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Purification and properties of a stable β-glucosidase from an extremely thermophilic anaerobic bacterium

Mark L. PATCHETT,* Roy M. DANIEL and Hugh W. MORGAN
School of Science, University of Waikato, Hamilton, New Zealand

A β-glucosidase (EC 3.2.1.21) was purified to homogeneity from cell-free extracts of an extremely thermophilic anaerobic bacterium. The enzyme has an M_r of 43000 as determined by molecular-exclusion chromatography, has a pI of 4.55 and shows optimum activity at pH 6.2. The enzyme is active against a wide range of aryl β-glycosides and β-linked disaccharides, with β-galactosidase activity only slightly less than β-glucosidase activity, and significant β-xylosidase activity. Lineweaver–Burk plots for *p*-nitrophenyl β-glucoside, *o*-nitrophenyl β-glucoside and cellobiose substrates are biphasic concave-downwards. Inhibition of the β-glucosidase by substrates and glucose is negligible. Thermal inactivation follows first-order kinetics, with $t_{\frac{1}{2}}$ (65 °C) 45 h, $t_{\frac{1}{2}}$ (75 °C) 47 min and $t_{\frac{1}{2}}$ (85 °C) 1.4 min and a deactivation energy of 380 kJ/mol at pH 6.2. At pH 7.0, which is the optimum pH for thermostability, $t_{\frac{1}{2}}$ (75 °C) is 130 min. At 75 °C, at pH 6.2, the thermostability is enhanced about 8-fold by 10% (w/v) glycerol, about 6-fold by 0.2 M-cellobiose and about 3-fold by 5 mM-dithiothreitol and 5 mM-2-mercaptoethanol.

INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyses the hydrolysis of substrate β-glucosidic linkages between glucose and alkyl, aryl or saccharide groups (Stone, 1961). The enzyme has been isolated from animals, plants and microbes. A large number of microbial β-glucosidases have been studied, the majority being fungal in origin, and the most recent review (Schliemann, 1983) lists properties of β-glucosidases from over 400 fungi, yeasts and bacteria. The enzyme from different microbial sources exhibits a high degree of variability with respect to substrate specificity, inducers and location (Meyer & Canevascini, 1981; Shewale, 1982). This variability extends to the β-glucosidase isoenzymes frequently produced by individual organisms, and has allowed the assignment of specific functions to β-glucosidases possessing particular properties (McHale & Coughlan, 1981; Woodward & Wiseman, 1982).

The principal role of β-glucosidase in cellulolytic micro-organisms is to catalyse the hydrolysis of cellobiose and cello-oligosaccharides, producing glucose. These soluble substrates of β-glucosidase are produced from insoluble cellulose by the action of other members of the cellulose system of enzymes, the β-glucanases. As β-glucanases (Berghem *et al.*, 1975; Bisset & Sternberg, 1978) and β-glucosidases (Hong *et al.*, 1981) are subject to product inhibition, an adequate level of β-glucosidase activity is required for the cellulase system to function efficiently (Sternberg *et al.*, 1977). Product inhibition and thermal inactivation of β-glucosidases constitute two major barriers to the realization of enzymic hydrolysis of cellulose as a commercial process (Woodward & Wiseman, 1982).

Studies on β-glucosidases from thermophilic microbes, including *Thermoascus aurantiacus* (Tong *et al.*, 1980; Shepherd *et al.*, 1981), *Mucor miehei* (Yoshioka & Hayashida, 1980), *Talaromyces emersonii* (McHale & Coughlan, 1981, 1982), *Clostridium thermocellum* (Ait *et al.*, 1979, 1982), *Thermomonospora* sp. (Hägerdal *et al.*, 1980) and *Thermoanaerobacter ethanolicus* (Mitchell *et al.*, 1982), have demonstrated enhanced thermostability of the enzyme relative to the β-glucosidases from mesophiles. The expectation of improved thermostability for enzymes of the cellulase system of Wai21W.2, an extremely thermophilic anaerobic Gram-negative short-rod-shaped non-motile bacterium isolated from a New Zealand geothermal pool, has already been demonstrated for endoglucanase activity (C. H. Sissons, K. R. Sharrock, R. M. Daniel & H. W. Morgan, unpublished work). It was therefore decided to see whether a similar increase would be observed for a β-glucosidase from Wai21W.2. In the present paper we report on the purification and some properties of this enzyme.

EXPERIMENTAL

Growth of bacteria and preparation of cell-free extract

The basal medium of Zeikus *et al.* (1979) was used with the following modifications; $FeSO_4$ solution and thioctic acid (in vitamin solution) were omitted, the resazurin concentration was halved, and cysteine hydrochloride (1 g/l) was used to reduce the medium. The basal medium was supplemented with trypticase peptone (2 g/l) and yeast extract (2 g/l), adjusted to pH 7.6 with NaOH, and autoclaved. Cellobiose was added as a filter-sterilized degassed solution to a final concentration of 2 g/l.

Growth in sealed 20-litre bottles at 72 °C was initiated by a 2% (v/v) inoculum of late-exponential-phase cells. The cells were harvested in early stationary phase, 20–24 h after inoculation, with a continuous-flow centrifuge (Sharples model 6) at approx. 12000 *g*, and stored in liquid N_2 before enzyme extraction.

Blocks of Wai21W.2 cells were thawed and mixed with

Abbreviations used: *o*NPG and *p*NPG, *o*- and *p*-nitrophenyl β-glucopyranoside respectively.
* To whom correspondence should be addressed.

cold 0.103 M-sodium phosphate (0.035 M-Na_2HPO_4/0.068 M-NaH_2PO_4) buffer, pH 6.5 at 20 °C, containing 10 mM-2-mercaptoethanol and 4 mM-EDTA, such that the cells represented 10% (w/v) of the suspension. After 15 min sonication on ice the solution was centrifuged at 13000 *g* for 30 min at 5 °C. The decanted supernatant was concentrated by ultrafiltration with a 10000-M_r-cut-off membrane.

Purification of the β-glucosidase

All column chromatography, including determination of column buffer pH values, was carried out at room temperature. All solutions used in this section and subsequent experiments were made up to volume with deionized reagent-grade water from a Milli-Q-water-purification system (Millipore).

Concentrated cell-free extract was loaded on to a Sephadex G-100 column (90 cm × 5 cm) equilibrated with 0.103 M-sodium phosphate buffer, pH 6.5, containing 0.02% NaN_3. Fractions were assayed with *p*NPG and cellobiose substrates, and those with high activity towards *p*NPG were concentrated, equilibrated in a 0.08 M-NaCl/0.04 M-histidine solution adjusted to pH 6.0 with conc. HCl and applied to a DEAE-Sepharose CL-6B column (29 cm × 2.5 cm). β-Glucosidase activity was eluted with a 600 ml linear salt gradient increasing to 0.28 M-NaCl. After concentration and equilibration of active fractions with a 0.0265 M-Na_2HPO_4/0.0513 M-NaH_2PO_4 buffer, pH 6.5, by ultrafiltration, the sample was loaded on to a Bio-Gel HTP hydroxyapatite column (58 cm × 1.6 cm). The enzyme was eluted with a 300 ml linear gradient increasing to 0.171 M-sodium phosphate (0.058 M-Na_2HPO_4/0.113 M-NaH_2PO_4) buffer, pH 6.5, and active fractions were concentrated by ultrafiltration. The resulting sample (approx. 2 ml) was injected on to a 60 cm × 2.15 cm TSK-GEL G3000SWG column (Toyo Soda Manufacturing Co., Tokyo, Japan) running at a flow of 5 ml/min with 0.103 M-sodium phosphate buffer, pH 6.5. Fractions with β-glucosidase activity were concentrated by ultrafiltration and equilibrated with the preparative-polyacrylamide-gel-electrophoresis sample buffer.

A discontinuous anionic buffer system operating at pH 7.5 was used for preparative polyacrylamide-gel electrophoresis. The resolving gel was formed from equal volumes of a 16% (w/v) acrylamide (2% bisacrylamide) solution and 0.12 M-HCl adjusted to pH 7.15 with triethanolamine. The stacking gel consisted of equal volumes of a 7% (w/v) acrylamide (23% bisacrylamide) solution and 0.12 M-HCl adjusted to pH 5.5 with histidine, and was polymerized with riboflavin and *NNN'N'*-tetramethylethylenediamine. The cathode buffer was 0.035 M-Mops adjusted to pH 6.5 with histidine, and the anode buffer was 5 mM-histidine adjusted to pH 6.4 with HCl. The sample buffer consisted of 30 mM-HCl, 15% (v/v) glycerol and 1 mM-EDTA adjusted to pH 5.5 with histidine. An LKB 2001 vertical electrophoresis unit was used to support a 3 mm-thick gel, and electrophoresis was carried out at 17 °C with a current of 75 mA for 2 h. *p*NPG (0.5 mM) was incorporated into the resolving gel, and the active enzyme band, located by formation of the yellow *p*-nitrophenoxide ion, was soaked for 24 h at 5 °C in 10 mM-histidine/HCl buffer, pH 6.0. The resulting solution was stored in liquid N_2 and used to characterize the β-glucosidase.

Protein determination and assay of enzymic activity

A modification of the dye-binding method of Bradford (1976) was used for determination of 2–25 μg of protein, with bovine serum albumin (catalogue no. A 7638, Sigma Chemical Co.) as a standard.

In all assays, 1 unit of β-glucosidase activity was defined as the amount of enzyme required to produce 1 nmol of nitrophenol or glucose/min. All substrates except for sophorose (Koch–Light Laboratories), lactose (BDH Chemicals) and sucrose (Ajax Chemicals) were obtained from Sigma Chemical Co. A 50 μl volume of enzyme solution was added to 0.45 ml of buffered substrate (0.1 M-sodium phosphate buffer, pH 6.2, unless otherwise stated) equilibrated at 75 °C, and incubated for up to 6 min at this temperature. All assay buffers were adjusted to the appropriate pH at the temperature of use, by using a combination electrode calibrated at the required temperature and an automatic temperature-compensation probe, both from Orion Research (Cambridge, MA, U.S.A.).

Assays with nitrophenyl β-glycopyranoside substrates were stopped by the addition of 2 ml of ice-cold 0.25 M-Na_2CO_3, and the absorbances of the *p*- or *o*-nitrophenoxide ions were measured at 400 nm and 420 nm respectively. A molar absorption coefficient of 18500 $M^{-1} \cdot cm^{-1}$ was determined for *p*-nitrophenol (Merck) under stopped assay conditions, in agreement with Jones (1969), and a value of 21300 $M^{-1} \cdot cm^{-1}$ from the same reference was used to calculate *o*-nitrophenol concentrations.

For all other substrates, β-glucosidase activity was determined by enzymic analysis of the glucose formed using the Test-Combination Glucose GOD-Perid reagent (Boehringer Mannheim). Assays with *p*NPG as substrate at 75 °C, at pH 6.2, were linear for at least 10 min over the range of substrate concentrations employed. Assays with 200 mM-cellobiose were also linear for this time. Assays (6 min) with 0.8 mM-*p*NPG, 40 mM-*p*NPG and 200 mM-cellobiose as substrate were linear over β-glucosidase concentrations of 37–370 ng/ml.

Cell pellets were assayed for β-glucosidase activity by suspending the pellet in 0.1 ml of toluene/acetone (1:1, v/v) for 5 min at room temperature, then transferring the suspension to a buffered substrate solution.

Determination of pI and M_r

Narrow-range isoelectric focusing, pH 4.5–5.0, was performed at 10–12 °C on polyacrylamide gels constructed according to the method of Sharrock (1985), with AG4.5–5.0 Servalyt ampholytes. β-Glucosidase activity was detected by briefly overlaying a *p*NPG-impregnated polyacrylamide gel buffered at pH 7, and incubating the overlay for 1–2 min at 70 °C. Protein was located by the silver staining method of Merril *et al.* (1981).

Discontinuous SDS/polyacrylamide-gel electrophoresis was performed by using a modification of the Laemmli system described in the LKB Laboratory Manual for the LKB 2001 Vertical Electrophoresis Unit (LKB Produkter, 1980). Gels of 0.75 mm thickness were run for 4 h with a current of 30 mA at 17 °C. M_r standards (kit MM-SDS-70L, Sigma Chemical Co.) and β-glucosidase samples were prepared by heating protein solutions in sample buffer [2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol in 0.0625 M-Tris/HCl buffer, pH 6.8) at 100 °C for 3 min. Analytical

Table 1. Purification of Wai21W.2 β-glucosidase

Assays were conducted with 5 mM-*p*NPG as substrate at 75 °C at pH 6.2 (approx. 0.1 M-sodium phosphate buffer).

Purification step	Total activity (kunits)	Total protein (mg)	Specific activity (kunits/mg)	Recovery (%)	Purification (fold)
Extract from 10 g of cells	805	890	0.91	100	1
1. Sephadex G-100 chromatography	460	220	2.1	57	2.3
2. DEAE-Sepharose chromatography	220	14	16	27	18
3. Hydroxyapatite chromatography	90	4.0	23	11	25
4. Molecular-exclusion h.p.l.c.	82	1.9	43	10	47
5. Preparative discontinuous polyacrylamide-gel electrophoresis	76	0.51	149	9.4	164

molecular-exclusion h.p.l.c. was performed at 20 °C on a TSK-GEL G3000SW column (600 mm × 7.5 mm) equilibrated with 0.3 M-ammonium acetate buffer adjusted to pH 6.5 with acetic acid and calibrated with M_r standards (kit KR-137, Sigma Chemical Co.).

Carbohydrate chromatography

Samples taken from assay mixtures were diluted in water, and 20 μl portions were injected on to a Waters Sugar-Pak I column. Filtered vacuum-degassed water formed the mobile phase. The flow rate was 0.5 ml/min, the column temperature was 90 °C and a refractive-index detector operating at 40 °C detected product saccharides.

RESULTS AND DISCUSSION

Production of β-glucosidase

Wai21W.2 grown in 20-litre containers reached stationary phase within 17–20 h, with cell wet-weight yields of about 0.75 g/l. No attempt was made to optimize cell yields. During exponential and early stationary phase, all β-glucosidase activity was cell-associated, increasing to a maximum in early stationary phase of up to 60 units/ml of culture as assessed at 75 °C, at pH 6.2, by the toluene/acetone assay of a cell pellet from 1 ml of culture with 5 mM-*p*NPG as substrate. This value equates to about 0.9 kunit/mg of protein (see Table 1), which is within the range of amounts of β-glucosidase found in other bacteria (e.g. Ait *et al.*, 1979; Berg *et al.*, 1980; Stoppok *et al.*, 1982). From 22 h onwards, cell-associated activity declined rapidly, with a concomitant increase in extracellular activity. Similar observations have been made for the cellulolytic bacteria *Acetivibrio cellulolyticus* (Saddler & Khan, 1981) and *Thermoanaerobacter ethanolicus* (Mitchell *et al.*, 1982). In the latter organism, and also in *Trichoderma reesei* (Inglin *et al.*, 1980), release of intracellular β-glucosidase resulted in its rapid inactivation. Wai21W.2 β-glucosidase activity persisted after release into the culture medium.

Enzyme purification

The β-glucosidase was purified 164-fold in a 9.4% yield (Table 1). Fractionation of cell-free extract on Sephadex G-100 demonstrated the presence of high-M_r and low-M_r β-glucosidase isoenzymes, which displayed high activity towards *p*NPG and cellobiose as substrate respectively. The high-M_r isoenzyme represented more than 60% of total cellobiase activity and about 20% of total aryl β-glucosidase (*p*NPGase) activity. The low-M_r peak, with high activity against *p*NPG, was purified further, and in all subsequent steps aryl β-glucosidase and cellobiase activities were co-eluted, indicating that the enzyme chosen for further purification had both activities. During steps 4 and 5 only a single active β-glucosidase peak was seen, showing that no aggregation to higher-M_r forms takes place under these conditions. The active step-5 band had R_F 0.37. In this final step, the ratio of aryl β-glucosidase to cellobiase activity remained unchanged.

Rapidly thawed samples of purified enzyme frozen in liquid N_2 showed a 15% decrease in specific activity due to the freeze–thaw process, but were completely stable during storage. Thawed solutions lost 1–2% activity per week at 4 °C.

The purity of the β-glucosidase was assessed by narrow-range isoelectric focusing, pH 4.5–5.0. Loadings of 0.5, 1 and 2 μg of protein all produced a single silver-stained (and Coomassie-dye-stained) band that was also active against *p*NPG. Silver-stained discontinuous-SDS/polyacrylamide-gel-electrophoresis gels also displayed single bands for 2 μg loadings of the purified β-glucosidase. Allowing for the problems involved in assessing the purity of β-glucosidase preparations (Shewale, 1982; Sprey & Lambert, 1983), the enzyme is homogeneous. The degree of purification achieved is within the range reported for other bacterial β-glucosidases and the yield is very similar, but the specific activity of the purified enzyme is higher than that of other bacterial β-glucosidases (e.g. Han & Srinivasan, 1969; Ohmiya *et al.*, 1985).

Physical properties

A pI of 4.55 was determined by narrow-range isoelectric focusing. This value is similar to the pI values of many microbial β-glucosidases (e.g. Ait *et al.*, 1979; McHale & Coughlan, 1982), which range from 3.3 (Bodenmann *et al.*, 1985) to 5.85 (Wood & McCrae, 1982).

An M_r of 43000 was determined by molecular-exclusion h.p.l.c. All β-glucosidase activity loaded was recovered in a single protein peak. Discontinuous SDS/polyacrylamide-gel electrophoresis indicated an apparent M_r of 50000. Similar differences in results from the two methods have been observed for the glycoprotein β-glucosidases of *Talaromyces emersonii* (McHale &

Coughlan, 1982) and *Ruminococcus albus* (Ohmiya *et al.*, 1985). Although the glycoprotein nature of the Wai21W.2 enzyme is unproven, the higher apparent M_r (50000) obtained by SDS/polyacrylamide-gel electrophoresis could be due to the lower electrophoretic mobility of glycoproteins compared with protein standards in such systems, an effect that results from the decreased binding of SDS to glycoproteins (Pitt-Rivers & Impiombato, 1968). In view of this possibility, the preferred M_r value is that obtained by molecular-exclusion chromatography. The similar M_r values calculated by the two methods show that the enzyme is monomeric.

The M_r of the Wai21W.2 β-glucosidase is the same as that of the *Clostridium thermocellum* enzyme (Ait *et al.*, 1982). The M_r values of β-glucosidases range from 32000 (Bodenmann *et al.*, 1985) to 440000 (Meyer & Canevascini, 1981).

Optimum pH and buffer ion effects

The optimum pH of the enzyme at 75 °C was 6.2 for both 40 mM-*p*NPG (Fig. 1) and 200 mM-cellobiose substrates. The pH optima of bacterial β-glucosidases, which usually lie in the range 5.5–7.0, are generally higher than those of most fungal β-glucosidases. A number of bacteria produce β-glucosidases with a pH optimum similar to that of the Wai21W.2 enzyme (e.g. Han & Srinivasan, 1969; Ait *et al.*, 1982; MacKenzie & Bilous, 1982). Wai21W.2 β-glucosidase activity on 40 mM-*p*NPG was unaffected by variations in sodium phosphate buffer concentration between 0.015 M and 0.6 M at 75 °C at pH 6.2, indicating an insensitivity to phosphate ions and ionic strength. As shown in Fig. 1,

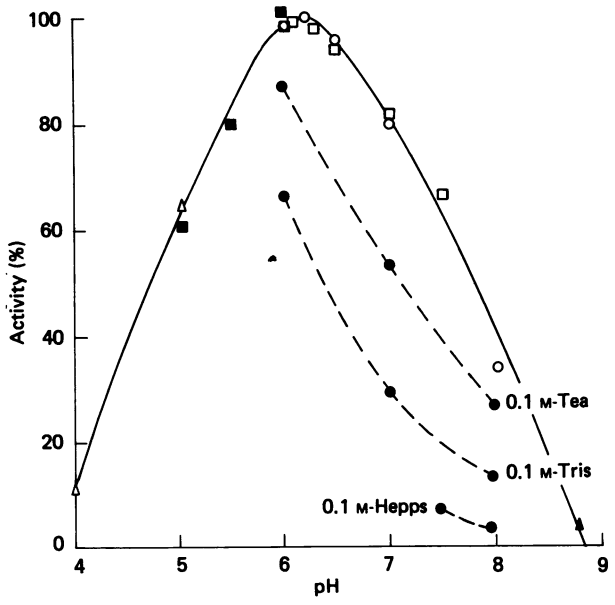

Fig. 1. pH optimum and buffer ion inhibition

Assays were conducted with 40 mM-*p*NPG in 0.1 M-acetate/NaOH (△), 0.1 M-Mes/NaOH (■), 0.1 M-Mops/NaOH (□), 0.1 M-sodium phosphate (○), 0.1 M-boric acid/NaOH (▲), and three inhibitory amine buffers (pH adjusted with HCl) at 75 °C. Activity is expressed as a percentage of activity in 0.1 M-sodium phosphate at pH 6.2. By shortening assay times to 2 min at pH values less than 6 and greater than 8 all assays were linear with time. Abbreviation: Tea, triethanolamine.

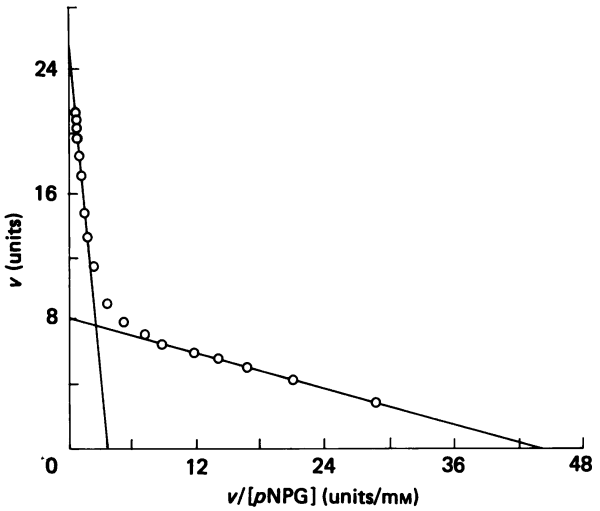

Fig. 2. Woolf–Hofstee plot of Michaelis–Menten data

β-Glucosidase (approx. 0.2 μg/ml) was assayed with 0.1–40 mM-*p*NPG at 75 °C at pH 6.2.

acetate, Mes and Mops appeared to have little effect on activity, but Tris, triethanolamine and Hepps buffers inhibited the enzyme. Imidazole was also inhibitory, whereas histidine caused a slight activation (results not shown). The inhibition by Tris has been observed for several glycosidases, and has been attributed to changes in conformation or charge distribution (Jorgensen & Jorgensen, 1967). As the percentage inhibition by amine buffers of the Wai21W.2 β-glucosidase increases with increasing pH, it may be that the unprotonated amine group complexes more readily with enzyme residues to cause inhibition. The enzyme is less sensitive to Tris than are some other β-glucosidases (McHale & Coughlan, 1981; Ait *et al.*, 1982).

Effect of substrate concentration

Graphing the effect of variations in substrate concentration by using Lineweaver–Burk, Hanes and Woolf–Hofstee (Fig. 2) inversions resulted in biphasic plots of the same form for *p*NPG, *o*NPG and cellobiose substrates. For homogeneous enzymes, two possible explanations for the non-linear plots are homotropic negative co-operativity (Koshland, 1970) and hysteretic co-operativity (Frieden, 1979). Homotropic co-operativity has been proposed to account for some non-linear reciprocal plots of β-glucosidases (e.g. Hirayama *et al.*, 1980; Bodenmann *et al.*, 1985). However, the explanation of hysteretic co-operativity is consistent with monomeric enzymes that display this form of plot. Nari *et al.* (1984) has demonstrated strong negative co-operativity in a hysteretic monomeric β-glucosyltransferase (which also possessed β-glucosidase activity) with *p*NPG as a substrate. Other kinetically based mechanisms cannot be discounted.

An alternative explanation for this type of kinetic behaviour is that two isoenzymes with different kinetic parameters are present. This is apparently ruled out by the homogeneity of the purified β-glucosidase and the similar half-life values for the enzyme when residual activity from incubation at 75 °C is assayed with high or low *p*NPG concentrations (see Table 4). It is possible, however, that small modifications (either natural or

sustained during purification) to the enzyme could produce two populations with differing K_m values (Cleland, 1970) at 75 °C that would not possess different thermostabilities or be separable by the methods used to establish homogeneity. On the basis of this assumption of two enzymes, the method of Spears *et al.* (1971) was used to determine two apparent K_m and $V_{max.}$ values for *p*NPG and cellobiose substrates (Table 5).

The low apparent K_m values found for the Wai21W.2 β-glucosidase activity are similar to those of many fungal β-glucosidases (Schliemann, 1983). The high apparent K_m values are more typical of bacterial enzymes (e.g. Ait *et al.*, 1982; Mitchell *et al.*, 1982; Ohmiya *et al.*, 1985). No substrate inhibition was observed for the three substrates, in contrast with many fungal enzymes (Hong *et al.*, 1981), but consistent with bacterial β-glucosidases. The general observation that K_m (*p*NPG) < K_m (cellobiose) for β-glucosidase is also seen for the Wai21W.2 enzyme.

Inhibitors and activators

Table 2 shows the effects of various agents on β-glucosidase activity. Inhibition by group I and II metal ions is small and consistent with other studies. In comparison with other microbial β-glucosidases, the Wai21W.2 enzyme is equally or more strongly inhibited by heavy-metal ions. The strong inhibition by Co^{2+} is not typical, although some sensitivity to this ion was also seen for the *Candida pelliculosa* β-glucosidase (Kohchi *et al.*, 1985). Heavy-metal-ion inhibition may be due to complex-formation with and/or catalysis of oxidation of specific residues, e.g. thiol groups, or simply the result of non-specific salt formation. Inhibition by the thiol-specific inhibitor *p*-chloromercuribenzenesulphonic acid is comparatively strong and, as there is no preincubation of enzyme and inhibitor, inactivation must occur rapidly at 75 °C, indicating the presence of essential thiol groups in the enzyme. Similar results with thiol-specific inhibitors have led to the same conclusion for many β-glucosidases (e.g. Han & Srinivasan, 1969; Sano *et al.*, 1975; Ait *et al.*, 1982; Ohmiya *et al.*, 1985). The reducing agents 2-mercaptoethanol, dithiothreitol and cysteine hydrochloride all activated the enzyme, possibly by reducing oxidized thiol groups in a proportion of the enzyme molecules that would otherwise be less active. The effects of 2-mercaptoethanol and EDTA were not additive. Similar activation was observed for β-glucosidases from the anaerobic bacteria *Ruminococcus albus* (Ohmiya *et al.*, 1985) and *Bacteroides succinogenes* (Forsberg & Groleau, 1982). The effects of glycerol can be attributed to β-glucosyltransferase activity, but ethanol is either an unsuitable acceptor for this reaction or causes simultaneous inactivation. Resistance to SDS-induced inactivation seen here has been observed in other thermostable β-glucosidases (Yoshioka & Hayashida, 1980; McHale & Coughlan, 1981).

Table 2. Inhibitors and activators of Wai21W.2 β-glucosidase

All assays were started by addition of enzyme to a solution containing the modifier and 40 mM-*p*NPG equilibrated at 75 °C at pH 6.2. When metal ions were included in the assay tube, 0.1 M-Mops/NaOH was substituted for 0.1 M-sodium phosphate in an attempt to minimize chelation of the metal ions by buffer salts. The following had no effect: 3 mM-Na^+, 3 mM-K^+, 3 mM-NH_4^+, 5 mM-Cl^-, 5 mM-NO_3^-, 5 mM-SO_4^{2-}.

Modifier	Concentration	Activity (%)
Ag^+, Hg^{2+}, Cu^{2+}	1 mM	0
Co^{2+}	1 mM	2
Ni^{2+}	1 mM	20
Cd^{2+}	1 mM	21
Al^{3+}	1 mM	30
Mn^{2+}	1 mM	85
Ba^{2+}	3 mM	80
Ca^{2+}	3 mM	83
Mg^{2+}	3 mM	89
Br^-	5 mM	115
I^-	5 mM	140
EDTA (disodium salt)	5 mM	120
2-Mercaptoethanol	1 mM	130
	5 mM	134
Dithiothreitol	1 mM	125
	5 mM	132
Cysteine hydrochloride	5 mM	125
Glycerol	1% (w/v)	111
	10% (w/v)	125
Ethanol	5% (w/v)	80
	25% (w/v)	5
SDS	1% (w/v)	13
Triton X-100	1% (w/v)	75
p-Chloromercuribenzenesulphonic acid (sodium salt)	1 mM	3
	0.05 mM	70

At low *p*NPG concentrations (0.2–0.8 mM) glucose was a poor inhibitor, with glucose/*p*NPG molar ratios of the order of 10^3:1 required for 50% inhibition. At *p*NPG concentrations greater than 10 mM up to 1.5 M-glucose failed to inhibit the enzyme. Wai21W.2 β-glucosidase is unusual in this respect, as most microbial β-glucosidases show a K_i (glucose) of 0.5–10 mM when assayed with *p*NPG. Exceptions include the *Clostridium thermocellum* enzyme, with a K_i (glucose) of 135 mM (Ait *et al.*, 1982).

β-Glucosyltransferase activity

Glycerol caused activation of the enzyme when assayed with *p*NPG (Table 2); no activation was seen with 200 mM-cellobiose as a substrate. It has been suggested that glycerol and other alcohols can act as alternatives to a water molecule in accepting the β-glucosyl moiety from the enzyme–β-glucosyl complex that forms during β-glucosidase catalysis (Umezurike, 1981). Thus, provided that the breakdown of this complex is rate-limiting (Umezurike, 1978), enhanced reaction rates could be expected if an acceptor superior to water were added to the assay solution. This effect has been demonstrated for a few β-glucosidases (Jermyn, 1966; Umezurike, 1978, 1981; Blondin *et al.*, 1983; Gondé *et al.*, 1985). The absence of any increase in the number of glucose molecules formed from cellobiose can be explained by proposing that half the glucose produced from the faster β-glucosyltransferase reaction is incorporated into a glyceryl β-D-glucopyranoside. This glucose is unmeasured by the enzymic assay. In addition, the ability of cellobiose to act as an acceptor (see below) may contribute to the lack of glycerol activation.

The enzyme was incubated with 200 mM-cellobiose at 75 °C at pH 6.2 for 1 h, and the products were analysed by h.p.l.c. Apart from glucose, the other major product was a trisaccharide, indicating that cellobiose is a suitable acceptor for the β-glucosyl group. A similar

observation was made for the β-glucosidase from *Fusarium solari* (Wood, 1971) and many others (Meyer & Canevascini, 1981). This result shows that the β-glucosyltransferase activity can mask some cellobiase or other glucose-releasing activity (as determined by the enzymic assay of glucose) when the substrate acts as an acceptor. In this way β-glucosyltransferase activity may contribute to the non-linearity of Lineweaver–Burk plots for cellobiose.

Substrate specificity

β-Glucosidases may be divided into three groups on the basis of substrate specificity. Aryl β-glucosidases hydrolyse exclusively, or show a great preference towards, aryl β-glucosides (Meyer & Canevascini, 1981), cellobiases hydrolyse oligosaccharides (including cellobiose) only (e.g. Bucht & Eriksson, 1969; Lusis & Becker, 1973; Rodionova *et al.*, 1977), and members of the third group, termed broad-specificity β-glucosidases, show significant activity on both substrate types and are the most commonly observed group in cellulolytic microbes (Schliemann, 1983; Sharrock, 1985).

The pattern of substrate specificity seen for the Wai21W.2 enzyme (Table 3) includes an inability to hydrolyse α-linkages or cleave glucose from Avicel or CM-cellulose. These results are typical of purified β-glucosidases, although some are capable of releasing glucose from CM-cellulose and other highly polymerized cellulosic substrates (e.g. Saddler & Khan, 1981; Beldman *et al.*, 1985). Some β-glucosidases from bacteria are able to hydrolyse all four of the β-linked glucose dimers, sophorose (β-1,2), laminaribiose (β-1,3), cellobiose (β-1,4), and gentiobiose (β-1,6), albeit at different rates. β-1,2- or β-1,3-linkages are usually most susceptible, and β-1,6-bonds the least (e.g. Ait *et al.*, 1982), although in some cases gentiobiose is not hydrolysed at all (Han & Srinivasan, 1969; Blondin *et al.*, 1983). The Wai21W.2 β-glucosidase is capable of cleaving all β-linkages with the order of rates of hydrolysis β-1,2 > β-1,4 > β-1,6. The enzyme's ability to cleave terminal β-1,3-linkages is indicated by the release of glucose from the mixed gluco-polysaccharide laminarin, which consists predominantly of β-1,3-linkages, with a few β-1,6-linkages. Other β-glucosidases can hydrolyse laminarin (e.g. Shepherd *et al.*, 1981; Bodenmann *et al.*, 1985).

The enzyme was active on nitrophenyl β-xyloside and β-galactoside substrates. The galactose epimers *o*-nitrophenyl β-galactoside and lactose were hydrolysed at 80% and 88% of the rate seen for the glucose epimers *o*NPG and cellobiose respectively. This result is similar to that found for the *Clostridium thermocellum* enzyme (Ait *et al.*, 1982), although greater discrimination between the two substrate epimers was observed in that case. Many other microbial β-glucosidases show limited β-xylosidase (e.g. Beldman *et al.*, 1985; Uziie *et al.*,

Table 3. Substrate specificity of Wai21W.2 β-glucosidase

Assay solutions contained 40 mM concentrations of all soluble substrates of known M_r and a known concentration of β-glucosidase in 0.5 ml of assay buffer (0.1 M-sodium phosphate buffer, pH 6.2). Laminarin, CM-cellulose and Avicel (crystalline cellulose) were included at 1.37% (w/v) concentrations. Arbutin interfered with the enzymic assay of glucose. Assays were conducted at 70 °C so that they could be run for longer times on some potentially poor substrates without significant denaturation. Rates of substrate hydrolysis were calculated assuming a reaction stoichiometry of 1 mol of measured product per mol of substrate hydrolysed, with the exception of the glucose dimers, for which a 2:1 ratio was used. Such assumptions may not be completely valid if significant β-glucosyltransferase activity occurs. Actual rates of hydrolysis for *p*NPG and cellobiose substrates were 183 nmol/min per μg and 56 nmol/min per μg respectively.

Substrate	Activity (% of that on cellobiose)	Activity (% of that on *p*NPG)
p-Nitrophenyl β-D-glucopyranoside	325	100
p-Nitrophenyl β-D-xylopyranoside	19	6
o-Nitrophenyl β-D-glucopyranoside	96	30
o-Nitrophenyl β-D-galactopyranoside	77	24
Methyl β-D-glucopyranoside	0	0
Phenyl β-D-glucopyranoside	16	5
Salicin	16	5
Esculin	21	6.5
Sophorose	121	37
Cellobiose	100	31
Gentiobiose	19	6
Laminarin	6.5	2
Lactose	88	26
Maltose	0	0
Sucrose	0	0
CM-cellulose (sodium salt)	0	0
Avicel (crystalline cellulose)	0	0

Table 4. $t_{\frac{1}{2}}$ values for Wai21W.2 β-glucosidase

β-Glucosidase (1.5–3.75 μg/ml) was equilibrated at room temperature for about 30 min in the solutions described below. After a zero-time sample had been taken for assay, the tube was immersed in a water bath of appropriate temperature and further samples were subsequently assayed. Residual activity was measured at 75 °C at pH 6.2 (0.105 M-sodium phosphate), with 40 mM-*p*NPG as substrate unless otherwise indicated. Temperatures were controlled to within ±0.2 °C.

Conditions of enzyme incubation	$t_{\frac{1}{2}}$
0.105 M-Phosphate buffer, pH 6.2, 65 °C	45 h
0.105 M-Phosphate buffer, pH 6.2, 75 °C	47 min
Assayed with 0.8 mM-*p*NPG	50 min
Assayed with 0.2 M-cellobiose	54 min
0.1 M-Mops/NaOH buffer, pH 6.2, 75 °C	49 min
0.015 M-Phosphate buffer, pH 6.2, 75 °C	50 min
0.10 M-Phosphate buffer, pH 6.2, 75 °C	
+10% (w/v) glycerol	400 min
+0.2 M-Cellobiose	330 min
+5 mM-Dithiothreitol	180 min
+5 mM-2-Mercaptoethanol	130 min
+5 mM-EDTA	95 min
0.1 M-Mops/NaOH buffer, pH 7.5, 75 °C	72 min
0.1 M-Mops/NaOH buffer, pH 7.0, 75 °C	130 min
0.1 M-Mes/NaOH buffer, pH 5.5, 75 °C	10 min
0.1 M-Acetic acid/NaOH buffer, pH 5.0, 75 °C	1.5 min
0.105 M-Phosphate buffer, pH 6.2, 85 °C	1.4 min

Table 5. Summary of β-glucosidase properties

Data on the β-glucosidases come from the following references: Ait *et al.* (1979, 1982) for *Clostridium thermocellum*; Han & Srinivasan (1969) for *Alcaligenes faecalis*; McHale & Coughlan (1981, 1982) for *Talaromyces emersonii*. The β-glucosidase from the last-mentioned microbe is β-glucosidase IV. To calculate the ratio of rates of hydrolysis for two substrates, individual hydrolytic rates were calculated as for Table 3.

Property	Wai21W.2	*Clostridium thermocellum*	*Alcaligenes faecalis*	*Talaromyces emersonii*
Multiplicity	2	1	1	4
Location	Cell-associated	Periplasmic	Cell-associated	Intracellular
pI	4.55	4.68	–	(4.41) 4.47 (4.50)
M_r	43000	~ 43000	120000–160000	52500
Optimum pH	6.2	6.0–6.5	6.0–7.0	5.7
K_m (mM)				
*p*NPG	0.15/11	2.6	0.125	0.81
Cellobiose	0.73/49	83	–	1.47
$V_{max.}$ (units/μg)				
*p*NPG	67/186	14.3	–	19.9
Cellobiose	35/228	7.1	–	79.8
Substrate inhibition	No	No	–	2.5 mM-Cellobiose
Glucose inhibition	No	Yes	Yes	Yes
K_i (mM)	–	135	3	52
Essential thiol groups	Yes	Yes	Yes	Possibly
Glucosyltransferase activity	Yes	–	–	Yes
Glucose dimer hydrolysis	β-1,2 > β-1, 4 > β-1,6; action on β-1,3 likely	β-1,3 > β-1,2 > β-1,4 > β-1,6	β-1,4 > β-1,3 > β-1,2; no action on β-1,6	–
Ratio of the rates of hydrolysis for:				
*p*NPG/cellobiose	3.2	15.5	Active on both substrates	0.52
Lactose/cellobiose	0.88	0.82	0.17	–
E_a (kJ/mol) (*p*NPG)	60	44.3	39.7	157
$t_{\frac{1}{2}}$				
58 °C	–	–	5 min	–
65 °C	45 h	–	–	–
68.5 °C	10.5 h	63 min	–	–
70 °C	5.5 h	–	–	2 min
75 °C	47 min	–	–	–
85 °C	1.4 min	–	–	–

1985) and/or β-galactosidase activity (e.g. Han & Srinivasan, 1969; Blondin *et al.*, 1983). The order of rates of hydrolysis of the aryl β-glucoside substrates roughly parallels the electron-withdrawing power of the aryl group with respect to the developing phenoxide anion during the first step of catalysis. This would explain the enzyme's inactivity towards methyl β-glucoside, but also requires that this first step be rate-limiting.

The results demonstrate that the enzyme is a broad-specificity β-glucosidase. Although the β-1,4-glucan glucohydrolase activity (EC 3.2.1.74) of the enzyme was not examined, the hydrolysis of laminarin suggests that some action on cello-oligosaccharides is likely.

Effects of temperature on activity

Arrhenius-plot data were determined from 40 °C to 75 °C for three substrate conditions at pH 6.2. When assayed with 40 mM-*p*NPG, the Arrhenius plot was linear with an E_a (activation energy) of 60 kJ/mol. With 0.8 mM-*p*NPG and 200 mM-cellobiose substrates, the plots were apparently biphasic, with high-temperature E_a values (assessed from 60–75 °C data points) of 31 kJ/mol and 41 kJ/mol respectively. Below 60 °C the E_a of the Wai21W.2 β-glucosidase was very similar for all substrate conditions. The concave-downward shape of the plots was not due to denaturation, which was insignificant over the period of the assays. E_a values for microbial β-glucosidases with *p*NPG as a substrate typically range from 29.4 kJ/mol (Kilian *et al.*, 1985) to 79.5 kJ/mol (Mahadevan & Eberhart, 1964).

β-Glucosidase thermostability

Under all conditions tested inactivation of β-glucosidase followed first-order kinetics, and half-life values (Table 4) were calculated from linear plots of log(percentage activity remaining) versus time. Similar linear decay plots have been observed for some purified β-glucosidases (e.g. Han & Srinivasan, 1969; Ait *et al.*, 1982), although in other cases non-linearity has been seen (Lusis & Becker, 1973; Garibaldi & Gibbins, 1975). Comparisons of the thermostability of different β-glucosidases are complicated by the variety of methods used to assess this property. Also, although thermostability is often determined at the optimum pH for activity, in some cases greater stability can be observed at a higher pH (e.g. Sternberg *et al.*, 1977; Tong *et al.*,

1980; Macris, 1984). This is the case for the Wai21W.2 β-glucosidase, which exhibited greatest thermostability at pH 7, 0.8 unit above the optimum pH for activity. At this pH $t_{\frac{1}{2}}$ (75 °C) was 130 min, indicating a thermostability second only to that of the partially purified *Mucor miehei* enzyme, which does not show first-order inactivation kinetics (Yoshioka & Hayashida, 1980). Half-life values for β-glucosidases from other thermophiles include $t_{\frac{1}{2}}$ (68.5 °C) 63 min for *Clostridium thermocellum* (Ait *et al.*, 1982), $t_{\frac{1}{2}}$ (65 °C) 600 min for *Thermoanaerobacter ethanolicus* (Mitchell *et al.*, 1982) and $t_{\frac{1}{2}}$ (70 °C) 410 min for extracellular β-glucosidase I from *Talaromyces emersonii* (McHale & Coughlan, 1981). The β-glucosidase from *Thermoascus aurantiacus* retained approx. 68% activity after 60 min at 75 °C (Tong *et al.*, 1980) and is therefore only slightly less stable than the Wai21W.2 enzyme is at 75 °C at pH 7.0. The endo-β-1,4-glucanase activity of Wai21W.2 (assayed with CM-cellulose) has a $t_{\frac{1}{2}}$ (85 °C) of 120 min in culture supernatant at pH 7 (C. H. Sissons, K. R. Sharrock, R. M. Daniel & H. W. Morgan, unpublished work). Thus the thermostability of the β-glucosidase component is consistent with the observation that β-glucosidase is typically the least-stable member of the cellulolytic enzyme system (Hägerdal *et al.*, 1980; Reese & Mandels, 1980).

A deactivation energy of 380 kJ/mol for *p*NPG hydrolysis at pH 6.2 was calculated from a plot of $\log k_d$ versus $1/T$. Similar calculations for the β-glucosidases of the yeasts *Dekkera intermedia* and *Candida molischiana* demonstrated deactivation energies of 218 kJ/mol (Blondin *et al.*, 1983) and 280 kJ/mol (Gondé *et al.*, 1985) respectively.

The solution found to be most effective in stabilizing the enzyme was 10% (w/v) glycerol. This could be due to increased ordering of solvent-sheath water molecules by glycerol, which decreases the ability of water–protein complexes to unfold (Gekko & Timasheff, 1981). Similar effects within the cell environment could increase the stability of the β-glucosidase *in vivo*, as was found for the *Thermomonospora* enzyme (Hägerdal *et al.*, 1980). Cellobiose (0.2 M) was almost as effective as glycerol, possibly because of the protection of active-site residues that would otherwise be susceptible to inactivating reactions, although this concentration of substrate could also have a significant structuring effect on water. Substrate stabilization by cellobiose has also been observed for β-glucosidases from *Streptomyces* strains (Moldoveanu & Kluepfel, 1983) and *Bacteroides succinogenes* (Groleau & Forsberg, 1981). In the latter case it was suggested that cellobiose and dithiothreitol both diminished inactivation by protecting thiol groups against oxidation (Forsberg & Groleau, 1982). This is also a possibility for the Wai21W.2 enzyme, as dithiothreitol and 2-mercaptoethanol both caused a transitory stabilization of the enzyme for 1 h, after which a rapid decline in residual activity occurred. This decline is probably due to the rapid oxidation of the protective agents that would occur under experimental conditions (Stevens *et al.*, 1983), rendering them ineffective. However, the transitory stabilization indicates that oxidation of thiol groups contributes to the inactivation.

Table 5 summarizes some of the properties of the Wai21W.2 enzyme, and compares it with β-glucosidases from a mesophilic bacterium, *Alcaligenes faecalis*, a thermophilic bacterium, *Clostridium thermocellum*, and a thermophilic fungus, *Talaromyces emersonii*. A low apparent K_m for cellobiose at physiological concentrations of substrate and the high apparent $V_{max.}$ values would suggest a role in cellobiose hydrolysis. A high $V_{max.}$ and lack of substrate and product inhibition would also lower the amount of enzyme necessary to produce acceptable rates of cellobiose cleavage, and the increase in $V_{max.}$ at higher substrate concentrations would allow full use of the enzyme's catalytic potential (Cleland, 1970).

Apart from a high thermostability, which is consistent with the organism's environment, unusual kinetic plots and a lack of glucose inhibition, the Wai21W.2 enzyme displays properties that are similar to those of β-glucosidases from many other microbial sources.

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