrophoresis demonstrated that the *Monilia* sp. β-D-glucosidase was not dissociated into sub-units, and hence consisted of a single polypeptide chain. Similar observations have also been reported for other β-D-glucosidases, such as those of *S. rolfsii*[24].

Several β-D-glucosidases have been demonstrated to be glycoproteins and were shown to contain mannose, glucose, and glucosamine residues[27,28]. As extra-cellular *Monilia* β-D-glucosidases were capable of binding to Con A–Sepharose, they too were considered to be glycoproteins. Likewise, β-D-glucosidases that were not recognized by concanavalin A have also been reported, e.g. *T. koningii*[2] and *T. viride*[21].

The specificity of the monilial β-D-glucosidase towards β-D-glucosides containing (1→2)-, (1→4)-, and (1→6)-linkages was similar to those observed from β-D-glucosidases of fungal origin, as from *T. koningii*[2], *S. thermophile*[25], and *Geotrichum candidum*[27]. The monilial β-D-glucosidase, like those of most other

cellulolytic fungi, was capable of hydrolyzing both cellobiose and aryl β-D-glucosides[3]. An exception was an intracellular β-D-glucosidase produced by *S. thermophile* (enzyme A, mol. wt. 400,000) which, although degrading *p*-nitrophenyl β-D-glucopyranoside, was not capable of hydrolyzing cellobiose[25]. Several fungal β-D-glucosidases, including that of *Monilia* sp., were also capable of hydrolyzing nitrophenyl β-D-xyloside[22,29], indicating that the absence of a substituent on C-5 did not hinder enzyme binding and the hydrolysis of this glycoside.

Many fungal β-D-glucosidases are inhibited strongly by D-glucose, the end-product of cellobiose (and cellulose) hydrolyis, and in this respect the *Monilia* sp. enzyme was similar. This type of inhibition was usually competitive with the substrate[3].

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