Identification of the two essential groups in the family 3 β-glucosidase from *Flavobacterium meningosepticum* by labelling and tandem mass spectrometric analysis

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β-Glucosidase from *Flavobacterium meningosepticum* (Fbgl) catalyses the hydrolysis of β-1,4-glucosidic bonds via a two-step double-displacement mechanism in which two amino acid residues act as nucleophile and acid/base catalyst. Definitive identification of these two residues is provided by the two active-site-directed inactivators, 2′,4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucoside (2FDNPG) and *N*-bromoacetyl-β-D-glucosylamine (NBGN), which stoichiometrically label the nucleophile and the acid/base catalyst of Fbgl, respectively. Pseudo-first-order inactivation rate constants (k_i) of 0.25 ± 0.01 and 0.05 ± 0.01 min^{-1} and dissociation constants (K_i) of 90 ± 15 and 4.4 ± 0.2 mM are determined for 2FDNPG and NBGN, respectively. Proteolytic digestion of the labelled proteins, followed by peptide mapping and tandem MS analysis identify Asp-247 and Glu-473 as the catalytic nucleophile and acid/base residues, respectively, of Fbgl. This study confirms that the catalytic nucleophile of family 3 glycohydrolase is conserved across sub-families. However, different sub-families may have unique general acid/base catalysts.

Key words: electrospray ionization, general acid/base, nucleophile, peptide mapping.

INTRODUCTION

β-Glucosidases (EC 3.2.1.21) catalyse the hydrolysis of β-1,4-glucosidic bonds of a variety of glucosides. This group of enzymes has been classified into glycohydrolase families 1 and 3 based on amino acid sequence similarities [1,2]. Enzymes in both families are known to retain glycosidases using a two-step, double-displacement mechanism involving the formation and breakdown of a covalent glycosyl-enzyme intermediate via oxocarbenium-ion-like transition states. Two active-site carboxylic acids are involved in catalysis. One residue functions as the catalytic nucleophile, attacking at the substrate anomeric centre to form a covalent α-D-glucosyl enzyme intermediate. The other carboxyl group acts as an acid/base catalyst, protonating the glycosidic oxygen in the first step and deprotonating the nucleophilic water molecule in the second step. Considerable evidence has been accumulated in support of these mechanisms [3–7].

Although β-glucosidases have been known about and studied for several decades, studies of the mechanism [8–11], identification of essential groups [12–15] and X-ray structure determinations [16–18] have largely focused on family 1 enzymes. The mechanism of family 3 β-glucosidases has been the subject of much less study [19,20]. Recently, the first structure of a family 3 enzyme, the β-glucosidase from barley, was solved at 2.2 Å resolution [21,22], providing valuable insights into the active sites of family 3 glycohydrolases. Several highly conserved residues, including Asp-95, Arg-158, Lys-206, His-207 and Asp-285 (barley enzyme numbering), are present in the active site in appropriate positions to interact with hydroxyl groups on the sugar ring, while Asp-285, in the conserved sequence SDW, is located appropriately to serve as the catalytic nucleophile. Chemical identification of the catalytic nucleophile in family 3 glycosidases has only been reported in a few cases, these being barley β-glucosidase [22] and two fungal β-glucosidases, from *Aspergillus wentii* [23] and *Aspergillus niger* [24]. The residues so assigned are consistent with the barley enzyme structure. Although we demonstrated recently by site-directed mutagenesis and kinetic analysis that Asp-247 (in the sequence TDY) in *Flavobacterium* β-glucosidase may function as the nucleophile [19], the first labelling study on the nucleophile of this bacterial family 3 β-glucosidase was performed in this study and is reported herein. In general, the identification of the acid/base catalyst of glycohydrolase is even more difficult than the identification of the nucleophile. No successful labelling study on the acid/base catalyst of a family 3 enzyme has been demonstrated so far.

2-Deoxy-2-fluoro-β-D-glycosides have been used successfully in the identification of the nucleophilic residues of many retaining glycosidases [24–27] since they form stable glycosyl-enzyme intermediates that are amenable to analysis. The fluorine substituent at position C-2 of the glycoside substrate slows down both the glycosylation and deglycosylation steps, whereas the good leaving group (typically 2,4-dinitrophenol or fluoride) speeds up the glycosylation step, allowing accumulation of the intermediate, and thus labelling of the nucleophile. The general acid/base catalyst in retaining glycosidases has been labelled in several cases using conduritol epoxides [28] or *N*-bromoacetyl-β-D-glycosylamines (NBGNs) [29,30]. Although conduritol epoxides, which incorporate an endocyclic epoxide within a cyclitol ring, are reasonable mimics of the sugar ring, these irreversible inhibitors are not fully specific [31–33], most likely due to the absence of a C5-hydroxymethyl substituent. NBGNs are a little more reliable and have been used to identify the acid/base catalyst in a cellulase [29], but identified a methionine in β-galactosidase [34]. In this study, 2′,4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (2FDNPG) and NBGN

Abbreviations used: Fbgl, *Flavobacterium meningosepticum* β-glucosidase; DNPG, 2′,4′-dinitrophenyl-β-D-glucopyranoside; 2FDNPG, 2′,4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside; NBGN, *N*-bromoacetyl-β-D-glucosylamine; 2FG, 2-deoxy-2-fluoro-β-D-glucosyl; NAGA, *N*-acetylglucosylaminyl; ES/MS, electrospray MS; MS/MS, tandem MS; a.m.u., atomic mass units.

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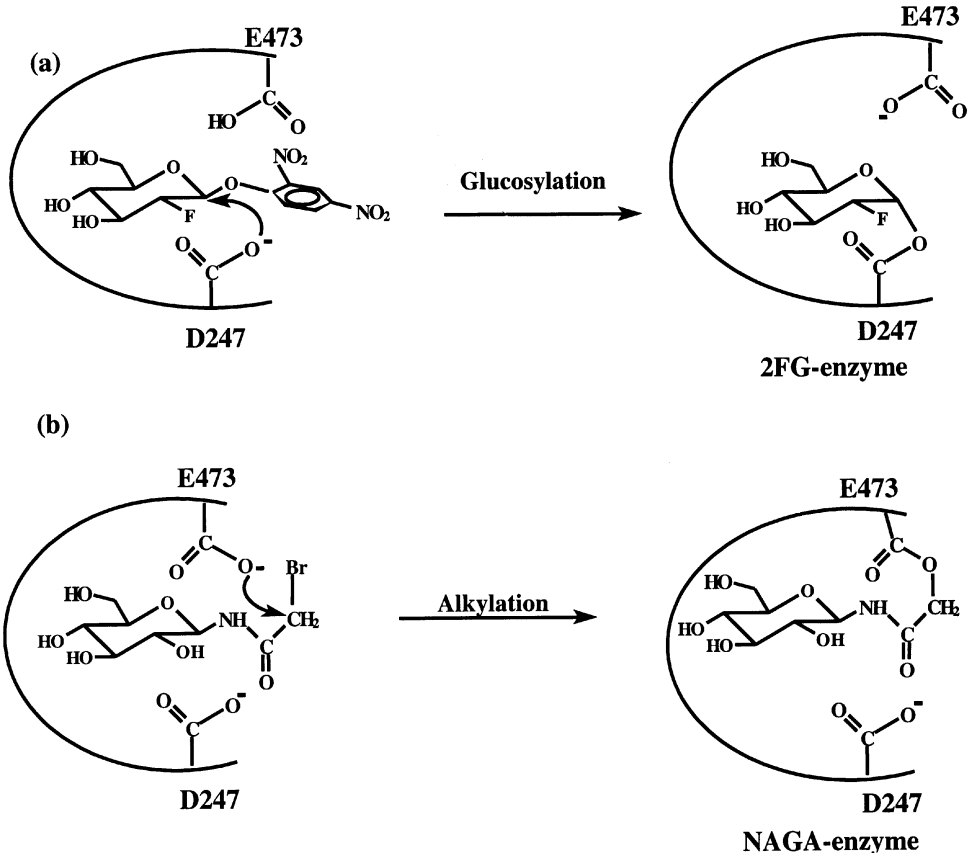

Scheme 1 Proposed inactivation mechanism of Fbgl by (a) 2FDNPG and (b) NBGN

are employed as active-site-directed inhibitors of *Flavobacterium meningosepticum* β-glucosidase (Fbgl), presumably functioning as shown in Scheme 1.

MATERIALS AND METHODS

General and synthesis

All micro-organisms were obtained from the Culture Collection and Research Center, Hsinchu, Taiwan. Buffers were purchased from either Sigma or from E. Merck Co. Sodium phosphate buffer (2.0 M, pH 1.7) was prepared by the addition of a concentrated NaOH solution to an aqueous solution of orthophosphoric acid until the desired pH was reached, as measured by a standard combination pH electrode. Sequencing-grade pepsin (Boehringer-Mannheim, Lewes, East Sussex, U.K.), trypsin and lysine C (Promega, Madison, WI, U.S.A.) were purchased as salt-free lyophilizates, and were freshly dissolved in double-distilled water prior to use. The syntheses of 2′,4′-dinitrophenyl-β-D-glucopyranoside (DNPG) [35] and the two inactivators, 2FDNPG [36] and NBGN [29], were carried out according to previous reports as referenced. The procedure for recombinant Fbgl purification involved $(NH_4)_2SO_4$ fractionation and cation-exchange chromatographic steps, as described in a previous paper [37].

Enzyme kinetics

Kinetic studies were performed at 37 °C, in 50 mM phosphate buffer, pH 7.0. A continuous spectrophotometric assay based on the hydrolysis of DNPG was used to monitor enzyme activity by measurement of the rate of 2,4-dinitrophenolate release ($\lambda = 400$ nm, $\epsilon = 1.07 \times 10^4$ $M^{-1} \cdot cm^{-1}$) using an HP8452 UV-visible spectrophotometer. The inactivation of Fbgl by 2FDNPG and NBGN was monitored by incubation of the enzyme under the above conditions in the presence of several concentrations (1.5, 2.0, 2.5, 4.0 and 5.0 mM) of 2FDNPG and (0.5, 1.25, 2.5, 5 and 7.5 mM) of NBGN. Residual enzyme activity was determined at appropriate time intervals by addition of an aliquot (10 μl) of the inactivation mixture to an assay solution (500 μl) containing DNPG (0.2 mM), pH 7.0. Pseudo-first-order rate constants at each inactivator concentration (k_{obs}) were determined by fitting the residual activity ($\ln V/V_o$; where V and V_o represent the maximum initial velocity of the catalytic reaction in the presence and the absence of inhibitor, respectively) versus time with the program Enzfitter [38]. Values for the inactivation rate constant (k_i) and the dissociation constant for the inactivator (K_i) were determined by fitting to the equation: $k_{obs} = k_i[I]/(K_i + [I])$.

Labelling and pepsin digestion of Fbgl

Fbgl (100 μl; 3.5 μg/μl) in buffer (50 mM sodium phosphate, pH 7.0) was incubated for 90 min at room temperature with 20 μl of 2FDNPG (20 mM) or NBGN (100 mM). The residual enzyme activity was measured using a stopped-enzyme assay with DNPG as a substrate. Pepsin (40 μl; 0.5 μg/μl in sodium phosphate, 2.0 M, pH 1.7) was then added and the reaction mixture incubated at 25 °C for 30 min. Control digestion on unlabelled enzyme was carried out under identical conditions. The candidate

labelled peptide from the pepsin hydrolysate of the inactivated enzyme was isolated by HPLC using electrospray MS (ES/MS) detection as described below.

ES/MS

Mass spectra were recorded using a PE-Sciex API 300 triple-quadrupole mass spectrometer. Protein samples were injected into the mass spectrometer via an HPLC system equipped with a PLRP-S column (5 μm, 300 Å, 1 mm × 50 mm). As an eluent a 5–90 % acetonitrile gradient system containing 0.1 % trifluoroacetic acid was used over 3 min. Proteins used for molecular-mass measurement were normally in the range 5–10 μg. Peptides were separated by reversed-phase HPLC interfaced with the mass spectrometer. For HPLC/MS experiments, proteolytic digests of labelled or control enzyme were loaded on to a C_{18} column (Nova-Pak, 3.9 mm × 150 mm; Waters, Milford, MA, U.S.A.) and eluted with a gradient of 0–60 % solvent B over the course of 60 min at a flow rate of 0.5 ml/min (where solvent A is 0.05 % trifluoroacetic acid/2 % acetonitrile in water and solvent B is 0.045 % trifluoroacetic acid/80 % acetonitrile in water). A post-column flow splitter was used to direct 90 % of the sample into a fraction collector, while the remainder was sent to the mass spectrometer for analysis. The quadrupole mass analyser was scanned over a mass-to-charge ratio range of 300–2400 atomic mass units (a.m.u.), with a step size of 0.5 a.m.u. and a dwell time of 1 ms/step. In all ES tandem MS (MS/MS) peptide sequencing experiments, the MS/MS daughter-ion spectrum was obtained in the triple-quadrupole scan mode. Peptides previously fractionated by HPLC were introduced into the mass spectrometer either via a small column (Reliasil, 1 mm × 150 mm; Michrom BioResources) with a flow rate of 0.05 ml/min or via a nanospray ion source (Protana, Staermosegaardvej, Demark). Following mass selection in the first quadrupole (Q1), the peptide of interest was fragmented by collision with nitrogen gas in the second quadrupole (Q2) and the resulting daughter ions were analysed in the third quadrupole (Q3). In a typical experiment, the following settings were used: Q3 scan range of m/z 100–2400 a.m.u., step size of 0.5 a.m.u., dwell time of 1 ms/step, orifice potential of 50 V, focusing ring voltage of 400 V, Q0 potential of −10 V, Q2 potential of −55 V (collision energy = Q0 − Q2 = 45 V) and source voltage of 5 kV. The collision gas thickness was varied to obtain optimal fragmentation of the parent peptide.

RESULTS AND DISCUSSION

Inactivation kinetics

Incubation of Fbgl with either 2FDNPG or NBGN resulted in inactivation of the enzyme in a time-dependent manner according to pseudo-first-order kinetics. The data for the inactivation of Fbgl with 2FDNPG and with NBGN are shown in Figures 1 and 2, respectively. The inactivation rate constants (k_i) and dissociation constants (K_i) for the reaction of each inactivator with Fbgl are as follows: 2FDNPG, $k_i = 0.25 \pm 0.01$ min^{-1}, $K_i = 90 \pm 15$ mM; NBGN, $k_i = 0.05 \pm 0.01$ min^{-1}, $K_i = 4.4 \pm 0.2$ mM. Incubation with 2FDNPG (5 mM) in the presence of a competitive inhibitor δ-gluconolactone (1.5 μM, equal to the K_i value) reduced the apparent rate constant for inactivation from 0.014 ± 0.001 min^{-1} to 0.006 ± 0.001 min^{-1} (Figure 1c).

At the same concentration of δ-gluconolactone, a similar rate reduction was observed for the reaction of Fbgl with NBGN (7.5 mM), the inactivation rate changing from 0.038 ± 0.001 min^{-1} to 0.024 ± 0.001 min^{-1} (Figure 2c). This is the expected degree of protection against inactivation if both ligands compete with δ-gluconolactone at the same site, indicating that the inactivation is active-site-directed. Confirmation that, in both cases, inactivation is caused by reaction with a single inactivator molecule is provided by the ES/MS analysis of the protein: the molecular mass of wild-type β-glucosidase increased by 164 a.m.u. and 219 a.m.u. after incubation with 2FDNPG (results not shown) and NBGN (Figure 3A), respectively. These

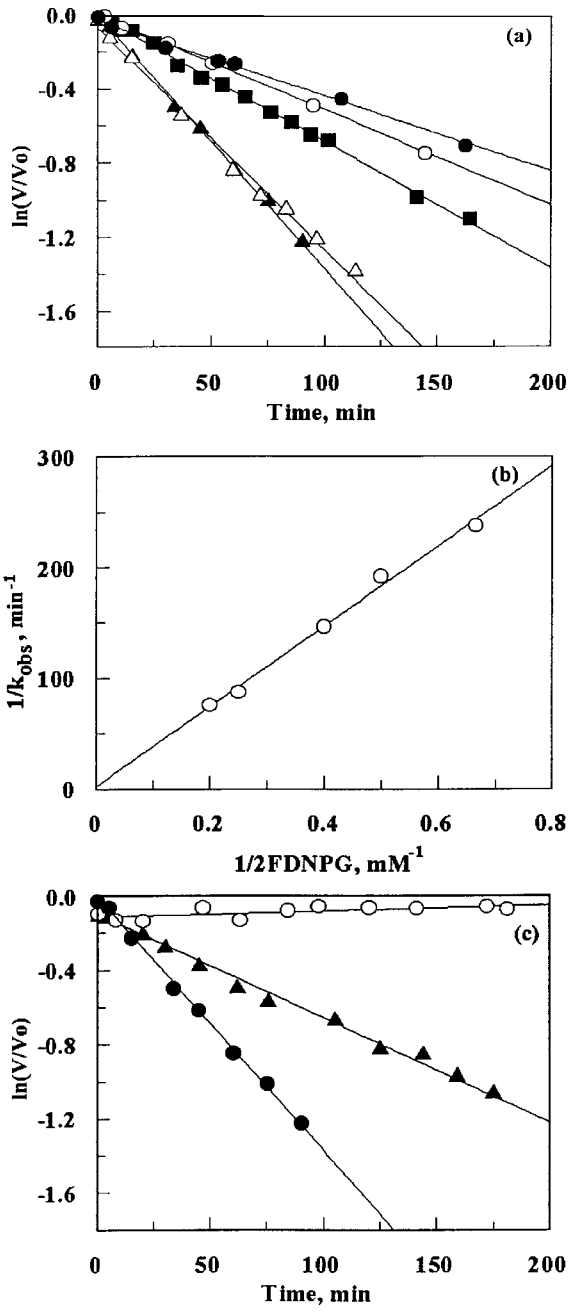

Figure 1 Inactivation of Fbgl by 2FDNPG

(**a**) Semi-logarithmic plot of residual activity versus time at the indicated inactivator concentrations: ●, 1.5 mM; ○, 2.0 mM; ■, 2.5 mM; △, 4.0 mM; ▲, 5.0 mM. (**b**) Double-reciprocal plot of the first-order rate constants from (**a**) versus 2FDNPG concentration. (**c**) Inactivation with 5.0 mM 2FDNPG in the presence (▲) and absence (●) of 1.5 μM δ-gluconolactone. The control reaction (○) was carried out under identical conditions but without inhibitors being added.

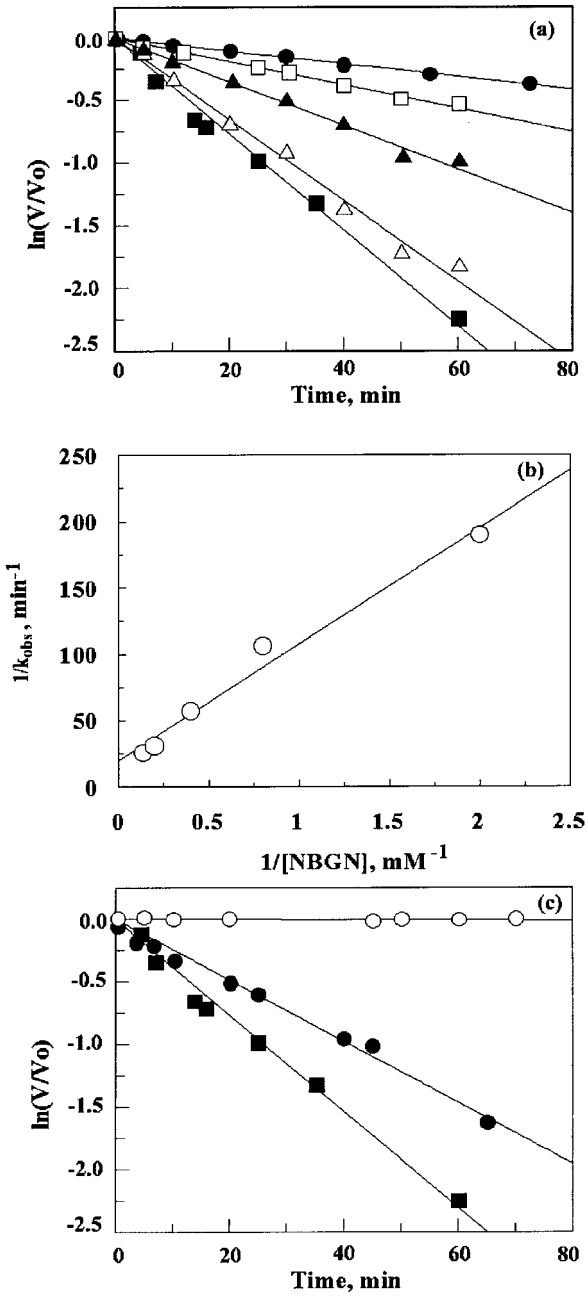

Figure 2 Inactivation of FbgI by NBGN

(**a**) Semi-logarithmic plot of residual activity versus time at the indicated inactivator concentrations: ●, 0.5 mM; □, 1.25 mM; ▲, 2.5 mM; △, 5.0 mM; ■, 7.5 mM. (**b**) Double-reciprocal plot of the first-order rate constants from (**a**) versus NBGN concentration. (**c**) Inactivation with 7.5 mM NBGN in the presence (●) and absence (■) of 1.5 μM δ-gluconolactone. The control reaction (○) was carried out under identical conditions, but without the addition of inhibitors.

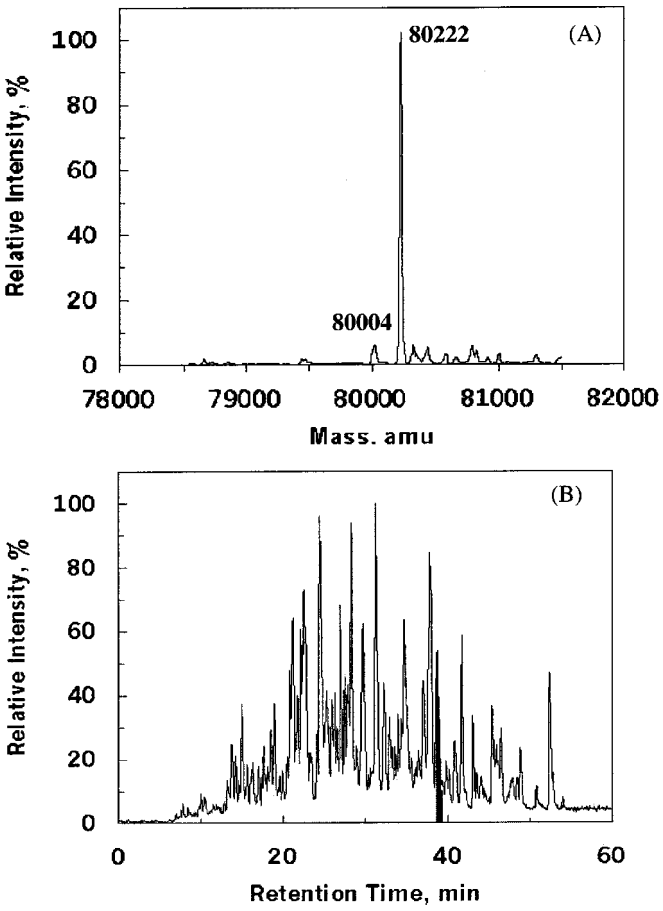

Figure 3 Reconstructed ES mass spectrum of wild-type enzyme (3.5 μg/μl, 100 μl) incubated with NBGN (100 mM, 20 μl) for 90 min

(**A**) The species with a molecular mass of 80222 Da corresponds to the wild-type enzyme (80004 a.m.u.) plus the inhibitor (219 a.m.u.). (**B**) Wild-type β-glucosidase labelled with NBGN followed by pepsin digestion and HPLC separation. Through comparison with the control digest, the shaded portion was found to contain the labelled fragment (retention time 37–39 min).

are the expected mass differences for reaction with 1 mol of reagent.

Identification of the labelled active-site peptides

Peptic digestion of the two modified enzymes [modified by 2-deoxy-2-fluoro-β-D-glucosyl (2FG) and *N*-acetylglucosaminyl (NAGA)] yielded a mixture of peptides in each case. These were separated by reversed-phase HPLC using the ES mass spectrometer as a detector. As can be seen in the total ion chromatogram of the digest of the NAGA-labelled enzyme (Figure 3B), a large number of peaks was observed, each peak corresponding to at least one peptide in the digest mixture. The labelled peptides were located within the chromatogram by comparison of the peptides present within digests of labelled and unlabelled enzyme, using the expected mass difference between the labelled and unlabelled peptides as confirmation of the locations of the peptides of interest. As shown in Figure 3(B), the shaded region (retention time within 37–39 min) contains the NAGA-labelled fragment. The 2FG-labelled peptide was located by the same strategy (results not shown).

Peptide sequence of the 2FG-labelled peptide by LC/MS/MS

On the basis of peptide mapping and mass analysis, doubly-charged peptides of m/z 645 and 563 were obtained by HPLC separation of the labelled and unlabelled samples, respectively. The m/z values of 645 and 563 correspond to peptides of mass 1289 Da (645 × 2-H) and 1125 Da (563 × 2-H), the 164 Da mass difference between the two peptides being the mass of the 2FG moiety. Upon searching the amino acid sequence

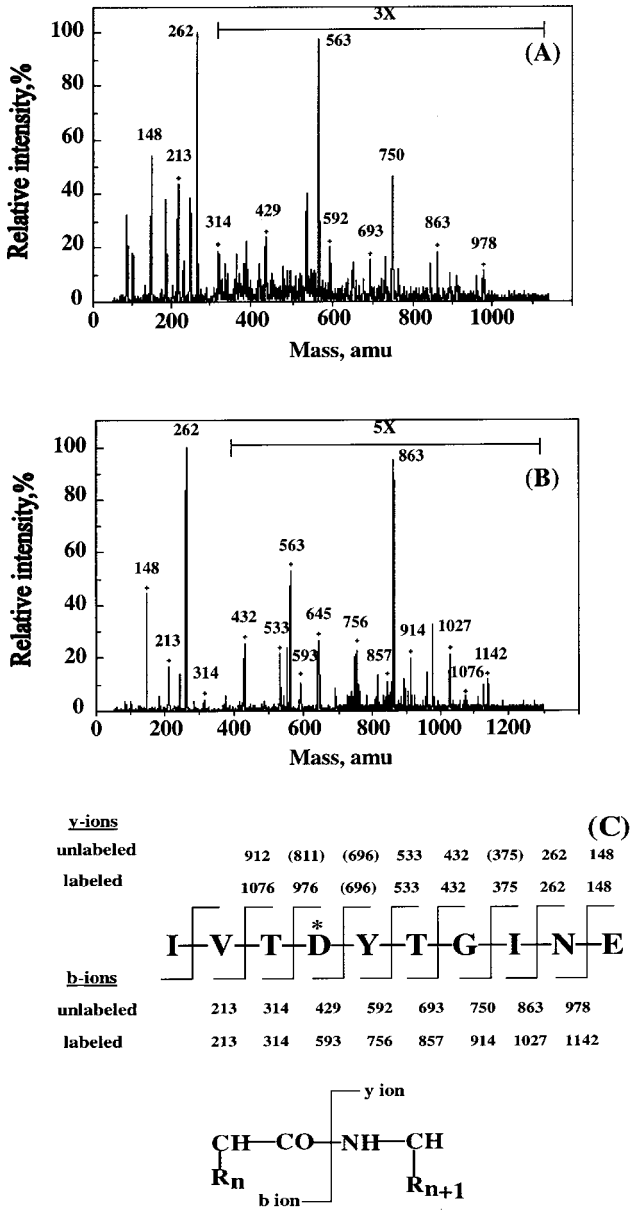

Figure 4 MS/MS analysis of the peptide labelled with 2FDNPG

(**A**) MS/MS daughter-ion spectrum of the unlabelled peptide (*m/z* 563 in the doubly-charged state); (**B**) MS/MS daughter-ion spectrum of the labelled peptide (*m/z* 645 in the doubly-charged state); (**C**) rationalization of the observed singly-charged y- and b-ions, defined as above. Masses shown in parentheses are those of expected peptides that were not found in the mass spectra.

of Fbgl for all possible peptides with the mass 1125 ± 1 Da, eight peptides could be found to meet this criterion (results not shown). Identification of the true peptide sequence was achieved through further MS/MS analysis. The parent ion of *m/z* 563 was subjected to collision-induced dissociation, yielding the spectrum shown in Figure 4(A). The presence of b-ions with masses of 213, 314, 429, 592, 693, 750, 863 and 978 and of y-ions with masses of 912, 533, 432, 262 and 148 unequivocally confirmed the sequence of this peptide as IVTDYTGINE, corresponding to residues 244–253 of Fbgl. Identification of the point of attachment of the sugar moiety was achieved through inspection of the daughter ions of the 2FG-labelled peptide (*m/z* = 645, doubly charged). A series of b-ions with masses of 593, 756, 857, 914, 1027 and 1142 were observed, corresponding to the 2FG-labelled fragments IVTD, IVTDY, IVTDYT, IVTDYTG, IVTDYTGI and IVTDYTGIN, respectively (Figure 4B). The IV and IVT fragments were clearly unlabelled because the masses were identical with those of the unlabelled peptides, allowing us to conclude unequivocally that the 2FG moiety was attached to the aspartic acid residue (Asp-247) immediately adjacent in the sequence. The series of y-ions observed was also consistent with this result: all ions found and their assignments are summarized in Figure 4(C). Corroboration of this assignment of Asp-247 as the nucleophile was obtained from attempts to label a mutant modified at this position. Incubation of the Asp-247Asn mutant with 2FDNPG for 2 h resulted in no modification of the enzyme, as analysed by ES/MS.

Sequence of the peptide labelled by NBGN

Identification of the labelled residue in this case was not as straightforward as was the case for labelling by 2FDNPG. Comparative scanning of the LC/MS profiles of labelled and unlabelled digests for peptides differing in mass by that of the NAGA moiety (219 a.m.u.) or for multiply-charged versions thereof revealed a peptide of *m/z* 1182 (doubly charged, along with the triply-charged peptide of *m/z* 788.5) in the labelled digest, and a corresponding peptide of *m/z* 1073 (doubly charged, along with a triply-charged peptide of *m/z* 715.5) in the unlabelled digest. This corresponds to a peptide of mass 2144 ± 1 (1073×2-H or 715.5×3-2H), for which several candidates exist when the full sequence of the protein is scanned. MS/MS analysis of the *m/z* 1073 and 716 peptides allowed assignment of the sequence SGESSSRANIEIPQAQKDLL, corresponding to residues 471–490 of Fbgl (results not shown). To identify the residue bearing the NAGA moiety, the labelled peptide (*m/z* 789, triply charged) was subjected to collision-induced dissociation within the tandem mass spectrometer, yielding the fragments shown in Figure 5(A). Unfortunately, only a few b-ions (1338, 1451, 1874, 2118, 2232 and their corresponding doubly-charged states) and y-ions (913 and 132) could be observed, making full assignment impossible. The b-ions of *m/z* 1338, 1451 and 1874 correspond to ions of *m/z* 1119, 1239 and 1655 found in the unlabelled peptide, but are 219 Da heavier in each case. This indicates that the NAGA moiety is present within the fragment SGESSSRANIE, thereby ruling out the possibility that the aspartate in the IPQAQKDLL fragment is the labelled residue.

To obtain shorter fragments, the labelled peptide was digested further by trypsin and the resulting mixture separated by HPLC (C_{18} column), with ES/MS detection. Although three fragments, SGESSSR, ANIEIPQAQK and DLL, were expected, only the last two fragments were observed, with masses ($M\text{-}H^+$) of 1112 and 361 Da. These masses correspond to the unlabelled fragments, indicating that the label resides on the SGESSSR fragment. Presumably this highly hydrophilic peptide, bearing the sugar moiety, was eluted through the C_{18} column along with the salt and buffer and consequently not detected. Further evidence for the site of labelling was derived by digesting the full, labelled peptide SGESSSRANIEIPQAQKDLL with lysine C protease, yielding a doubly charged, labelled peptide with a mass (*m/z* 1011) corresponding to the anticipated sequence of SGESSSRANIEIPQAQK. Further MS/MS analysis of this peptide is shown in Figure 5(B). A series of singly charged b-ions with masses of 1875, 1747, 1676, 1548, 1451, 1338, 1208, 1095 and 911, corresponding to unlabelled peptides of masses 1656,

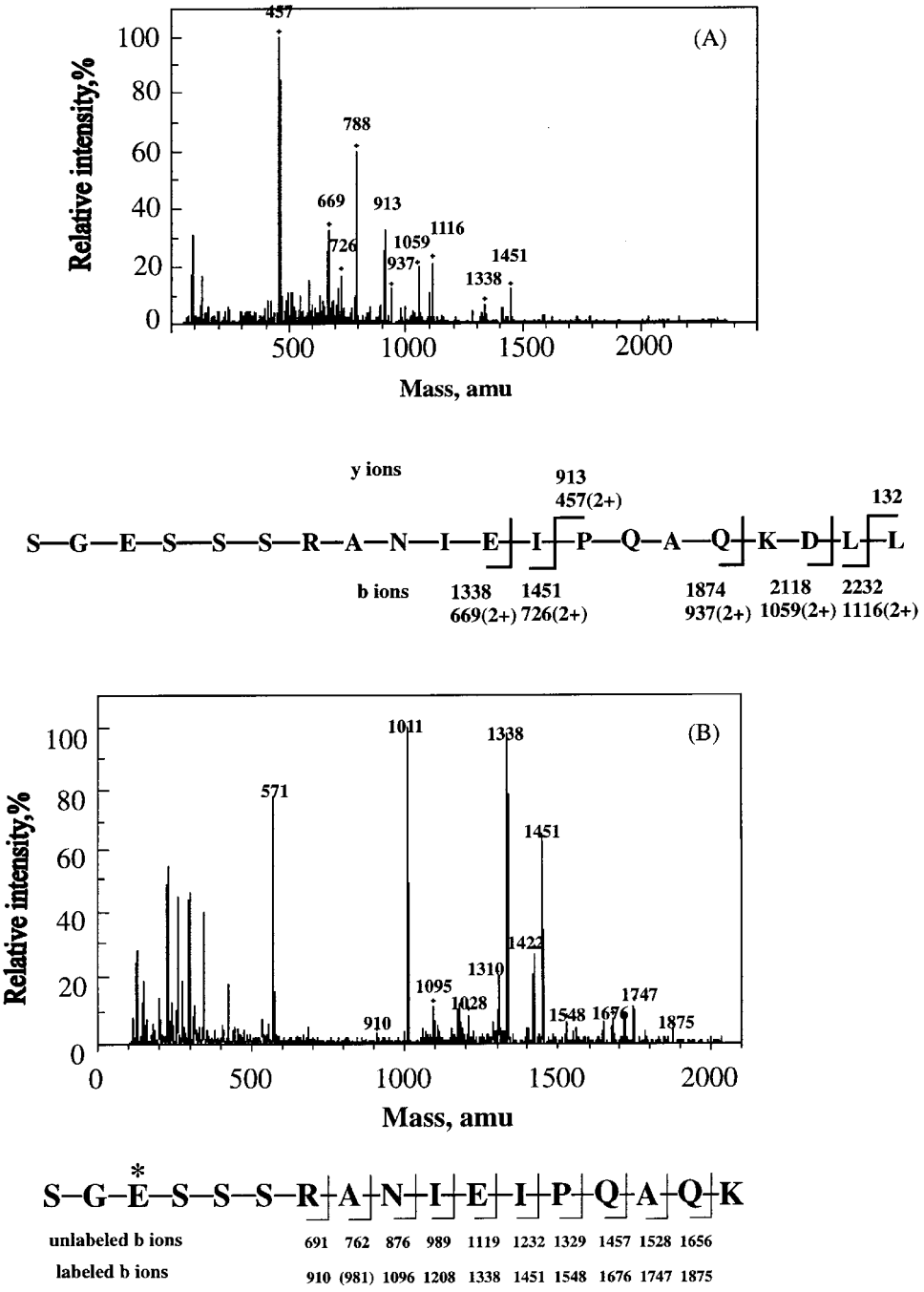

Figure 5 MS/MS analysis of the peptide labelled with NBGN

(**A**) The MS/MS daughter-ion spectrum of the labelled peptide (*m/z* 789 in the triply-charged state) and the rationalization of the observed singly- and doubly-charged y- and b-ions. (**B**) The MS/MS daughter-ion spectrum of the peptide of *m/z* 1011 (doubly charged) obtained from lysine C protease digestion of the labelled peptic peptide (2362 a.m.u.), along with a rationalization of the fragmentation pattern.

1528, 1457, 1329, 1232, 1119, 989, 876 and 691, are observed. Since all these fragments still carry the NAGA moiety, and since the acid/base catalyst is expected to be an aspartic or glutamic acid, we can conclude that the NAGA moiety is located on the glutamate residue (Glu-473) of the SGESSSR peptide. In confirmation of this conclusion, the Glu-473Gly mutant was incubated with the affinity label NBGN to look for labelling. After 3 h of incubation no labelling was detected by MS analysis, suggesting that Glu-473 is indeed the residue labelled in the wild-type enzyme.

Conclusions

Around 150 family 3 glycohydrolases have been cloned and sequenced. Although all of them bear the common conserved sequences, enzymes within this family can be further classified into six sub-families [39]. The aspartate residue in the SDW conserved sequence has been shown to be the nucleophile of this family in several cases. However, the identity of the general acid/base catalyst is very uncertain. Even though Glu-491 is located in the appropriate position to function as the general

acid/base catalyst of barley β-glucosidase [22], this residue is not fully conserved in family 3. In fact, Fbgl (this study) is very different from the barley β-glucosidase in terms of molecular size and phylogenetics. In this study, we have provided proof that the catalytic nucleophile in Fbgl (Asp-247) is the same residue as that identified in other enzymes of this family, thereby confirming that the nucleophile is conserved across sub-families. More importantly, we provide a clear identification of Glu-473 as the acid/base catalyst within this sub-family through covalent modification with an affinity label targeted at the acid/base catalyst. This outcome agrees completely with the conclusion arrived at on the basis of detailed kinetic analysis of mutants modified at this position [40]. Although this glutamate is conserved within a subgroup of family 3 enzymes containing similar phylogenetics with Fbgl [40], no related residue can be found in the barley enzyme. It is very likely that different sub-families may have unique general acid/base catalysts. This argument can only be evaluated through studies on enzymes from both the same and different sub-families.

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