A cell-associated oligo-1,6- α -glucosidase from an extremely thermophilic anaerobic bacterium, *Thermoanaerobium* Tok6-B1

Adrian R. PLANT, Susan PARRATT, Roy M. DANIEL* and Hugh W. MORGAN

Thermophile Research Unit, Department of Biological Sciences, University of Waikato, Private Bag, Hamilton, New Zealand

Cell-associated oligo-1,6- α -glucosidase (EC 3.2.1.10) was isolated from *Thermoanaerobium* Tok6-B1 grown on starch-containing medium. Activity was purified 11.4-fold by salt precipitation, gel filtration, hydroxyapatite and anion-exchange chromatography. Molecular mass was determined as 30000 by SDS/polyacrylamide-gel electrophoresis and 33000 by analytical gel filtration. The probable order of specificity was *p*-nitrophenyl- α D-glucose > -isomaltose > -isomaltotriose > -panose > -nigerose and no activity was shown against malto-oligosaccharides, melezitose, melibiose, raffinose, cellobiose, sophorose, gentiobiose, lactose, pullulan, dextran or amylose. The optima for activity and stability were between pH 5.6 and 7.0 and the half-life at pH 6.5 was 1000 min at 70 °C and 20 min at 76 °C. Activity was stabilized by substrate, Mg²⁺, Mn²⁺ and Ca²⁺, but was destabilized by Zn²⁺ and EDTA. *N*-Ethylmaleimide, glucose and 1-*O*-methyl- α D-glucose were inhibitory but 1-*O*-methyl- β D-glucose stimulated activity. The activation energy (E_a) was 109 kJ/mol.

INTRODUCTION

α -Glucosidases are produced by many microbes in response to growth on starch-containing media and are involved in the degradation of oligosaccharides produced by the action of amylolytic enzymes on starch. The occurrence and properties of bacterial α -glucosidases have been reviewed [1,2] and two types of activity are recognized. Firstly, α -D-glucosidase (EC 3.2.1.20) hydrolyses terminal, non-reducing, 1–4-linked (and to a lesser extent, 1–6-linked) α D-glucose residues of disaccharides, oligosaccharides and aryl-glucosides. Enzymes showing preferential specificity towards maltose and malto-oligosaccharides, but little activity towards aryl glucosides have been termed ‘maltases’ and have been reported in mesophilic [3,4,5] and thermophilic bacteria [6].

Oligo-1,6- α -glucosidase (EC 3.2.1.10) is a smaller and less well known group of enzymes undertaking the exo hydrolysis of 1–6-linked α D-glucose residues from the non-reducing terminals of isomaltosaccharides, certain disaccharides, oligosaccharides and synthetic glucosides, but have no activity towards 1–4-linked α D-glucose residues in malto-oligosaccharides [3,7]. This enzyme has been reported from the thermophilic bacterium *Bacillus thermoglucosidius* KP1006 and is apparently unique in demonstrating high specificity towards isomaltose [8,9].

Thermoanaerobium Tok6-B1 is an anaerobic, extremely thermophilic bacterium isolated from a New Zealand geothermal environment [10]. When grown on starch-containing medium the organism elaborates α -amylase, pullulanase and cell-associated aryl-glucosidase [11–13].

In this paper we report the isolation and partial characterization of the aryl-glucosidase. The *Thermoanaerobium* Tok6-B1 enzyme appears to be an oligo-1,6- α -glucosidase of the *B. thermoglucosidius* type [8,9] with

specificity directed towards the hydrolysis of aryl-glucosides and the α 1–6-glucosidic linkages of isomaltose, isomaltotriose and panose. However, unlike the *B. thermoglucosidius* enzyme, the *Thermoanaerobium* activity requires a free thiol group for full activity and is stabilized by bivalent cations.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade, obtained from Sigma Chemical Co., with the exception of D-sophorose which was obtained from Koch–Light (Slough, Berks., U.K.).

Enzyme isolation

Thermoanaerobium Tok6-B1 was grown on starch-enriched media as previously described [11]. Washed cells (11.1 g) were suspended in 50 ml of 100 mM-Mops/1 mM-CaCl₂, pH 7.0, and sonicated on ice for 15 min at 50 % setting on a Dynatech sonic disintegrator. Cell debris was removed by centrifugation (14000 *g* for 15 min) and the supernatant made 80 % saturated with (NH₄)₂SO₄ at 4 °C. Precipitated protein was collected by centrifugation, then taken up in and dialysed against 0.2 M-ammonium acetate buffer, pH 7.2, containing 1 mM-CaCl₂. Aliquots (2.0 ml) of the resulting sample were injected onto a 60 cm $\times$ 2.15 cm TSK-G3000 SW preparative gel-filtration h.p.l.c. column equilibrated in the same buffer. Active fractions from successive experiments were combined, dialysed against H₂O, freeze-dried, suspended in 2.0 ml of buffer and reappplied to the h.p.l.c.

Active fractions were pooled and exhaustively dialysed against 10 mM-sodium phosphate, pH 6.8, and the sample (55 ml) was applied to a 9 cm $\times$ 1.5 cm column of

Abbreviations used: Epps, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulphonic acid; GlcNAc, *N*-acetylglucosamine; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

* To whom correspondence should be addressed.

Bio-Rad Bio-Gel HTP hydroxyapatite. The column was washed with 10 mM-sodium phosphate, pH 6.8, until no more protein was eluted and the enzyme was then eluted with a linear 50 ml gradient of 10–400 mM buffer. Active fractions were pooled and exhaustively dialysed against 20 mM-Mops/1 mM-CaCl₂, pH 6.9, and 20 ml was applied to a 15 cm × 2.1 cm column of DEAE-Sephacrose CL-6B equilibrated in the same buffer. After washing with 50 ml of buffer, enzyme activity was eluted with a linear 50 ml gradient of 0–0.6 M-NaCl. Activity was dialysed against 10 mM-Mops/3 mM-CaCl₂, pH 6.9, and stored at –70 °C.

Protein was estimated by dye binding [14] or a modification of the Lowry method [15]. SDS/polyacrylamide-gel electrophoresis (PAGE) [16] and analytical gel-filtration h.p.l.c. [13] were performed as previously described.

Enzyme assays

Unless otherwise stated, assays were performed at 70 °C with buffers adjusted at 70 °C using a temperature-compensated Pye pH electrode. Capped tubes containing 0.5 ml of 1 mM-*p*-nitrophenyl- α D-glucose in 100 mM-Mes, 1 mM-CaCl₂, 1 mM-mercaptoethanol, pH 5.6, and 0.5–2 μ g of enzyme in 5–20 μ l were held at 70 °C for 10 min and the reaction was stopped by adding 0.5 ml of 1 M-Na₂CO₃. Reaction products were determined from the absorbance at 420 nm using *p*-nitrophenol standards.

Analysis of products of enzyme action

Monosaccharide products of enzyme action on putative di-, tri- and poly-saccharide substrates were identified by h.p.l.c. using a 6.5 mm × 300 mm Waters Sugar-Pak I column fitted with Bio-Rad Micro Guard ion-exclusion (125-0129) and anion/OH cartridge (125-0130) guard columns operated at 90 °C. Solvent was sterile-filtered Milli Q water at a flow rate of 0.5 ml/min supplied by a Waters model 5-10 pump. Saccharide elution was monitored with an Erma refractive-index detector (ECR-7510). Substrates (1 mg/ml) in 100 mM-Mes/1 mM-CaCl₂, pH 5.6, were incubated with 3 μ g of enzyme for 15 h, cooled and 20 μ l was injected onto the column via a Rheodyne valve (model 7125).

RESULTS AND DISCUSSION

The purification data are summarized in Table 1. A purification factor of only 11.4 was achieved and SDS/PAGE of the final preparation revealed one major band and three minor bands of material staining with

Coomassie Blue. The molecular mass of the major band was determined as 29500 by reference to standard molecular-mass marker proteins. Analytical gel-filtration h.p.l.c. indicated a molecular mass of 32600 for the active enzyme in solution.

The activity data in Table 1 were determined in the absence of 2-mercaptoethanol. It was subsequently found that addition of 2-mercaptoethanol or dithiothreitol caused a large increase in activity, which indicated the presence of oxidation-sensitive thiol group(s) on the enzyme. The presence of 1 mM-2-mercaptoethanol elevated specific activity from 5.19 to 12.15 μ mol/min per mg of protein and 2-mercaptoethanol was present in all subsequent experiments unless specifically excluded.

A broad peak of activity occurred between pH 5.6 and 7.0 (Fig. 1), decreasing rapidly at more extreme pH values. Similarly, slightly acid pH optima are a feature of many α -D-glucosidases [1,2,8].

At high concentrations of *p*-nitrophenyl- α D-glucose the enzyme demonstrated non-linear kinetics (Fig. 2) characteristic of substrate inhibition. A K_m value of 0.51 mM was estimated using substrate concentrations of < 0.3 mM for which Michaelis-Menten kinetics were obeyed.

Although the enzyme was able to hydrolyse *p*-nitrophenyl- α D-glucose, it had no activity on *p*-nitrophenyl- β D-glucose and was, therefore, strictly an α -glucosidase. *p*-Nitrophenyl-linked α D-galactose, β D-galactose, β D-xylose, α L-arabinose and α D-*N*-acetylglucosamine were not substrates when tested at 1 mM under standard assay conditions. Analysis on the Sugar-Pak I h.p.l.c. column revealed that isomaltose (Glc α 1–6Glc) and isomaltotriose (Glc α 1–6Glc α 1–6Glc) were hydrolysed to glucose, whilst panose (Glc α 1–6Glc α 1–4Glc) was hydrolysed to glucose and maltose. Glucose was only slowly released from nigerose (Glc α 1–3Glc) or palatinose (Glc α 1–6Fru). We were not able to determine the K_m values for the sugar substrates directly, but instead we treated each sugar as a competitive inhibitor of *p*-nitrophenyl- α D-glucosidase and determined the competitive inhibition constant, K_i (Table 2). Inhibition was competitive in all cases and the competing inhibitor may also be regarded as a competing substrate, hence the value of K_i is likely to be similar to K_m [18]. Compared with K_m (0.51 mM for *p*-nitrophenyl- α D-glucose) the K_i values for saccharide substrate/inhibitors were high (Table 2), suggesting that they were relatively poor substrates.

For dimeric glucose substrates, specificity favoured α 1–6 linkages of isomaltose (K_i 32 mM) over the α 1–3

Table 1. Purification of α -glucosidase

Step	Total protein (mg)	Total activity (μ mol/min)*	Specific activity (μ mol/min per mg of protein)	Yield (%)	Purification (-fold)
Sonication supernatant	280	128	0.46	100	1
(NH ₄) ₂ SO ₄ pellet	142	136	0.96	106.7	2.1
First TSK-G3000 SW column	24.8	44.7	1.80	35.0	4.0
Second TSK-G3000 SW column	8.1	24.1	2.98	18.9	6.5
Bio-Gel HTP column	3.0	16.7	5.62	13.0	12.3
DEAE-Sephacrose CL-6B column	1.7	8.6	5.19	6.7	11.4

* Activity was determined in 100 mM-Mes/1 mM-CaCl₂, pH 5.6. 2-Mercaptoethanol was not present.

Fig. 1. pH-activity profile for *Thermoanaerobium* Tok6-B1 α D-glucosidase

Enzyme activity was determined from 10 min incubations at 70 °C in 100 mM-sodium acetate (●), Mes (○), Mops (□) or Epps (△). All buffers contained 1 mM- CaCl_2 and 1 mM-2-mercaptoethanol. Inset: influence of pH on thermostability; plot of log half-life against pH. Enzyme (2.5 $\mu\text{g}/\text{ml}$) was incubated at 70 °C or 76 °C with 100 mM-sodium acetate, (●); Mes, (○); Mops, (□) or Epps, (△) containing 1 mM- CaCl_2 . Aliquots were withdrawn at intervals, cooled and residual activity was determined at 70 °C under standard assay conditions. Values of $t_{1/2}$ were calculated from plots of log (% residual activity) against time [13].

linkages of nigerose ($K_i > 70$ mM). The slightly higher value of K_i for isomaltotriose compared with isomaltose (Table 2) suggests that specificity decreased with increasing chain length of isomaltosaccharides, as has been found for *Bacillus thermoglucosidius* KP1006 α -glucosidase [9], and suggests that the *Thermoanaerobium* enzyme was also an oligo-1,6- α -glucosidase. Enzyme action on panose yielded glucose and maltose, but no isomaltose and was consistent with activity involving an exo attack at the non-reducing terminal of saccharide substrates. Enzyme had no activity on α 1-4-glucosidic linkages as h.p.l.c. was unable to detect any hydrolysis products of maltosaccharides (of chain length 2-7). Melezitose, melibiose, raffinose, cellobiose, sophorose, β -gentiobiose, lactose and the polysaccharides dextran, amylose and pullulan were also ineffective as substrates.

Product inhibition was apparent as 110 mM-glucose inhibited by 70.1 % under standard assay conditions, but 110 mM-galactose had no effect. At 62 mM, 1-O-methyl- α D-glucose caused 42 % inhibition, but 1-O-methyl- α D-galactose, 1-O-methyl- β D-galactose and 3-O-methyl-glucose had no effect. 1-O-Methyl- β D-glucose (62 mM) caused 52 % activation. Product inhibition has often

Fig. 2. Effect of *p*-nitrophenyl- α D-glucose concentration on α -glucosidase activity

Double-reciprocal plot; at 70 °C in 100 mM-Mes, 1 mM- CaCl_2 , 1 mM-2-mercaptoethanol, pH 5.6.

Table 2. K_i and $t_{1/2}$ values in the presence of di- and trisaccharides

Values of K_i were obtained from Dixon plots [17] using *p*-nitrophenyl- α D-glucose as substrate and inhibitor concentrations of up to 60 mM. Half-life values were determined at 76 °C as detailed in Fig. 1 inset using each sugar at 0.7 mg/ml in 0.1 M-Mes, 1 mM- CaCl_2 , 1 mM-2-mercaptoethanol, pH 5.6. For the control, $t_{1/2}$ was determined in the absence of any added sugar.

Inhibitor	K_i (mM)	$t_{1/2}$ (min)
Isomaltose	32	21
Isomaltotriose	36	29
Panose	46	21
Palatinose	70	16
Nigerose	> 70	15
Cellobiose*	—	13
Control	—	14

* Cellobiose is not an inhibitor.

been noted for α -glucosidases [1,3,8] whilst 1-O-methyl- α D-glucose is a poor substrate for *B. thermoglucosidius* α -glucosidase [8] and might be expected to be a competitive inhibitor of the *p*-nitrophenyl- α D-glucosidase activity in this study.

An Arrhenius plot of log(initial reaction velocity) against the reciprocal of temperature at pH 5.6 was apparently linear below 65 °C (Fig. 3) enabling the activation energy (E_a) of 109 kJ/mol to be calculated. Deviation from linearity increased above 65 °C until at 74 °C, major denaturation occurred resulting in loss of activity.

Inspection of the inset in Fig. 1 also reveals that a major drop in thermostability occurred between 70 °C and 76 °C [e.g. at pH 6.5 when the half-life ($t_{1/2}$) dropped from about 1000 min at 70 °C to about 20 min at 76 °C]. The pH-dependence of thermal denaturation was similar at 70 °C and 76 °C (Fig. 1 inset) and similar to the pH-dependence of activity (Fig. 1).

Thermostability was promoted in the presence of a substrate/competitive inhibitor (Table 2). Effective substrates/inhibitors such as isomaltose or panose with lower K_i values, stabilized the enzyme more than did poor substrates/inhibitors, such as palatinose or nigerose,

Fig. 3. Arrhenius plot of log[initial velocity (v_0)] against the reciprocal of temperature for *p*-nitrophenyl- α -D-glucosidase in 100 mM-Mes, 1 mM- CaCl_2 , 1 mM-2-mercaptoethanol, pH 5.6

Incubation times were kept short (10 min) to limit thermal denaturation at higher temperatures.

Fig. 4. Thermostability of *p*-nitrophenyl- α -glucosidase activity

Enzyme (10 $\mu\text{g}/\text{ml}$) was held at 76 °C in 100 mM-Mes, pH 5.6, containing 1 mM- CaCl_2 (▲), MnCl_2 (■), MgCl_2 (●), ZnCl_2 (□) or sodium EDTA (○). Samples were withdrawn at the times shown and residual activity determined using standard assay conditions.

which had high values of K_i and which only increased $t_{1/2}$ by a small amount (Table 2). Cellobiose was not a substrate or inhibitor of the enzyme and had no significant effect on its thermostability.

In the presence of EDTA at pH 5.6, rapid loss of activity occurred at 76 °C (Fig. 4) with a $t_{1/2} < 4$ min, suggesting that metal ions are involved in maintaining thermostability. Addition of Ca^{2+} , Mn^{2+} or Mg^{2+}

stabilized the enzyme and elevated $t_{1/2}$ values to 14–16 min (Fig. 4). Activity was rapidly destroyed in the presence of Zn^{2+} , possibly through interaction with free thiol groups present in the active enzyme. When enzyme was incubated with 3.3 mM-*N*-ethylmaleimide at room temperature, only 30% residual activity remained after 10 min and inactivation could not be prevented by inclusion of 6 mM-isomaltose or isomaltotriose in the incubation. These data suggest that the enzyme requires a free thiol for full activity and that this thiol group is not located at the substrate-binding site.

Activity was destabilized by detergents and chaotropic agents, as inclusion of SDS (1 mg/ml) or urea (4 M) into standard assays resulted in 96% and 26% inhibition respectively.

Overall, the properties of the enzyme isolated from an extremely thermophilic bacterium, *Thermoanaerobium* Tok6-B1, were similar to those of the oligo-1,6- α -glucosidase from the moderately thermophilic *B. thermoglucosidius* KP1006 [8,9]. The strict specificity for α 1-6-glucosidic bonds and lack of activity against α 1-4-glucosidic bonds has hitherto been considered a unique feature of the *B. thermoglucosidius* enzyme [9]. Both enzymes are thermostable but are rapidly inactivated at temperatures above 70 °C. Unlike the *Bacillus* activity, the *Thermoanaerobium* enzyme was stabilized by bivalent cations and was sensitive to thiol-active reagents.

The authors thank Y. P. Casey and R. M. Clemens for able technical assistance. This work was financially supported by Pacific Enzymes Ltd and the New Zealand University Grants Committee.

REFERENCES

- Kelly, C. T. & Fogarty, W. M. (1983) *Process Biochem.* **18**, 6–12
- Fogarty, W. M. & Kelly, C. T. (1979) *Prog. Ind. Microbiol.* **15**, 87–151
- Thirunavukkarasu, M. & Priest, F. G. (1984) *J. Gen. Microbiol.* **130**, 3135–3141
- McWethy, S. J. & Hartman, P. A. (1979) *Appl. Environ. Microbiol.* **37**, 1096–1102
- Suzuki, A. & Tanaka, R. (1981) *Eur. J. Appl. Microbiol. Biotechnol.* **11**, 161–165
- Suzuki, Y., Tsuji, T. & Abe, S. (1976) *Appl. Environ. Microbiol.* **32**, 747–752
- Urlaub, H. & Wöber, G. (1978) *Biochim. Biophys. Acta* **522**, 161–173
- Suzuki, Y., Yuki, T., Kishigami, T. & Abe, S. (1976) *Biochim. Biophys. Acta* **445**, 386–397
- Suzuki, Y., Ueda, Y., Nakamura, N. & Abe, S. (1979) *Biochim. Biophys. Acta* **556**, 62–66
- Morgan, H. W., Patel, B. K. C. & Daniel, R. M. (1985) *FEMS Microbiol. Lett.* **30**, 121–124
- Plant, A. R., Patel, B. K. C., Morgan, H. W. & Daniel, R. M. (1987) *Syst. Appl. Microbiol.* **9**, 158–163
- Plant, A. R., Clemens, R. M., Morgan, H. W. & Daniel, R. M. (1987) *Biochem. J.* **246**, 537–541
- Plant, A. R., Clemens, R. M., Daniel, R. M. & Morgan, H. W. (1987) *Appl. Microbiol. Biotechnol.* **26**, 427–433
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Plant, A. R., Morgan, H. W. & Daniel, R. M. (1986) *Enzyme Microb. Technol.* **8**, 668–672
- Dixon, M. (1953) *Biochem. J.* **55**, 170–171
- Teipel, J. W., Hass, G. M. & Hill, R. L. (1968) *J. Biol. Chem.* **243**, 5684–5694