Partial Purification and Characterization of a New Intracellular β -Glucosidase of *Trichoderma reesei*

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(Received 2 August 1979)

A new intracellular β -glucosidase was isolated from *Trichoderma reesei*. It was sequentially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography and rechromatography on Sephadex G-150. The enzyme has a mol.wt. of 98 000, optimal activity at pH 6.5, pI 4.4 and K_m values of 6.7 mM and 3.3 mM for sophorose and cellobiose respectively. Possible functions of the enzyme may be regulation of cellulase induction and/or to serve as a proenzyme.

Sophorose (2-O- β -glucopyranosyl-D-glucose) is the most potent inducer of the cellulase enzyme for the fungus *Trichoderma reesei* QM 9123 (formerly *Trichoderma viride*), one of the best producers of cellulase (Nisizawa *et al.*, 1971a,b) and a common source of commercial cellulase. The synthesis of cellulase *de novo* has been directly correlated with the presence of sophorose (Sternberg & Mandels, 1979). Although sophorose is the most potent inducer, it is catabolized to CO_2 and water with a subsequent decrease in cellulase production (Loewenberg & Chapman, 1977). Furthermore, before catabolism the sophorose inducer is intracellularly hydrolysed to glucose, which itself is a known repressor of cellulase formation (Nisizawa *et al.*, 1972). The enzyme that hydrolyses sophorose to glucose is thus important for two reasons: (1) it eliminates the major source of induction and (2) the glucose produced serves as a catabolite repressor of the cellulase enzyme. More generally, the more efficiently the fungus destroys sophorose, the less remains for induction. The question remains as to which intracellular enzyme(s) is responsible for sophorose hydrolysis, and it is to this question that the present work is addressed.

Other inducers of cellobiose production such as cellobiose and lactose are as much as 3 orders of magnitude less effective than sophorose for induction (Mandels *et al.*, 1962). More importantly, the hydrolysis of cellobiose apparently takes place extracellularly (Mandels & Reese, 1960). On 1.0% cellobiose, *R. reesei* extracellularly consumes 90% of the cellobiose rapidly and the last 10% slowly. In contrast, essentially no sophorose is hydrolysed in the external medium, which is at a low pH. Also, there is no evidence as yet that cellobiose hydrolyses sophorose. Normally the hydrolysis of sophorose occurs intracellularly (Sternberg & Mandels, 1979; Loewenberg & Chapman, 1977).

Up to now, the only evidence concerning the intracellular enzymic hydrolysis of sophorose has come from the study of intact mycelia, and no isolation or purification of the enzyme involved has been accomplished. The studies with intact mycelia indicate that sophorose induction occurs only after cellular uptake of sophorose and, more critically, a certain minimum concentration (4 mg/l) is necessary in the medium to maintain induction; cellulase induction ceases on removal of external sophorose. It is important to note that sophorose has not been shown to be the natural inducer, and, although cellobiose is not as potent, it is still considered to be the natural inducer (Mandels *et al.*, 1962).

It would appear that the intracellular enzyme that hydrolyses the inducer sophorose exerts metabolic control over cellulase synthesis. Until now, no isolation or characterization of such a β -glucosidase has been reported. Indeed, only the extracellular *T. reesei* β -glucosidases have been studied. In the present paper we report the isolation and characterization of a new soluble intracellular β -glucosidase that hydrolyses sophorose and cellobiose.

Experimental

Materials

Sophorose was synthesized by following published procedures (Coxon & Fletcher, 1961). Cellobiose was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), lactose from Allied Chemicals (Morristown, NJ, U.S.A.) and Bistris {2[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol} from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); Calbiochem–Behring single-vial reagent (Elk Grove Village, IL, U.S.A.) was used for glucose analyses. Ampholines were obtained from LKB Produkter (Stockholm, Sweden), and density-gradient-quality sucrose from Schwarz–Mann

(Orangeburg, NY, U.S.A.) was used for the sucrose gradient in isoelectrofocusing. Only double-distilled water and 20mM-Bistris buffer were used throughout except where noted otherwise. Solka floc SW40 was provided by Brown Co. (Berlin, NH, U.S.A.). Stock cultures of *Trichoderma reesei* (formerly *Trichoderma viride*) strain QM 9123 (obtained from M. Mandels and E. T. Reese, Quartermaster Research and Engineering Center, Natick, MA, U.S.A.) were maintained on Bacto potato dextrose (Difco) agar slants at 25°C.

Growth of mycelia and preparation of crude homogenate

A spore suspension prepared from a potato/dextrose slant and sterile water was used as inoculum for liquid cultures. The approximate number of spores/ml was determined by measuring the absorbance of the suspension at 550nm; an A_{550} value of 1.0 represents approx. 6.7×10^7 spores/ml. The inoculum used contained 1.0×10^5 – 3.0×10^5 spores/ml of medium. Liquid cultures were grown on cellulose (Solka floc) media in 125ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 27°C. The medium described here is a slight modification of one previously reported (Mandels *et al.*, 1962); urea was not used and 0.2% Trypticase replaced proteose peptone.

The mycelia from liquid cultures were harvested by filtration on a glass-fibre filter with suction, and washed with a volume of distilled water double that of the media. The mycelial mat was peeled from the filter and resuspended in 100ml of 20mM-Bistris/HCl buffer, pH 6.5, at 4°C. One or more drops of GE-60 antifoam were added before homogenization of the suspension with three 30s bursts in an Ultra-Turrax homogenizer. The homogenized mat was lysed by passage through a French press with a maximum pressure of 34 MPa (340 atm). Two passes proved sufficient to produce a lysed homogenate. The suspension was centrifuged at 26 000g for 20min at 0°C, and the supernatant was saved for determination of protein, assay of enzymic activity and subsequent purification.

Determination of protein and assay of enzymic activity

A protein-dye binding method (Bradford, 1976) was used for determining microgram amounts of protein. Enzymic activity was determined by measuring the rate of production of glucose. The reaction was quenched by immersion of reaction tubes in boiling water for 15s. In a typical assay 25 μ l of substrate (2mg/ml) was incubated with 25 μ l of enzyme solution (approx. 300 μ g of protein/ml) for 10min at 28°C. The units of measured activity

are expressed in nmol of glucose/min per mg of protein.

Partial purification of intracellular β -glucosidase

$(\text{NH}_4)_2\text{SO}_4$ fractionation. The supernatant from the above preparation was adjusted to 1.5M- $(\text{NH}_4)_2\text{SO}_4$ (28% saturation) and centrifuged at 26 000g for 20min at 0°C. The pellet was discarded and the supernatant adjusted to 3.0M- $(\text{NH}_4)_2\text{SO}_4$ (57% saturation), and centrifuged at 26 000g for 20min at 0°C. The supernatant was discarded and the pellet redissolved in buffer. This constitutes the crude enzyme preparation.

Sephadex G-150 fractionation. The dissolved pellet was chromatographed on a Sephadex G-150 (10–120 mesh) column (2.5cm $\times$ 52cm) equilibrated with 20mM-Bistris/HCl buffer, pH 6.5, at 4°C. Fractions were collected, and those with activity were precipitated by 3.0M- $(\text{NH}_4)_2\text{SO}_4$ (57% saturation). The precipitates were collected by centrifugation, and the resulting pellet resuspended in a small amount of buffer was rechromatographed on a new Sephadex G-150 column as described above and the precipitation procedure repeated. The enzyme isolated from the second Sephadex G-150 chromatography was used for subsequent assays, kinetic studies, isoelectrofocusing and determination of other biophysical characteristics.

Isoelectrofocusing

A 110ml LKB isoelectrofocusing column was used for isoelectrofocusing at 4°C. LKB Ampholine (40%, w/v), pH 3.5–5.0, was used with a sucrose gradient with the anode at the bottom of the column.

For the pH3–10 gradient isoelectrofocusing experiments, LKB Ampholines (40%, w/v) were used with a glycerol gradient with the anode at the top of the column. To enhance the stability of the protein for the glycerol gradient, the sample was dialysed and concentrated against 1% glycine. In the sucrose-gradient experiments the protein was dialysed and concentrated against 2mM-Bistris/HCl buffer, pH 6.5. In both cases, suction filtration/dialysis was done with substituted cellulose bags (Schleicher and Schuell). The absorbance of the eluted fractions was monitored at 280nm with an LKB Uvicord instrument.

Determination of molecular weight

An approximate molecular weight of the enzyme was obtained by Sephadex G-150 chromatography of the enzyme along with molecular-weight markers bovine haemoglobin (mol.wt. 68 000) and glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 140 000). The latter protein did not come out at the front, thus indicating that it was retarded and served as an appropriate marker.

Table 1. Steps of purification of *T. reesei* intracellular β -glucosidase and activities with sophorose and cellobiose as substrates.

Experimental details are given in the text.

Fraction	Total activity (nmol/min)	Protein (mg/ml)	Specific activity (nmol/min per mg of protein)	Purification factor
Crude homogenate				
Sophorose	74 700	2500	30	—
Cellobiose	90 000		36	—
(NH ₄) ₂ SO ₄ fractionation				
Sophorose	45 500	460	99	3.3
Cellobiose	59 800		130	3.6
1st Sephadex G-150 chromatography				
Sophorose	42 000	150	281	9.4
Cellobiose	55 500		370	10.2
2nd Sephadex G-150 chromatography				
Sophorose	41 428	59	705	23.5*
Cellobiose	52 300		890	24.7*

* Increase of specific activity $890/36 = 24.7$ for cellobiose and $705/30 = 23.5$ for sophorose. Percentage recovery $52\,300/90\,000 = 58\%$ for cellobiose and $41\,428/74\,700 = 55\%$ for sophorose.

Fig. 1. Isoelectrofocusing of partially purified *T. reesei* intracellular β -glucosidase on a sucrose gradient. For experimental details see the text. O, Activity towards cellobiose; —, protein (A_{280}); ●, pH.

Results

Preparation of enzyme and enhancement of specific activity

The purification of the intracellular enzyme and its ability to hydrolyse both sophorose and cellobiose can be summarized in four separate stages: (1) crude homogenate (obtained by cellular disruption); (2) preparation from (NH₄)₂SO₄ fractionation; (3) preparation from first Sephadex G-150 chromatography; (4) preparation from second Sephadex G-150 chromatography.

The entire purification scheme is shown in Table 1. There is a steady rise in specific activity

with each step, with an eventual 24-fold enhancement. All attempts to use either DEAE-cellulose (Whatman DE-32) or ECTEOLA-cellulose (Sigma) resulted in complete loss of activity of the protein(s) that were eluted, indicating either denaturation or irreversible binding of the enzyme to the column.

Isoelectrofocusing

The enzyme preparation that had been twice chromatographed on Sephadex G-150 was isoelectrofocusing in the pH regions 3.5–5.0 and 3–10; the results for the narrow pH range are shown in Fig. 1, and the apparent pI of the active enzyme was 4.4 in both experiments. Although the activities were deter-

mined with cellobiose, the active fractions also hydrolysed sophorose. In the sucrose-gradient isoelectrofocusing, precipitation and lowered specific activity were observed. In the glycerol-gradient isoelectrofocusing, in which glycerol stabilizes the enzyme, there was a 150-fold enhancement of specific activity over that of the crude homogenate; however, the optimum pH for enzymic activity in both experiments was shifted to pH 5.5, and the proper-

ties of the enzyme were somewhat different from those of the enzyme obtained from Sephadex G-150 chromatography, as discussed below.

Biochemical characterization

The appropriate molecular weight of the enzyme determined by Sephadex G-150 chromatography was about 98 000. Fig. 2 shows the plot of the elution volume versus log (molecular weight) for the β -glucosidase, bovine haemoglobin (mol.wt. 68 000) and glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 140 000).

The pH optima with cellobiose and sophorose as substrates were determined for the enzyme that had been twice chromatographed on Sephadex G-150. The results are shown in Fig. 3. The pH optima were both 6.5. After the pH of the enzyme solution had been adjusted to a value different from the optimum, re-adjustment of the pH to 6.5 did not lead to a re-gain of the original specific activity, indicating that the enzyme is not stable at pH values much removed from the optimum. At the optimum pH the enzyme retained full activity for at least 24 h at 28°C and was stable indefinitely on being frozen. This intracellular enzyme also acts on 4-methylumbelliferyl β -D-glucoside. It does not act on lactose, sucrose or gentiobiose.

The Lineweaver-Burk plots were obtained for the enzymic reaction with cellobiose and sophorose as substrates and the K_m values were 6.7 mM and 3.3 mM for sophorose and cellobiose respectively.

The thermal stability of the Sephadex-purified enzyme was determined. After 1 h incubation at 40°C, the purified enzyme lost nearly all its activity (95%). Also, on storage at 4°C for 4–7 days, nearly all the activity (75%) of the Sephadex-purified enzyme was lost.

Discussion

An intracellular β -glucosidase has been isolated from *T. reesei* and partially purified, and this is the first time that an intracellular β -glucosidase has been isolated from this organism. This intracellular β -glucosidase has a mol.wt. of about 98 000, an apparent pI of 4.4, K_m values of 3.3 mM and 6.7 mM for cellobiose and sophorose respectively and optimum activity at pH 6.5. The properties of the twice-Sephadex G-150-chromatographed enzyme are compared with those of two other β -glucosidases in Table 2. Although the pI and K_m values are similar for the intracellular and extracellular (Ladisch *et al.*, 1977; Gong *et al.*, 1977) β -glucosidases, the molecular weights are significantly different, 98 000 versus 76 000, and the pH optima are dramatically different, i.e. pH 6.5 versus 4.8. It is clear from Table 2 that the cellobiase described by Berghem & Pettersson (1974) is quite different from the intra-

Fig. 2. Determination of the molecular weight of *T. reesei* intracellular β -glucosidase by Sephadex G-150 chromatography

For experimental details see the text. Key: 1, glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 140 000); 2, *T. reesei* intracellular β -glucosidase (mol.wt. 98 000); 3, bovine haemoglobin (mol.wt. 68 000).

Fig. 3. pH optima of the activity of partially purified *T. reesei* intracellular β -glucosidase towards sophorose (●) and cellobiose (○)

For experimental details see the text.

Table 2. Comparison of the properties of the intracellular β -glucosidase with those of extracellular β -glucosidases from *T. reesei*

	Intracellular β -glucosidase (present work)	Cellobiase (Ladisich <i>et al.</i> , 1977; Gong <i>et al.</i> , 1977)	Cellobiase (Berghem & Pettersson, 1974)
Mol. wt.	98 000	76 000	47 000
Optimum pH	6.5	4.8	~5
pI	4.4	4.4	5.8
Thermal stability			
40°C, 1 h	95% loss	No loss	—
60°C, 1 h	100% loss	5% loss	—
K_m (mM)			
Cellobiose	3.3	2.6	1.5
Sophorose	6.7	—	—

cellular β -glucosidase. From these comparisons it appears that the isolated intracellular β -glucosidase is distinct from previously isolated extracellular β -glucosidases.

Two possible functions of this intracellular β -glucosidase would be: (1) metabolic control of cellulase induction by destruction of inducer and production of the repressor glucose; (2) to serve as a pro-enzyme before transport through the cell membrane into the external environment. The high molecular weight of the intracellular enzyme provides some evidence for the second function.

One possible reason that this intracellular enzyme has not been detected previously is that, even if it were released into the external environment, for example by cell lysis, the generally low pH of growth medium would inactivate this β -glucosidase.

Conclusions

An apparently new intracellular β -glucosidase has been isolated from *T. reesei* and partially characterized. Its properties are sufficiently different from those of other β -glucosidases for it to be considered a new enzyme or possibly a proenzyme.

B. A. F. acknowledges the Science and Education Administration of the U.S. Department of Agriculture

under Grant no. 5901-0410-8-0156-0 from the Competitive Research Grants Office and the University of Wisconsin-Milwaukee Graduate School Research Committee for partial support of this work.

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