Induction, Localization and Characterization of β-D-Glucosidases Produced by a Species of *Monilia*

BY ROBERT F. H. DEKKER†

Department of Biochemistry, University of Natal, Pietermaritzburg, Republic of South Africa

(Received 2 October 1980; revised 3 March 1981)

A species of the imperfect fungus *Monilia* produced extracellular, intracellular and mycelial-bound β-glucosidases when grown on cellulose. The extracellular enzyme was induced on cellulose but was repressed by cellobiose and glucose. Derepression of this enzyme occurred when cellobiose and glucose were nearly exhausted from the growth medium. Intracellular and mycelial-bound β-glucosidases were induced on cellulose and cellobiose but appeared to be repressed on glucose and lactose. One extracellular (EG-1) and two intracellular (IG-1, IG-2) β-glucosidases were produced when *Monilia* sp. was grown on cellulose. The molecular weights of EG-1 and IG-2 were 37500 and that of IG-1 was 480000. The $K_m$ of EG-1 and IG-2 was $8 \times 10^{-3}$ M for $p$-nitrophenyl-β-D-glucoside (PNPG), and $5-7 \times 10^{-3}$ M for cellobiose; the $K_i$ for glucose, a competitive inhibitor, was $6-7 \times 10^{-4}$ M. The $K_m$ of IG-1 was $1-67 \times 10^{-3}$ M for PNPG and $2-0 \times 10^{-2}$ M for cellobiose; the $K_i$ for glucose, a non-competitive inhibitor, was $1-63 \times 10^{-3}$ M. β-Glucosidases EG-1 and IG-2 showed optimal activity at pH 4.5 whilst the pH optimum for IG-1 was 4.2. Temperature optima were 50 °C for EG-1 and IG-2, and 60–65 °C for IG-1. Their respective isoelectric points were 8.3, 8.3 and 4.4.

INTRODUCTION

At least three classes of enzymes are involved in the complete degradation of cellulose. These include several endo-cellulases of different specificity, the exo-cellulases (i.e. the cellobiohydrolases) and the β-D-glucosidases, or cellobiases, (Dekker & Lindner, 1979; Eriksson, 1978; Pettersson, 1975; Wood & McCrae, 1977). Together these enzymes constitute the 'cellulase complex' and synergistically degrade native cellulose to glucose. β-D-Glucosidases (β-D-glucoside glucohydrolases; EC 3.2.1.21) hydrolyse the end-products of cellulase attack on cellulose, i.e. cellobiose and cello-oligosaccharides, to glucose and also help alleviate the inhibition of cellulase by cellobiose. β-Glucosidases therefore play an important role in the complete saccharification of cellulose.

In addition to extracellular β-glucosidases, intracellular (Eberhart & Beck, 1970, 1973; Hägerdal et al., 1978; Kwang & Suzuki, 1976; O'Day & Paterno, 1979; Smith & Gold, 1979; Wilson & Niederpruem, 1967) and cell- or mycelial-bound (Berg & Pettersson, 1977; Deshpande et al., 1978; Eberhart & Beck, 1970; Kwang & Suzuki, 1976; Lusis & Becker, 1973; Smith & Gold, 1979) β-glucosidases have been found in micro-organisms. Intracellular β-glucosidases have been implicated in the germination of microcysts of *Polysphondylium pallidum* (O'Day & Paterno, 1979) and basidiospores of *Schizophyllum commune* (Wilson & Niederpruem, 1967), and, together with the extracellular enzyme and possibly the mycelial-bound enzyme, appear also to be involved in cellulose degradation.

† Present address: CSR Research Laboratories, 28 Barcoo St, Roseville, Sydney, N.S.W. 2069, Australia.
Recently researchers in this laboratory isolated from decomposing bagasse a cellulolytic Monilia species, tentatively identified as Monilia sitophila (Mont.) Sacc. (Dekker, 1980). Because of its strong cellulolytic activity and potential for bagasse bioconversion, this organism was chosen for studies on cellulases and xylanases. The present paper examines the induction, localization and characterization of β-D-glucosidases elaborated by this Monilia sp.

METHODS

Organism and culture medium. A species of Monilia (Pers. ex Fries), tentatively identified as Monilia sitophila (Mont.) Sacc., was isolated from decomposing bagasse obtained from a site at a sugar mill (Dekker, 1980). The organism was grown in submerged culture in nutrient medium containing 1% (w/v) carbon source, 0-20% (w/v) Tween 80 and 0-075% (w/v) proteose peptone as described by Mandels & Reese (1957) and Reese & Maguire (1971). Cultures were grown in shake-flasks (250 ml and 2-5 l capacity containing 75 ml and 750 ml nutrient medium, respectively) inoculated with 1-5 x 10⁹ spores and grown at 28 °C in a New Brunswick G-25 shaker operating at 120 rev. min⁻¹. Cell-free culture fluid containing the extracellular enzyme was obtained by centrifugation (20000 g, 0-5 h). In small scale experiments (75 ml medium), extracellular, intracellular and mycelial bound enzymes were isolated as described by Dekker (1980).

Biochemicals. Sophorose monohydrate was obtained from Carl Roth, Karlsruhe, West Germany. Xylan (larchwood), galactomannan (locust bean), carboxymethylcellulose (low viscosity), peroxidase (horseradish), glucose oxidase (Aspergillus niger), o-dianisidine dihydrochloride, glucono-1,5-lactone, cellobiose and p-nitrophenyl-β-D-glucopyranoside were purchased from Sigma. Avicel was supplied by Merck. Amorphous cellulose was prepared according to Walseth (1952).

Assays. Soluble and mycelial protein concentrations were determined by the Lowry method as modified by Hartree (1972) and Sandermann & Strominger (1972), respectively, using bovine serum albumin as the standard. In monitoring column chromatography effluents, protein was determined by measuring the absorbance at 280 nm.

β-D-Glucosidase activity was assayed by measuring the amount of p-nitrophenol liberated from p-nitrophenyl-β-D-glucoside (PNPG) as substrate. In a typical assay, PNPG (0-9 ml, 2 mm in 50 mm-sodium acetate buffer, pH 4-5) and enzyme (0-1 ml) were incubated together at 45 °C for 10 min. The reaction was stopped by adding 0-25 M-Na₂CO₃ (4 ml) and the amount of p-nitrophenol produced was measured spectrophotometrically at 401 nm. One unit (U) of β-D-glucosidase activity is defined as the amount liberating 1 pmol p-nitrophenol min⁻¹ under the conditions of assay. Results are expressed as mU (ml enzyme)⁻¹ or mU (mg protein)⁻¹. In assaying for mycelial-bound β-glucosidase activity, disrupted mycelial suspension (0-1 ml) prepared as described by Dekker (1980) was used. The reaction was terminated by adding 0-25 M-Na₂CO₃ (4 ml) and the mixture was centrifuged prior to measuring the absorbance at 401 nm.

Cellobiase activity was determined with cellobiose as substrate by measuring the amount of glucose produced using the glucose oxidase method (Dekker & Richards, 1971). The reaction mixture contained cellobiose (0-1 ml, 10 mm in water), 50 mm-sodium acetate buffer, pH 4-5 (0-7 ml) and enzyme preparation (0-2 ml). After 10 min incubation at 45 °C, a freshly made solution of glucono-1,5-lactone (0-05 ml, 20 mm in 150 mm-sodium citrate/sodium phosphate buffer, pH 6-6) was added which totally inhibited cellobiase. To this mixture was immediately added glucose oxidase reagent (2.0 ml of a solution containing glucose oxidase (3 units), peroxidase (6 units) and o-dianisidine dihydrochloride (0.2 mg) in 500 mm-Tris/HCl/glycerol buffer, pH 7-0) followed by incubation for a further 30 min at 45 °C. The reaction was terminated by adding 5 m-HCl (4 ml) and the absorbance was measured at 540 nm.

Total carbohydrate was determined by the phenol/sulphuric acid method of Dubois et al. (1956).

Gel permeation chromatography. Extracellular or intracellular fluid was concentrated 20-fold by ultrafiltration using a Diaflo PM-10 membrane (Amicon Corp., Lexington, Mass., U.S.A.). The retentate (approx. 7 ml) was applied to a column (2-6 x 60 cm) of Sepharose 4B equilibrated in 150 mm-citrate/phosphate buffer (pH 6-6) and eluted with the same buffer. Fractions of 3 ml were collected and assayed for β-glucosidase and cellobiase. Those fractions containing enzyme activity were combined, concentrated by ultrafiltration and applied to a Biogel P-100 column (2-6 x 94-5 cm) equilibrated in 50 mm-potassium phosphate buffer (pH 7-0). Both columns were calibrated with molecular weight marker proteins as described by Andrews (1970).

Polyacrylamide gel electrophoresis and analytical isoelectric focusing. Slab electrophoresis and analytical isoelectric focusing were performed in polyacrylamide gels using a Multiphor unit (LKB-Produkter) as described in LKB Application Notes 250 and 306. Commercial LKB polyacrylamide gel Ampholine plates of pH 3-5-9-5 were used in analytical isoelectric focusing. Following electrophoresis and isoelectric focusing, β-glucosidase activity was detected by a zymogram method. This consisted of soaking the gel in 4 mm-PNPG (50 mm-acetate buffer, pH 4-5) for 10-15 min at room temperature, or 37 °C, followed by spraying of the gel with 0-25 m-Na₂CO₃.
**RESULTS**

*Induction of extracellular β-glucosidases*

The extracellular β-glucosidase elaborated by *Monilia* sp. appeared to be an inducible enzyme. Activities were highest when the organism was cultured on cotton, Avicel and filter paper, all cellulosic substrates that are rather resistant to attack by exo- and endo-cellulases (Fig. 1, Table 1). The time course of formation of extracellular β-glucosidase by *Monilia* sp. grown on several cellulosic substrates or bagasse is shown in Fig. 1. With the exception of carboxymethylcellulose, maximum enzyme production resulted after 5 d growth, with cotton being the best inducer. Despite its high content of lignin (20–30%), bagasse, which consists of 40–60% cellulose (Dekker & Lindner, 1979), also caused the production of high extracellular β-glucosidase activity. Carboxymethylcellulose and amorphous phosphoric acid-swollen cellulose, although more easily degraded by cellulases, resulted in lower extracellular β-glucosidase activity (Fig. 1, Table 1). On the other hand, when the hemicelluloses xylan and galactomannan were used as growth substrates, rather low extracellular β-glucosidase activities resulted compared with the cellulosic substrates. Addition of phytone to the Avicel-containing culture medium resulted in significantly higher production of extracellular β-glucosidase than did proteose peptone which was used as a control. There was no significant increase in extracellular β-glucosidase activity when the surfactant Tween 80 was added. Only low extracellular β-glucosidase activities were induced when *Monilia* sp. was grown for 6 d on d-glucose, glycerol, cellobiose, lactose or sophorose (Table 1). In a separate experiment, during growth on either 0.5% cellobiose or d-glucose, extracellular β-glucosidase activity did not appear until the carbohydrate concentration in the medium had fallen to 0.5 mg ml⁻¹, i.e. 0.05% (w/v) (Fig. 2). Under these conditions maximum enzyme activity occurred after 10–12 d at which time activities were comparable with those obtained after 4–5 d growth on insoluble cellulosates.

These results indicate that at high concentrations cellobiose and glucose repressed enzyme synthesis, and that repression was relieved only when these carbohydrates were decreased to very low concentrations in the growth medium. This was confirmed in another experiment in which *Monilia* sp. was cultured on low carbohydrate media. Under these conditions enzyme synthesis was not repressed (Fig. 3) and extracellular enzyme activity was detectable 1 d after inoculation.

*Localization of the β-glucosidases*

*Monilia* sp. produced β-glucosidases which were located in the extracellular medium in which the fungus was grown, intracellularly, and in a form bound to the mycelium. The induction of each of these enzymes on different growth substrates is shown in Table 1. Growth on cotton, bagasse, amorphous cellulose and carboxymethylcellulose resulted in the production of highest activities of the intracellular enzyme. Growth on cellobiose or sophorose resulted in greater intracellular activity than did growth on gentiobiose, lactose, glycerol or glucose (Table 1). Although the amounts of enzyme were lower than when growth was on cellulose (Avicel being an exception), they were nevertheless significantly higher than the corresponding extracellular β-glucosidase activities. It appears that both glucose and lactose repressed the formation of intracellular β-glucosidases whereas cellobiose did not.

Appreciable mycelial-bound β-glucosidase was also produced when *Monilia* sp. was grown on most of the substrates tested, but as with the intracellular enzyme, accumulation was lower for growth on glucose and lactose. Carboxymethylcellulose and amorphous cellulose were the best substrates for the synthesis of mycelial-bound β-glucosidases (Table 1).
Fig. 1. Time course of formation of extracellular β-glucosidase by Monilia sp. grown in nutrient medium containing various cellulosic carbon sources (each at 1\% (w/v): cotton (●), Avicel (○), filter paper (□), bagasse (■), carboxymethylcellulose (▲).

Fig. 2. Production of extracellular β-glucosidase by Monilia sp. grown in nutrient medium containing high concentrations of glucose and cellobiose: 0.5\% (w/v) glucose (●), 0.5\% (w/v) cellobiose (○). Concentrations of carbohydrates remaining in the medium: glucose (□), cellobiose (■).

Table 1. Distribution and activity of β-glucosidases in Monilia sp. after 6 d growth on various carbon sources

<table>
<thead>
<tr>
<th>Carbon source*</th>
<th>Extracellular β-Glucosidase activity [mU ml(^{-1})](\dagger)</th>
<th>Intracellular β-Glucosidase activity [mU (mg protein(^{-1}))]</th>
<th>Mycelial β-Glucosidase activity [mU (mg protein(^{-1}))]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>159</td>
</tr>
<tr>
<td>Cotton</td>
<td>528 (1200)</td>
<td>360</td>
<td>ND</td>
</tr>
<tr>
<td>Bagasse</td>
<td>272 (555)</td>
<td>235</td>
<td>556</td>
</tr>
<tr>
<td>Avicel + phytone</td>
<td>244 (841)</td>
<td>116</td>
<td>465</td>
</tr>
<tr>
<td>Avicel</td>
<td>172 (491)</td>
<td>125</td>
<td>522</td>
</tr>
<tr>
<td>Amorphous cellulose</td>
<td>144 (620)</td>
<td>263</td>
<td>1044</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>84 (323)</td>
<td>230</td>
<td>1010</td>
</tr>
<tr>
<td>Xylan</td>
<td>52 (106)</td>
<td>24</td>
<td>520</td>
</tr>
<tr>
<td>Galactomannan</td>
<td>40 (85)</td>
<td>73</td>
<td>440</td>
</tr>
<tr>
<td>Cellulbiose</td>
<td>30 (88)</td>
<td>140</td>
<td>516</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>6 (14)</td>
<td>54</td>
<td>200</td>
</tr>
<tr>
<td>Sophorose</td>
<td>6 (21)</td>
<td>117</td>
<td>254</td>
</tr>
<tr>
<td>Glucose</td>
<td>6 (21)</td>
<td>12</td>
<td>91</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4 (12)</td>
<td>54</td>
<td>278</td>
</tr>
<tr>
<td>Lactose</td>
<td>3 (912)</td>
<td>11</td>
<td>92</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Carbon sources were present in the nutrient medium at 1\% (w/v) except for carboxymethylcellulose, cellobiose, glucose and glycerol which were present at 0.5\% (w/v), and gentiobiose and sophorose which were at 0.1\% (w/v).

\(\dagger\) Values in parentheses are specific activities [mU (mg protein\(^{-1}\)].
**β-D-Glucosidases of Monilia sp.**

![Graph](image.png)

Fig. 3. Production of extracellular β-glucosidase by Monilia sp. grown in nutrient medium containing low concentrations of glucose and cellobiose: 0-1% (w/v) glucose (○), 0.05% (w/v) glucose (□), 0-05% (w/v) cellobiose (●).

![Graph](image.png)

Fig. 4. Elution profile of Monilia sp. extracellular β-glucosidase on Sepharose 4B: absorbance at 280 nm (---), β-glucosidase activity (-- -- --), cellobiose activity (-----).

The β-glucosidase present in the mycelial enzyme fraction is probably a cell wall-bound enzyme, as less than 3% of the total activity was solubilized by Triton X-100 treatment of mycelium, thus ruling out the existence of membrane-bound β-glucosidases. The possibility that some β-glucosidase is physically adsorbed on to residual cellulose in the mycelial fraction cannot be excluded as some of these enzymes are known to adsorb on to insoluble cellulose (W. A. Lindner & R. F. H. Dekker, unpublished results).

**Fractionation of the β-glucosidases**

Concentrated cell-free culture fluid from a 7 d old Avicel-grown culture was fractionated by chromatography on Sepharose 4B. The elution profile (Fig. 4) showed only one peak (EG-1) of β-glucosidase activity corresponding to a molecular weight of 37 500. Chromatography on Biogel P-100 failed to resolve this enzyme further into other peaks of β-glucosidase activity. Similarly, polyacrylamide gel electrophoresis at pH 4.5 and 8.0, and analytical isoelectric focusing (pH 3.5–9.5) followed by the highly specific zymogram procedure for β-glucosidases failed to detect more than one extracellular β-glucosidase component.

When concentrated intracellular fluid (i.e. the cytosol fraction obtained after the maceration of mycelium) was applied to a column of Sepharose 4B, two peaks of β-glucosidase activity were obtained (Fig. 5). The component which eluted first (IG-1) had a molecular weight of 480 000 while that of the second component (IG-2) was 37 500. Subsequent chromatography on a Biogel P-100 column failed to resolve further either of these
enzymes. Neither polyacrylamide gel electrophoresis at pH 4.5 and 8.0, nor analytical isoelectric focusing (pH 3.5–9.5) demonstrated subcomponents within either IG-1 or IG-2.

Attempts to fractionate a mixture of EG-1 and IG-2 at equivalent activities by chromatography on Biogel P-100, polyacrylamide gel electrophoresis and analytical isoelectric focusing in polyacrylamide gels were not successful. It is tentatively concluded that β-glucosidases EG-1 and IG-2 are the same enzyme. Furthermore, it is thought that IG-2 is the intracellular form (i.e. nascent enzyme) of EG-1 prior to its secretion into the extracellular medium.

**Characterization of the extra- and intracellular β-glucosidases**

The physicochemical properties of the different β-glucosidases produced by *Monilia* sp. are shown in Table 2. The results are consistent with the above finding that β-glucosidases EG-1 and IG-2 are the same enzyme. All enzymes showed optimal activity at acid pH values (pH 4.2–4.5) and relatively high temperatures (50–60 °C, 10 min). β-Glucosidase EG-1/IG-2 also differed from IG-1 with respect to its affinity (i.e. the Michaelis constant $K_m$) for the substrates PNPG and cellobiose. Furthermore, EG-1/IG-2 hydrolysed cellobiose 3.5 times faster than PNPG: maximum velocities ($V_m$) for cellobiose and PNPG were 5.75 and 1.68 μmol min$^{-1}$ (mg protein)$^{-1}$, respectively.

EG-1 and IG-1 also displayed different inhibition characteristics as evidenced by their Dixon plots (data not shown). Glucose acted as a competitive inhibitor of PNPG in the case of β-glucosidase EG-1, while a non-competitive effect was observed for IG-1. All three β-glucosidases were totally inhibited by 1 mM-glucono-1,5-lactone.

**DISCUSSION**

Cellulosic materials that are generally less susceptible to attack by exo- and endo-cellulases appear to be the best growth substrates for the production of extracellular β-glucosidases in most cellulolytic micro-organisms including *Monilia* sp. (see, for example, Berg & Pettersson, 1977; Deshpande *et al.*, 1978; Smith & Gold 1979; Wilson & Niederpruem, 1967). This trend has also been observed in the production of extracellular cellulases and cellobiose dehydrogenase in *Monilia* sp. (Dekker, 1980). There are, however, exceptions in which no extracellular β-glucosidase (Hägerdal *et al.*, 1978), or very little β-glucosidase (Sternberg, 1976), was produced when cellulose was used as the carbon source in the nutrient medium.

Extracellular β-glucosidase synthesis was reported to be repressed by glucose in
Phanerochaete chrysosporium (Smith & Gold, 1979), Schizophyllum commune (Wilson & Niederpruem, 1967), Sporotrichum pulverulentum (Deshpande et al., 1978) and Trichoderma viride (Berg & Pettersson, 1977), and by cellobiose in T. viride (Berg & Pettersson, 1977) and S. pulverulentum (within the first 24 h) (Deshpande et al., 1978). In Monilia sp., extracellular β-glucosidase formation was strongly repressed by either glucose or cellobiose and derepressed when these carbohydrates were nearly exhausted from the nutrient medium. This is similar to the induction of extracellular β-glucosidases in P. chrysosporium when grown on cellulose in the presence of glucose (Smith & Gold, 1979). In Monilia sp., therefore, synthesis of extracellular β-glucosidase appears to be strictly regulated by the levels of glucose and cellobiose produced as a result of cellulose breakdown through the action of cellulases.

Cell wall-bound β-glucosidases are also extracellular since they exist external to the plasma membrane, in the periplasmic space. These enzymes are induced by cellobiose (Berg & Pettersson, 1977; Deshpande et al., 1978; Lusis & Becker, 1973) and cellulose (Berg & Pettersson, 1977; Kwang & Suzuki, 1976; Lusis & Becker, 1973), and repressed by glucose (Lusis & Becker, 1973), a trend which is also evident in Monilia sp. The few reports on intracellular β-glucosidases have indicated that these enzymes are induced mainly on cellobiose (Eberhart & Beck, 1973; Smith & Gold, 1979; Wilson & Niederpruem, 1967) although cellulose also promoted β-glucosidase synthesis but in lower amounts (see, for example Smith & Gold, 1979). In Monilia sp. the existence of at least two enzyme forms is a complicating factor. The induction pattern of intra- and extracellular β-glucosidases by the various carbon sources suggests that a different control mechanism is operative in the synthesis of each enzyme form.

The β-glucosidases from Monilia sp. resembled the enzymes from several other fungal sources, e.g. S. pulverulentum (Deshpande et al., 1978) and T. viride (Maguire, 1977), in that both PNPG and cellobiose were attacked, and in most cases the $K_m$ for PNPG was much lower than that for cellobiose. β-Glucosidase EG-1/IG-2 although showing a higher affinity (lower $K_m$) for PNPG, nevertheless hydrolysed cellobiose faster than PNPG and in this respect is very similar to a cellobiase produced by T. viride (Maguire, 1977). The Monilia sp. enzymes EG-1/IG-2 therefore appear to be of the cellobiase type and would thus constitute a part of the cellulase complex, whereas IG-1, which has a higher $K_m$ for cellobiose, is probably a non-specific β-glucosidase whose function is as yet unknown.

Glucose acted as a competitive inhibitor of the Monilia sp. β-glucosidase EG-1 which hence resembles enzymes from Chaetomium thermophile var. coprophile (Lusis & Becker, 1973), P. chrysosporium (Smith & Gold, 1979) and S. commune (Wilson & Niederpruem, 1967). Non-competitive inhibition by glucose was observed for the extracellular β-glucosidases of T. viride (Gong et al., 1977) and Lenzites trabea (Herr et al., 1978), whilst the intracellular β-glucosidase IG-1 showed this effect in Monilia sp.
Note added in proof. Recent work has shown that our Monilia sp. can ferment both glucose and xylose to ethanol, and Tsao's group at Purdue University (Biotechnology Letters 1981, 3, 77–82) have shown that their monilial isolate can directly ferment cellulose, in addition to glucose and xylose, to ethanol.

This work was supported by grants from the Council of Scientific and Industrial Research, Co-operative Scientific Programmes, Pretoria, South Africa, and the University of Natal Research Fund.

The author is grateful to Mr R. K. Berry, Ms N. E. Nilsen and Mrs S. L. Lines of this laboratory for their technical assistance.

REFERENCES


