The Preliminary Characterization of the β -Glucosidases of *Cellulomonas fimi*

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β -Glucosidase activity in *Cellulomonas fimi* is strictly intracellular. Cell-free extracts contain at least two β -glucosidases. One of the enzymes is constitutive and it hydrolyses *p*-nitrophenyl- β -D-glucoside (PNPG) but not cellobiose. A second enzyme is induced about fourfold by growth on Avicel, and about sevenfold by growth on cellobiose; it hydrolyses both PNPG and cellobiose.

INTRODUCTION

β -Glucosidase (β -D-glucoside glucohydrolase; EC 3.2.1.21) is frequently a rate-limiting enzyme in the microbial degradation of cellulose to glucose. Cellulose is converted to soluble products (mostly cellobiose with some cellodextrins) by extracellular cellulases. The β -glucosidases, which are usually cell-associated (Gong & Tsao, 1979) convert cellobiose to glucose. Although widely distributed in plants and fungi, very few bacterial β -glucosidases have been described. *Clostridium thermocellum* (Alexander, 1968) and *Cellulomonas fimi* (Sato & Takahashi, 1967) contain cellobiose phosphorylases. *Clostridium thermocellum* also contains a true β -glucosidase (Ait *et al.*, 1979), as does a *Flavobacterium* (Sano *et al.*, 1975), *Alcaligenes faecalis* (Han & Srinivasan, 1969) and *Escherichia adacarboxylata* (Armentrout & Brown, 1981).

We are attempting to characterize completely the enzymes used by *C. fimi* to convert cellulose to glucose. It is our intention to clone the genes determining these enzymes, and considerable progress has been made in this regard (Whittle *et al.*, 1982; Gilkes *et al.*, 1984). In this paper we describe the preliminary characterization of the β -glucosidases of *C. fimi*.

METHODS

Preparation of cell extracts. *Cellulomonas fimi* ATCC 484 was grown in basal salts medium (Whittle *et al.*, 1982) supplemented with 0.2% glucose, 0.2% cellobiose or 0.2% Avicel. Residual Avicel was removed from cultures by allowing it to settle out at 4 °C for 2–4 h. Cells were harvested by centrifugation for 15 min at 10000 *g* and 4 °C. The pellets were washed by centrifugation in 0.1 M-sodium/potassium phosphate buffer, pH 7.0. The final pellets were resuspended in the same buffer (at 1/25 the original volume). Pancreatic DNAase was added to give a final concentration of 50 μ g ml⁻¹. The cells were ruptured by two to three passages through a French pressure cell and debris was removed by centrifugation for 20 min at 50000 *g* and 4 °C. To prevent proteolysis, phenylmethylsulphonyl fluoride was added to the supernatant to give a final concentration of 50 μ g ml⁻¹. When the supernatant was stored at 4 °C, activity was stable for a month.

Enzyme assays. β -Glucosidase activity was measured in two ways. In the first, *p*-nitrophenyl- β -D-glucoside (PNPG) was used as substrate (Han & Srinivasan, 1969); the extinction coefficient of *p*-nitrophenol was assumed to be 18600 l mol⁻¹ cm⁻¹ at 400 nm. In the second, the release of glucose from cellobiose was determined using Diagnostic Kit 15-UV (Sigma). For assaying cell extracts the reaction mixture contained the following: 0.4 ml 0.1 M-sodium/potassium phosphate buffer, 0.1 ml substrate (5 mM-PNPG or 50 mM-cellobiose) and 0.1 ml cell

Abbreviations: MUG, 4-methylumbelliferyl- β -D-glucoside; PNPG, *p*-nitrophenyl- β -D-glucoside; X-Glc, 5-bromo-4-chloro-3-indolyl- β -D-glucoside.

Fig. 1. β -Glucosidase activities in *C. fimi* extracts using MUG. Extracts were prepared from cells grown on different carbon sources. These were analysed by non-denaturing PAGE. Active bands were visualized by soaking the gel in 1 mM-MUG. Extracts from cells grown in glucose (A), cellobiose (B) and Avicel (C) are shown.

Table 1. β -Glucosidase activity in *C. fimi* extracts

Activity of the cells is expressed as $\mu\text{mol } p\text{-nitrophenol released min}^{-1}$ by 10^8 washed cells. Activity of the extracts is expressed as $\mu\text{mol } p\text{-nitrophenol or glucose released min}^{-1} (\text{mg protein})^{-1}$.

Assay substrate	β -Glucosidase activity on growth substrate:					
	Glucose		Cellobiose		Avicel	
	Cells	Extract	Cells	Extract	Cells	Extract
PNPG	0.019	0.556	0.015	0.763	0.016	0.483
Cellobiose		0.029		0.214		0.109

extract. When gel slices were assayed they were placed directly into the 0.5 ml reaction mixture. PNPG hydrolysis was stopped by the addition of 0.5 ml 1 M- Na_2CO_3 . Cellobiose hydrolysis was stopped by steaming the reaction mixture for 5 min. A unit of activity is defined as that causing the release of 1 $\mu\text{mol } p\text{-nitrophenol or glucose min}^{-1}$.

Gel electrophoresis. Proteins were separated by non-denaturing electrophoresis in 10% (w/v) polyacrylamide slab gels, 1.5 mm thick and 10 cm long (Langsford *et al.*, 1984). Bands of β -glucosidase activity were visualized either by soaking the gel in 1 mM-4-methylumbelliferyl- β -D-glucoside (MUG) in 0.1 M-sodium/potassium phosphate buffer, pH 7.0 until fluorescent bands appeared under shortwave UV light (about 10 min at 37 °C) or by overlaying the gel with 0.5% agarose containing 40 μg 5-bromo-4-chloro-3-indolyl- β -D-glucoside (X-Glc) ml^{-1} and incubating the gel at 37 °C until blue bands appeared (usually 12–16 h). The MUG-treated gels were photographed

Fig. 2. β -Glucosidase activities in *C. fimi* extracts using X-Glc. Extracts were prepared from cells grown on different carbon sources. Active bands were visualized by incubating the gel with a substrate-containing overlay. Lanes are as for Fig. 1.

under shortwave UV light. X-Glc-treated gels were fixed in 10% (v/v) acetic acid overnight and dried onto Whatman 3MM filter paper under vacuum, then photographed. β -Glucosidase activities in a gel were assayed as follows. A lane was excised from the gel and cut into 3 mm slices. Each slice was incubated in a reaction mixture containing either PNPG or cellobiose for 2–4 or 24 h. All β -glucosidase substrates were obtained from Sigma.

RESULTS

Cell-free culture supernatants from *C. fimi* cultures on glucose, cellobiose or Avicel were devoid of β -glucosidase activity. The washed cells from these cultures exhibited negligible activity (Table 1); therefore, only cell extracts were examined in detail. β -Glucosidase activity with PNPG as substrate was not affected significantly by the carbon source used for growth of the cells; however, activity with cellobiose as substrate was markedly growth substrate-dependent (Table 1).

Non-denaturing PAGE revealed at least two β -glucosidase activities in *C. fimi* extracts; both appeared to use MUG and X-Glc as substrates, but with different efficiencies (Figs 1 and 2). Only the faster moving was active on cellobiose (Fig. 3); it also gave more activity with X-Glc (Fig. 2).

Total activity against PNPG and activity against cellobiose was optimal at pH 7.0; the former declined more than the latter below pH 7.0.

DISCUSSION

Cellulomonas fimi contains at least two β -glucosidases. One of them is active with PNPG but not with cellobiose, and appears to be an aryl- β -D-glucosidase. The other is more active with cellobiose, and appears to be a β -D-glucoside glucohydrolase (or true cellobiase). Several fungi and bacteria, such as *Sporotrichum thermophile* (Meyer & Canevascini, 1981), *Trichoderma*

Fig. 3. β -Glucosidase activity profiles in non-denaturing polyacrylamide gels. Cell-free extracts were run on 10% non-denaturing PAGE. The lanes were excised after electrophoresis and sliced into 3 mm fractions. The slices from one lane for each growth substrate were incubated with PNPg (open boxes; A_{400}), and those from an adjacent lane with cellobiose (hatched boxes; ΔA_{340}). Growth substrates were (a) Avicel, (b) cellobiose and (c) glucose.

koningii (Wood & McCrae, 1982), *Streptomyces venezuelae* (Chatterjee & Vining, 1982), and a *Pseudomonas* sp. (Hwang & Suzuki, 1976) also contain both aryl- β -D-glucosidases and cellobiases. *Cellulomonas uda* has constitutive β -glucosidase activity against PNPg; activity against cellobiose was not measured (Stoppok *et al.*, 1982). In an unidentified *Cellulomonas*, activity against both PNPg and cellobiose was induced by growth on cellobiose (Haggett *et al.*, 1978).

The cellulases of *C. fimi* are induced by growth on cellulosic substrates. The cellobiase activity is also induced by growth on Avicel and, to a greater extent, by growth on cellobiose. As it is probably the cellobiase that hydrolyses the cellobiose released from cellulose by the cellulases to glucose, it is the gene for this enzyme that must be cloned in order to reconstitute the complete cellulolytic system of *C. fimi*.

Although PNPg is a convenient substrate for assaying β -glucosidase activity, our results suggest that the detection of cellobiase activity requires the use of cellobiose as an assay substrate because PNPg may detect mainly aryl- β -D-glucosidase activity. This finding is similar to those reported in the literature for some fungal systems (Bucht & Eriksson, 1969; Jermyn, 1955a, b; Youatt, 1958).

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