Catalytic mechanism of a family 3 β-glucosidase and mutagenesis study on residue Asp-247

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A family 3 β-glucosidase (EC 3.2.1.21) from *Flavobacterium meningosepticum* has been cloned and overexpressed. The mechanistic action of the enzyme was probed by NMR spectroscopy and kinetic investigations, including substrate reactivity, secondary kinetic isotope effects and inhibition studies. The stereochemistry of enzymic hydrolysis was identified as occurring with the retention of an anomeric configuration, indicating a double-displacement reaction. Based on the k_{cat} values with a series of aryl glucosides, a Bronsted plot with a concave-downward shape was constructed. This biphasic behaviour is consistent with a two-step mechanism involving the formation and breakdown of a glucosyl–enzyme intermediate. The large Bronsted constant ($\beta = -0.85$) for the leaving-group-dependent portion (pK_a of leaving phenols > 7) indicates substantial bond cleavage at the transition state. Secondary deuterium kinetic isotope effects with 2,4-dinitrophenyl β-D-glucopyanoside, *o*-nitrophenyl β-D-glucopyanoside and *p*-cyanophenyl β-D-glucopyanoside as substrates were 1.17 ± 0.02, 1.19 ± 0.02 and 1.04 ± 0.02 respectively. These results support an S_N1-like mechanism for the deglucosylation step and an S_N2-like mechanism for the glucosylation step. Site-directed mutagenesis was also performed to study essential amino acid residues. The activities (k_{cat}/K_m) of the D247G and D247N mutants were 30000- and 200000-fold lower respectively than that of the wild-type enzyme, whereas the D247E mutant retained 20 % of wild-type activity. These results indicate that Asp-247 is an essential amino acid. It is likely that this residue functions as a nucleophile in the reaction. This conclusion is supported by the kinetics of the irreversible inactivation of the wild-type enzyme by conduritol-B-epoxide, compared with the much slower inhibition of the D247E mutant and the lack of irreversible inhibition of the D247G mutant.

Key words: Bronsted plot, family 3 glycohydrolase, *Flavobacterium meningosepticum*, secondary isotope effect, site-directed mutagenesis.

INTRODUCTION

β-Glucosidase (EC 3.2.1.21) catalyses the hydrolysis of the β-glucosidic linkages of glucosides. A large number of these enzymes from bacteria [1–3], fungi [4–7] and plants [8–10] have been purified and studied, and many of them have been cloned [11–15]. By computer analysis of protein sequences, β-glucosidase can be classified into families 1 and 3 of the glycoside hydrolases [16,17]. In addition to the biotechnological importance of this group of enzymes [18–20], study of their catalytic mechanisms provides a means of understanding the catalytic power of the enzyme.

Many mechanistic studies have been performed on β-glucosidase [21–25]. However, the studies have focused largely on the family 1 enzyme. Perhaps the most extensive study on a family 1 β-glucosidase (from *Agrobacterium faecalis*) was carried out by Withers' group. The techniques employed for elucidating the mechanism of the enzyme and the topology of the active site included pH-dependence, inhibition, secondary deuterium isotope effect and structure–reactivity studies [24], essential amino acid labelling with fluorosugars [26], reactions with deoxy substrate analogues [27], and site-directed mutagenesis [28,29]. On the basis of these studies, and those of many other groups, the general mechanistic framework of the family 1 enzymes was revealed. In summary, the members of this group are retaining enzymes, which catalyse the hydrolysis of their substrates with retention of the anomeric configuration. The catalytic reaction is a two-step, double-displacement mechanism involving two essential carboxylic acid residues, functioning as a general acid/base and a nucleophile. Although the action of family 3 enzymes is thought to be similar to that of family 1 enzymes, detailed kinetic investigations and site-directed mutagenesis of essential residues in the former have been much less reported, presumably, at least in part, due to the lack of availability of a suitable, cloned bacterial enzyme and the corresponding X-ray structure.

Recently, the potential for mechanistic studies on the family 3 hydrolases has increased greatly, owing to the complete resolution of the three-dimensional structure of a β-glucosidase from barley [30]. On the basis of this structure, Asp-285 and Glu-491 were suggested to be candidates for the nucleophile and the general acid/base catalyst respectively. Although the function as a nucleophile of Asp-285, which is conserved in the 'SDW' (Ser-Asp-Trp) sequences of family 3 enzymes, was supported by active-site affinity labelling [14,21], it has not yet been confirmed by means of site-directed mutagenesis. In order to gain a better understanding of the detailed mechanism and of the active-site topology of the family 3 hydrolases, a cloned and expressed enzyme is essential. The β-glucosidase from *Flavobacterium meningosepticum* is one suitable candidate. Complete purification of the *F. meningosepticum* β-glucosidase (denoted as fbgl) [31] and the cloning of its gene [32] have been reported. In the present paper we combine physical and chemical studies to investigate the catalytic mechanism of the enzyme, and propose Asp-247 as the essential nucleophile, as determined by site-directed mutagenesis.

Abbreviations used: CBE, conduritol-B-epoxide; CNP-Glc, 4-chloro-2-nitrophenyl β-D-glucopyranoside; DNP-Glc, dinitrophenyl β-D-glucopyranoside; fbgl, *Flavobacterium meningosepticum* β-glucosidase; MNP-Glc, *m*-nitrophenyl β-D-glucopyranoside; ONP-Glc, *o*-nitrophenyl β-D-glucopyranoside; PCP-Glc, *p*-cyanophenyl β-D-glucopyranoside; P-Glc, phenyl β-D-glucopyranoside; PKP-Glc, *p*-chlorophenyl β-D-glucopyranoside; PNP-Ara, *p*-nitrophenyl α-L-arabinopyranoside; PNP-Gal, *p*-nitrophenyl β-D-galactopyranoside; PNP-Glc, *p*-nitrophenyl β-D-glucopyranoside.

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EXPERIMENTAL

Materials

All micro-organisms were obtained from the Culture Collection and Research Center, Hsin-Chu, Taiwan. Buffers were purchased either from Sigma or from E. Merck. *p*-Nitrophenyl β-D-glucopyranoside (PNP-Glc) and *o*-nitrophenyl β-D-glucopyranoside (ONP-Glc) were purchased from Sigma Co. The rest of substrates used were synthesized from 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide (Aldrich Co.) via 2,3,4,6-tetra-*O*-acetylaryl-β-D-glucopyranoside and deacetylation to form the product [33,34]. The C-1-deuterated substrates were synthesized by reducing δ-gluconolactone to deuterated glucose with sodium amalgam [35], using methods described in the literature [33,34]. Each product was purified by silica-gel chromatography and obtained in crystalline form. All products were identified by NMR spectroscopy and characterized by melting point. Melting points determined for substrates were as follows: 2,5-dinitrophenyl β-D-glucopyranoside (2,5-DNP-Glc), 164–166 °C; 3,4-DNP-Glc, 150–151 °C; 4-chloro-2-nitrophenyl β-D-glucopyranoside (CNP-Glc), 158–159 °C; *m*-nitrophenyl-β-D-glucopyranoside (MNP-Glc), 165–167 °C; *p*-cyanophenyl β-D-glucopyranoside (PCP-Glc), 193–195 °C; *p*-chlorophenyl β-D-glucopyranoside (PKP-Glc), 174–176 °C. The ^{1}H-NMR spectra of C-1-deuterated 2,4-DNP-Glc, ONP-Glc and PCP-Glc are identical with those of the corresponding ^{1}H substrates, except for the absence of the anomeric proton and related coupling. No more than 5% contamination of the ^{2}H substrate with ^{1}H compound was estimated according to the integration of the anomeric proton region.

Construction of Asp-247 mutants

Mutagenesis was performed by the Quick Change method (Stratagene Co.), with the following oligonucleotide primers: D247N, 5′-CAATGGCTTTATCGTAACGAACTATACAGGAAT-3′ and 5′-ATTCCTGTATAGTTCGTTACGATAAAGCCATTG-3′; D247E, 5′-CAATGGCTTTATCGTAACGGAGTATACAGGAAT-3′ and 5′-ATTCCTGTATACTCCGTTACGATAAAGCCATTG-3′; D247G, 5′-CAATGGCTTTATCGTAACGGGCTATACAGGAAT-3′ and 5′-ATTCCTGTATAGCCCGTTACGATAAAGCCATTG-3′ (underlining shows the location of the mutation). DNA sequence analysis was performed by dye-terminator cycle sequencing on an ABI 310 sequencer.

Purification of cloned β-glucosidase and Asp-247 mutants

The processes for purification of the cloned wild-type β-glucosidase and of the D247N, D247E and D247G mutants were virtually identical. The procedure involved the application of ammonium sulphate fractionation and cation-exchange chromatographic steps. All purification steps were performed at ambient temperature (approx. 25 °C). The detailed procedure has been described previously [32].

Protein determination

The protein content of enzyme preparations was determined by the bicinchoninic acid (BCA) method, as described in the manufacturer's protocol (Sigma; BCA-1 kit for protein determination), combined with measurement of UV absorption at 280 nm. The $A^{1\%}$ (at 280 nm) is estimated to be 9.5.

Enzyme assays

β-Glucosidase activity was assayed with PNP-Glc or 2,4-DNP-Glc as substrate, by determining the amount of phenol released. All kinetic studies were performed by monitoring the production of phenols on a Hewlett Packard Model 8452A Diode Array Spectrophotometer, with a circulating water bath set at 37 °C.

NMR experiments

^{1}H-NMR spectra in ^2H_2O solution were obtained at 300 MHz on a Varian UNITY-300FT spectrometer. The enzyme for the NMR study was prepared by exchanging the buffer system into one containing deuteriophosphate (phosphate buffer exchanged with ^2H_2O; 50 mM phosphate; p^{2}H 7.1) via ultrafiltration. A 5 mM sample of DNP-Glc (substrate) was prepared in deuteriophosphate buffer (50 mM; p^{2}H 7.1). After recording the spectrum of the substrate (500 μl in a 5-mm NMR tube), 50 μl of deuteriophosphate-exchanged enzyme was added. The spectra were recorded subsequently.

CD spectra

CD spectra of wild-type and mutant β-glucosidases were obtained with a Jasco J-715 spectropolarimeter using a protein concentration of 25 μM. All spectra, with correction for the buffer background, were acquired from 200 to 250 nm.

Determination of K_m

The Michaelis constant was determined for each synthetic substrate from the Michaelis–Menten equation by non-linear regression analysis [36]. Rates were determined at 7–10 different substrate concentrations, ranging from approx. $0.2\times$ the K_m value determined ultimately to $4–6\times K_m$. The wavelengths employed and molar absorption coefficients ($\Delta\epsilon$; $M^{-1}\cdot cm^{-1}$, at pH 6.8) obtained at that wavelength for each glucoside were adapted from Withers' previous work [24], and were as follows: CNP-Glc, 425 nm, 3546; 2,4-DNP-Glc, 400 nm, 10910; 2,5-DNP-Glc, 440 nm, 4288; 3,4-DNP-Glc, 400 nm, 11009; PNP-Glc, 400 nm, 7280; ONP-Glc, 400 nm, 2170; MNP-Glc, 380 nm, 385; PCP-Glc, 270 nm, 3101; PKP-Glc, 278 nm, 580; phenyl β-D-glucopyranoside (P-Glc), 277 nm, 778.

Secondary kinetic isotope effect

Isotope effects were determined by measuring the ratio of the initial rates for ^{1}H and ^{2}H substrates. The initial rate applied for the isotope effect calculation was an average of at least seven rates measured carefully at high substrate concentrations ($8–10\times K_m$).

Reversible inhibition

Reactions were carried out with low substrate concentrations, which allow the Michaelis–Menten equation to be simplified to $v = V_{max}\cdot[S]/K_m$. Thus the reaction is first-order with regard to [S]. The apparent first-order rate constant, V_{max}/K_m, can be evaluated by a plot of $\ln(A_\infty - A_t)$ against t (where A_∞ and A_t are the absorbance of the aglycon product at the reaction end-point and at time t respectively), or by non-linear regression [36]. For mutant enzymes, owing to their low activities the initial velocities were obtained and employed for further calculation. Since inhibitors used for reversible inhibition were found to be competitive, the dissociation constant for the enzyme–inhibitor complex, K_i, was calculated from the effect of the inhibitor on the ratio of the two steady-state parameters:

$$(K/V)_+ = (K/V)_0\times(1+[I]/K_i)$$

where $(K/V)_+$ is the K_m/V_{max} ratio in the presence of inhibitor.

The parameter K/V was determined under first-order conditions ($[S] \ll K_m$).

Inactivation kinetics

Inactivation of the β-glucosidase by conduritol-B-epoxide (CBE) was performed by incubation of the wild-type enzyme (37.5 μM) with various concentrations of CBE (0–5 mM). Residual enzyme activity was determined at appropriate time intervals by addition of an aliquot of the inactivation mixture to phosphate buffer (50 mM, pH 6.8) containing 2,4-DNP-Glc and measurement of phenolate release. Pseudo-first-order rate constants at each CBE concentration (k_{obs}) were determined by fitting each curve to a first-order rate equation. Values for the inactivation rate constant (k_i) and the dissociation constant for the inactivators (K_i) were determined by fitting to the following equation (where I is CBE):

$$k_{obs} = k_i[I]/(K_i + [I]).$$

RESULTS AND DISCUSSION

Substrate specificity and reactivity

A variety of glycosides, including *p*-nitrophenyl β-D-mannopyranoside, *p*-nitrophenyl β-D-galactopyranoside (PNP-Gal), *p*-nitrophenyl β-D-*N*-acetylglucosaminide, *p*-nitrophenyl β-D-xylopyranoside, *p*-nitrophenyl α-L-arabinopyranoside (PNP-Ara) and α-PNP-Glc, and a series of aryl β-glucosides were employed for investigating the substrate specificity and the reactivity of the expressed wild-type enzyme. Of the substrates investigated, only the aryl β-D-glucopyranosides were hydrolysed effectively. The activities for non-glucoside substrates were less than 0.05% of that with PNP-Glc. This is in contrast with the substrate specificity of *Agrobacterium* β-glucosidase, a family 1 enzyme, which possesses noticeable activities towards PNP-Gal (2.5% of that with PNP-Glc) and PNP-Ara (4.6%) [24]. This broad glycoside specificity was also found in another family 1 β-glucosidase from sweet almond, which was considered to be a bi-functional enzyme with both β-glucosidase and β-galactosidase activities [37–39].

Although fbgl is highly specific with regard to the glycon moiety of the substrate, the enzyme shows a broad specificity for the aglycon portion. It accommodates β-D-glucosides with various aryl groups as the aglycon moiety. Kinetic parameters for the aryl β-D-glucopyranosides are summarized in Table 1. Although the K_m values for all substrates are comparable, the k_{cat} values differ by up to 2.6 orders of magnitude. Analysing these data allows us to investigate the catalytic mechanism of the enzyme by means of a linear free-energy relationship, namely the Bronsted relationship, which has been shown to be a valuable tool for rationalization of the mechanistic actions of enzymes [40–42]. Based on the k_{cat} values, an extended Bronsted plot can be constructed by plotting the logarithm of k_{cat} against the pK_a of the leaving phenol (Figure 1). A biphasic plot with a concave-downward trend was obtained, with a slope (β_{lg} value) of near zero at lower pK_a values (< 7.0) and one of -0.85 at higher pK_a values (> 7.0). Clearly, the enzymic reaction involves a two-step mechanism, with the formation of an intermediate, most likely a glucosyl–enzyme intermediate. Since substrates with good leaving phenols (such as DNP-Glc and CNP-Glc) show no significant dependence of their reactivity on the pK_a of the leaving phenol, the rate-limiting step of the reaction for good substrates must be the breakdown of the intermediate, whereas the rate-limiting step for poor substrates is the formation of the intermediate. The slope of the leaving-dependent portion of the Bronsted plot ($\beta_{lg} = -0.85$) indicates a large degree of glycosidic bond cleavage in the transition state.

Table 1 Michaelis constants and relative k_{cat} values for aryl β-D-glycopyransosides

K_m values were measured at pH 6.8.

Substrate	pK_a of leaving phenol	K_m (mM)	k_{cat} (s^{-1})	log k_{cat}
2,4-DNP-Glc	3.90	0.37	36.4	1.56
2,5-DNP-Glc	5.15	0.33	25.7	1.41
3,4-DNP-Glc	5.36	0.45	16.2	1.21
CNP-Glc	6.45	0.79	32.4	1.51
PNP-Glc	7.18	0.65	12.4	1.09
ONP-Glc	7.22	0.47	12.7	1.10
MNP-Glc	8.39	0.82	0.8	−0.10
PCP-Glc	8.49	0.75	0.7	−0.15
PKP-Glc	9.38	0.68	0.25	−0.61
P-Glc	9.99	1.10	0.09	−1.03

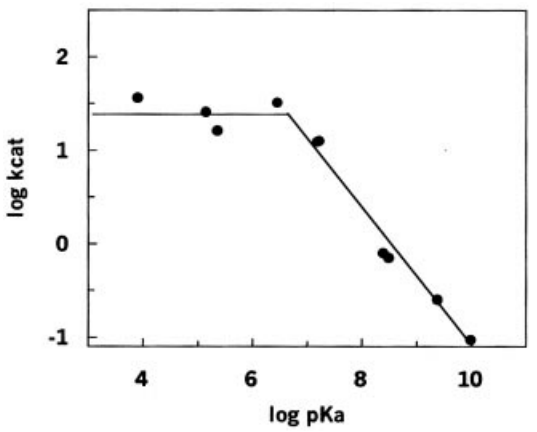


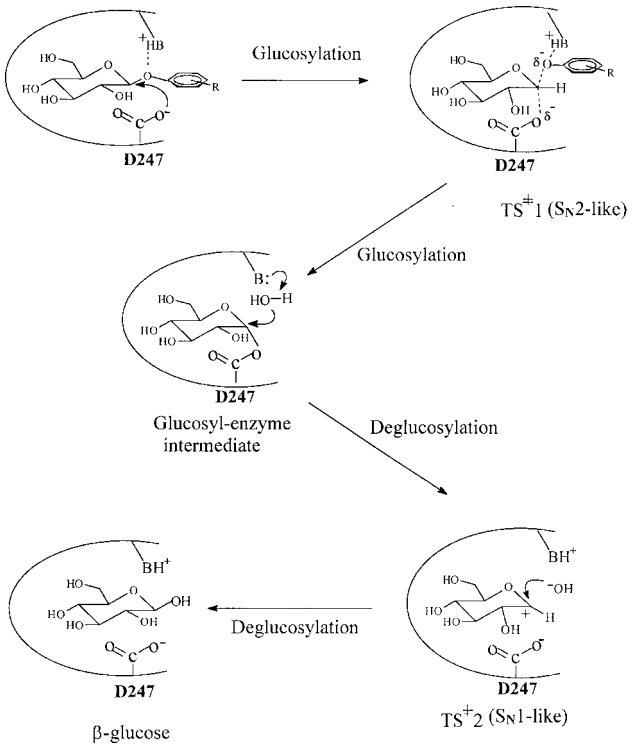
Figure 1 Bronsted plot of log k_{cat} against pK_a of the aglycon phenol

^{1}H-NMR analysis of stereoselectivity

^{1}H-NMR spectroscopy has been used successfully for investigation of the stereoselectivity of various glycohydrolases. Studies have revealed diversity in the mechanisms of glycohydrolase activity [40,43]. To understand better the catalytic action of fbgl, an NMR study of the stereoselectivity of the enzyme was carried out. In a time-course NMR study, spectra were recorded after the addition of fbgl in DNP-Glc. Based on analysis of the anomeric proton, it was revealed that the β-glucose (H-1 4.38 p.p.m., J 7.8 Hz) was formed first. The α-glucose (H-1 4.98 p.p.m., J 3.3 Hz) was detected later as a consequence of mutarotation. The experiment confirmed that fbgl is a 'retaining' enzyme, which is consistent with other family 3 β-glucosidases [43].

Secondary kinetic isotope effects

A kinetic α-deuterium isotope effect is considered as a useful tool for distinguishing between S_N1 and S_N2 mechanisms. It provides a means of investigating changes in hybridization at the substituted site when proceeding from the ground state to the transition state of the reaction. Isotope effects of $k_H/k_D > 1$ are expected for an S_N1-like mechanism (where k_H and k_D are values



Scheme 1 Proposed mechanism of family 3 β-glucosidases

See the text for details.

for ^{1}H and ^{2}H compounds respectively), whereas values near to unity are thought to indicate an S_N2-like reaction. Secondary deuterium kinetic isotope effects (k_H/k_D) upon deglycosylation of various glycosidases, such as β-galactosidase and β-glucosidase, have been shown to range from 1.25 to 1.09 [19–21]. For these enzymes, the transition state of the reactions was believed to possess substantial carbo-cation (or alternatively oxocarbonium ion) character.

In the present study, secondary ^{2}H kinetic isotope effects on the k_{cat} of the cloned β-glucosidase were measured carefully with DNP-Glc, ONP-Glc and PCP-Glc as substrates, and values of 1.17 ± 0.02, 1.19 ± 0.02 and 1.04 ± 0.02 respectively were obtained. The large isotope effect for good substrates provides strong evidence that the intermediate of the enzymic reaction is a covalent glucosyl–enzyme intermediate rather than an ion-pair complex, for which an inverse isotope effect would be expected. The large isotope effect also indicates that hydrolysis of the glucosyl–enzyme intermediate occurs via an S_N1-like mechanism, indicating that a relatively large amount of carbo-cation character is present at the transition state. In contrast with good substrates, a smaller isotope effect was observed for PCP-Glc (a poor substrate), suggesting that an S_N2-like mechanism (i.e. a relatively small amount of carbo-cation character at the transition state) is involved in the glucosylation step. This small kinetic isotope effect, combined with the observation of a large Bronsted coefficient ($\beta_{lg} = -0.85$) for leaving groups of $pK_a > 7$, suggest a relatively late transition state for the glucosylation step. There is substantial bond formation between the essential nucleophilic group on the enzyme and the C-1 position of substrate, and near-complete cleavage of the substrate's glycosidic bond in the transition state.

Table 2 Conserved sequence in family 3 β-glucosidases

Protein sequences are derived from the corresponding gene sequences in GenBank.

Species	Sequence	GenBank accession no.	Ref.
Kluyveromyces fragilis	D^{219}GMLM**SDW**FGT^{229}	X05918	[48]
Agrobacterium tumefaciens	D^{216}GVVM**SDW**FGS^{226}	M59852	[12]
Clostridium thermocellum	D^{226}GFVV**SDW**GAV^{236}	X15644	[13]
Barley	K^{279}GFVI**SDW**EGI^{289}	AF102868	–
Flavobacterium meningosepticum	N^{241}GFIV**TDY**TGI^{251}	AF015915	[32]

Scheme 1 illustrates the catalytic mechanism of fbgl. The glucosylation step involves an S_N2-like mechanism, with a relatively late transition state. The deglucosylation step, on the other hand, is more S_N1-like, with substantial carbo-cation (or its resonant oxocarbonium ion) character in the transition state ($TS^‡2$). This catalytic mechanism of fbgl is very similar to those of the family 1 enzymes. However, interestingly, as compared with the mechanism of *Aspergillus wentii* β-glucosidase, another well studied family 3 enzyme [44], a discrepancy is present with regard to substrate reactivity. A very small Bronsted constant (~ -0.05) and a large α-deuterium kinetic isotope effect (~ 1.1) were obtained for *A. wentii* β-glucosidase. These results indicated that the catalytic proton is completely transferred to the leaving phenols (pK_a 4–10) when the reaction approaches its transition state, and also that a glucosyl cation-like transition state is involved. Although this is consistent with the finding for fbgl when good substrates were employed, it is different from that with poor substrates. Since there are six subgroups within family 3, it is possible that fbgl and the *A. wentii* enzyme (for which the sequence is not available) may belong to very different subgroups, and therefore exhibit variations in catalytic mechanism.

Kinetics of Asp-247 mutant enzymes

The catalytic reaction of fbgl is identified as a two-step, double-displacement mechanism. Two amino acid residues, functioning as a general acid/base and a nucleophile, are involved in catalysis. Based on the multi-alignment of amino acid residues of some family 3 enzymes (Table 2), it is found that an 'SDW' sequence is highly conserved. The aspartate residue has been identified as the nucleophile in the *A. niger* [14] and *A. wentii* [21] β-glucosidases by active-site affinity labelling. Although the corresponding sequence in fbgl is 'TDY' (Thr-Asp-Tyr), it is similar in character to SDW. We therefore constructed a group of mutant enzymes in order to investigate the role of Asp-247 in fbgl. The aspartate residue was changed to asparagine, glutamate or glycine. These mutations had no significant effect on the protein's conformation, as indicated by the CD spectra (Figure 2).

All purified mutant enzymes were assayed against 2,4-DNP-Glc, and kinetic parameters are summarized in Table 3. The K_m values of all mutants were quite similar, with variation of only a few fold. The catalytic efficiencies (k_{cat}/K_m) of D247G and D247N were greatly decreased, by factors of 3×10^4 and 2×10^5 respectively. However, the D247E mutant retained at least 20 % activity as compared with the wild-type enzyme. These results provide evidence that Asp-247 plays an important role in the enzymic reaction catalysed by fbgl. It is possible that the low apparent activities might have been due to the physical contamination of mutants with a small quantity of wild-type enzyme. However, since new columns were used for the purification of each mutant, these low-level activities are unlikely to be due to

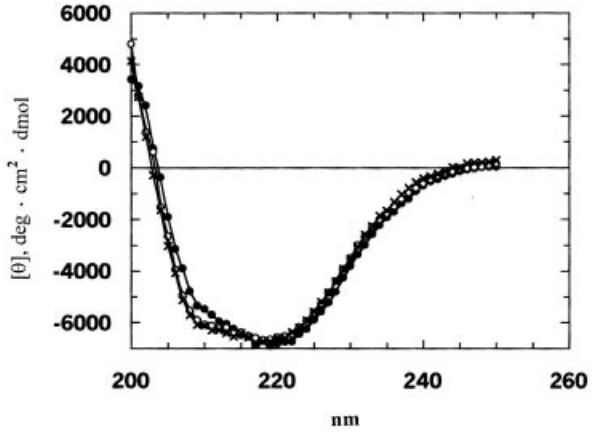


Figure 2 Comparison of the CD spectra of the native β-glucosidase ($\times$), and the D247N (○) and D247E (●) mutants

Table 3 Apparent activity of wild-type and mutant β-glucosidases towards 2,4-DNP-Glc

Measurements were carried out at pH 7.0 and 37 °C.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Wild type	0.37	36.4	98.4
D247E	0.24	4.8	20.0
D247N	1.50	7.0×10^{-4}	4.7×10^{-4}
D247G	0.23	7.1×10^{-4}	3.1×10^{-3}

Table 4 Inhibition constants (K_i) determined for wild-type and mutant β-glucosidases

	Inhibition constant		
Inhibitor	Wild type	D247E	D247G
δ-Gluconolactone	0.0015 mM	0.095 mM	0.012 mM
CBE	8.9 mM (K_i)†	9.4 mM*	6.2 mM*
	0.014 s^{-1} (k_i)†		

* Owing to the low inactivation rate, CBE was tested as a reversible inhibitor. The inhibition was competitive (results not shown).

† Values were obtained from analysis of the kinetic inactivation of CBE.

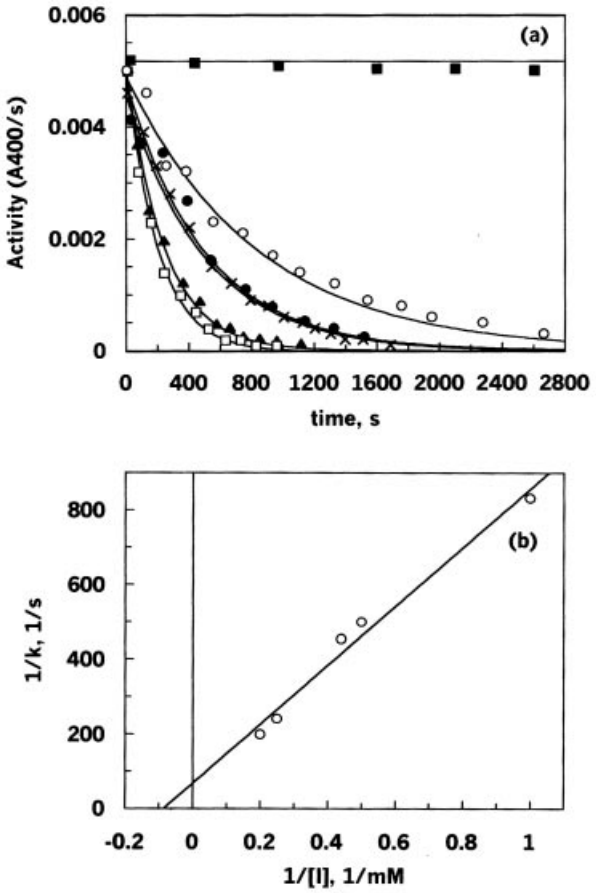


Figure 3 Inactivation of wild-type β-glucosidase and the D247E mutant by CBE

(**a**) Residual activity of the wild-type enzyme against time at various inactivator concentrations: 1 mM (○), 2 mM (●), 2.25 mM ($\times$), 4 mM (▲) and 5 mM (□). The inactivation of the D247E mutant by 8.5 mM CBE is also shown (■). (**b**) Double-reciprocal plot of the first-order rate constants obtained from the analysis of data in (**a**) by single exponential decay.

contamination by the wild-type enzyme through physical processes, although contamination resulting from translational misreading, or possibly spontaneous deamination, could not be ruled out, particularly for D247N. The activity of D247G was not caused by contamination by a minute amount of wild-type enzyme, because in this case the K_i values of the two enzymes with δ-gluconolactone would be comparable, which was not the case (see Table 4).

Inhibition study

CBE has been widely used as active-site-directed inhibitor of β-glucosidases [45–47]. In order to elucidate the catalytically essential amino acid residues, wild-type fbgl was inactivated by CBE. As shown in Figure 3(a), the inactivation is concentration-dependent. Based on the time-dependent inactivation of fbgl with 1–5 mM CBE, the kinetic parameters k_i, K_i and k_i/K_i were determined to be 0.014 s^{-1}, 8.9 mM and 1.6 $M^{-1} \cdot s^{-1}$ respectively (Figure 3b). However, on changing the Asp-247 residue into glutamate, the rate of inactivation of the D247E mutant was much lower than that of the wild-type enzyme, even with 8.5 mM CBE (shown at the top of Figure 3a). This suggests that Asp-247 is involved in catalysis, and most likely functions as the nucleophile. The substitution of this Asp residue in fbgl results in weaker binding of δ-gluconolactone (Table 4). In contrast, the reversible binding (K_i values) of CBE to the wild-type and mutant enzymes was nearly identical (Table 4), and comparable with the binding of glucose to fbgl (5 mM). It is interesting to note that, while the binding of δ-gluconolactone shows more sensitivity to the mutation of Asp-247 than does binding of CBE or glucose, this sensitivity does not parallel that of k_{cat}/K_m. Thus conversion of Asp-247 into Glu reduces the catalytic efficiency by a factor of 5, but the binding constant ($= 1/K_i$) of the lactone is reduced by a factor of 63. An even more dramatic difference is seen with the D247G mutant, where the catalytic efficiency is reduced by 3×10^4-fold and the binding constant for the lactone is reduced by only 8-fold. This lack of a parallel effect of mutation on k_{cat}/K_m and on inhibitor binding argues against the gluconolactone being a transition-state analogue.

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