Identification of Glu-277 as the catalytic nucleophile of *Thermoanaerobacterium saccharolyticum* β-xylosidase using electrospray MS

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Thermoanaerobacterium saccharolyticum β-xylosidase is a member of family 39 of the glycosyl hydrolases. This grouping comprises both retaining β-D-xylosidases and α-L-iduronidases. *T. saccharolyticum* β-xylosidase catalyses the hydrolysis of short xylo-oligosaccharides into free xylose via a covalent xylosyl–enzyme intermediate. Incubation of *T. saccharolyticum* β-xylosidase with 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-xyloside resulted in time-dependent inactivation of the enzyme (inactivation rate constant $k_i = 0.089\ min^{-1}$, dissociation constant for the inactivator $K_i = 65\ \mu M$) through the accumulation of a covalent 2-deoxy-2-fluoro-α-D-xylosyl–enzyme, as observed by electrospray MS. Removal of excess inactivator and regeneration of the free enzyme through transglycosylation with either xylobiose or thiobenzyl xyloside demonstrated that the covalent intermediate was kinetically competent. Peptic digestion of the 2-deoxy-2-fluoro-α-D-xylosyl–enzyme intermediate and subsequent analysis by electrospray ionization triple-quadrupole MS in the neutral-loss mode indicated the presence of a 2-deoxy-2-fluoro-α-D-xylosyl peptide. Sequence determination of the labelled peptide by tandem MS in the daughter-ion scan mode permitted the identification of Glu-277 (bold and underlined) as the catalytic nucleophile within the sequence IILNSHFPNLPFHIT**E**Y.

INTRODUCTION

Xylan is the major constituent of plant cell wall hemicelluloses and is composed of a β-1,4-linked D-xylopyranosyl backbone ornamented with various sugars including D-glucuronic acid and L-arabinose [1]. The enzymic degradation of xylan has attracted the interest of the pulp and paper industry because it has been shown to considerably facilitate pulp bleaching, decreasing both cost and environmental impact. Complete digestion of the xylan requires the action of several glycosyl hydrolases, including endo-β-1,4-xylanase, which hydrolyses the insoluble xylan backbone to yield shorter, soluble xylo-oligosaccharides, and β-xylosidase which hydrolyses the soluble xylo-oligosaccharides to yield free xylose.

There are two major mechanistic classes of glycosyl hydrolases that can be distinguished by the different stereochemical outcomes of the reactions they catalyse. Inverting glycosidases operate by a direct displacement mechanism in which water directly displaces the aglycone in a general acid/base-catalysed process. Retaining enzymes operate by a double-displacement mechanism involving the action of two active-site carboxylate residues to yield a product with the same anomeric stereochemistry as the substrate [2]. The first step involves the attack by one of the carboxylates on the sugar anomeric centre, along with general acid catalysis from the other carboxyl group, expediting the departure of the leaving group to yield an α-D-glycosyl–enzyme intermediate. In a second step, the intermediate is hydrolysed by general base-catalysed attack of water at the anomeric centre, resulting in cleavage of the glycosidic bond with net retention of the anomeric configuration (Scheme 1).

β-Xylosidases are currently classified into families 39 and 43 of glycosyl hydrolases according to amino acid sequence similarities [3]. *Thermoanaerobacterium saccharolyticum* β-xylosidase is a family 39 enzyme, members of which are defined by a section of high sequence similarity in a region encompassing the proposed acid/base catalyst, and is composed of both β-D-xylosidases and α-L-iduronidases. This family, by extension from stereochemical outcome studies with *T. saccharolyticum* β-xylosidase [4], operates by a retaining mechanism. Only five members of family 39 are currently known, none of which have had their structure solved or their nucleophile identified. The catalytic nucleophile of *T. saccharolyticum* β-xylosidase has been predicted to be Glu-277 [5] on the basis of hydrophobic cluster analysis.

2,4-Dinitrophenyl 2-deoxy-2-fluoro-β-D-glycopyranosides have proven extremely successful reagents for derivatization of active-site nucleophiles in a number of β-retaining glycosidases [6–8]. These inactivators are mechanism-based, as the C-2 fluorine destabilizes the transition states for both the glycosylation and deglycosylation steps, while the excellent leaving group, 2,4-dinitrophenol, accelerates the first step (xylosylation) so as to permit the trapping of a 2-deoxy-2-fluoro-α-D-glycosyl–enzyme [9]. Proteolytic digestion of the labelled enzyme yields short peptides, one of which has the fluorosugar attached. The sole ester in the complex, the glycosyl–enzyme linkage, is uniquely fragile to fragmentation in the collision cell of a mass spectrometer. Thus, neutral-loss tandem MS can be used to localize the labelled peptide. Further tandem mass spectrometric analysis permits identification of the nucleophile. In this paper, we will use this procedure to identify the *T. saccharolyticum* β-xylosidase catalytic nucleophile.

Abbreviations used: 2F-DNPX, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-xylopyranoside; 2F-xylosyl, 2-deoxy-2-fluoro-α-D-xylosyl; BTX, benzyl β-D-thio-xylopyranoside; pNPX, 4-nitrophenyl β-D-xylopyranoside; ISV, ion-source voltage; OR, orifice energy; LC/MS, tandem liquid chromatography MS; MS/MS, tandem MS; ESMS, electrospray MS; TFA, trifluoroacetic acid; TIC, total ion chromatogram.

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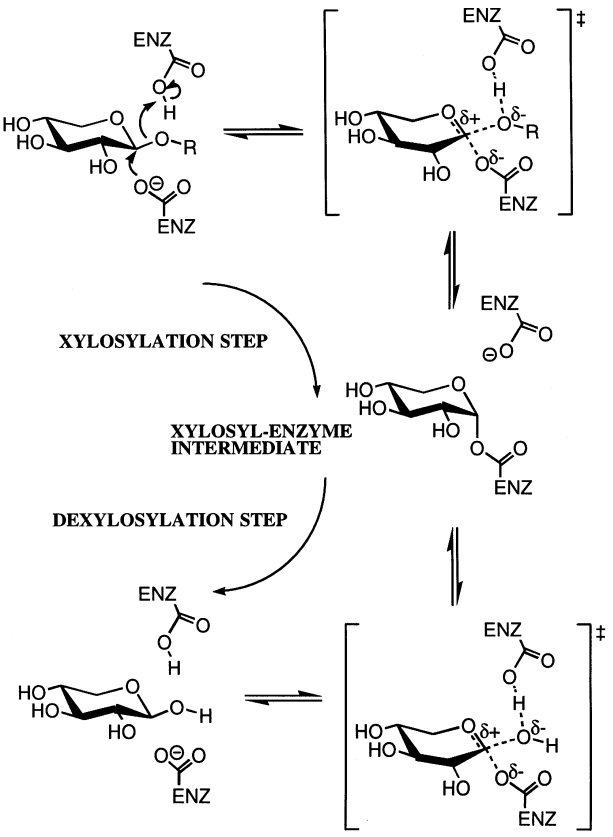

Scheme 1 Proposed mechanism for a retaining β-xylosidase

EXPERIMENTAL

General procedures

T. saccharolyticum β-xylosidase was purified as a recombinant enzyme from *Escherichia coli* R1360. Expression and purification were performed as previously described [10]. All buffer chemicals and other reagents were obtained from Sigma unless otherwise noted. The synthesis of 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-xylopyranoside (2F-DNPX) and benzyl β-D-thioxylopyranoside (BTX) have been described [11].

Enzyme kinetics

Kinetic studies were all performed at 37 °C in 50 mM sodium phosphate, pH 6.5, containing 0.1 % BSA (buffer A). A continuous spectrophotometric assay based on the rate of release of *p*-nitrophenolate upon hydrolysis of *p*-nitrophenyl β-D-xyloside (pNPX) was used. The resulting absorbance change was measured at 400 nm ($\Delta\epsilon_{400}$ 4610 $M^{-1}\cdot cm^{-1}$) using a Pye-Unicam UV/VIS spectrophotometer equipped with a circulating-water bath. Michaelis–Menten parameters for the substrate, previously undetermined with this enzyme, were $K_m = 26\ \mu M$ and $k_{cat} = 242\ min^{-1}$ at 37 °C in buffer A.

The inactivation of β-xylosidase by 2F-DNPX was monitored by incubation of the enzyme (0.68 mg/ml) in buffer A at 37 °C in the presence of various concentrations of the inactivator (35.5–532.3 μM). Residual enzyme activity was determined at several time intervals by addition of an aliquot (5–10 μl) of the inactivation mixture to a solution of *p*-nitrophenyl β-D-xyloside (267 μM, 750 μl) in buffer A, and measurement of the rate of *p*-nitrophenolate release. Pseudo-first-order rate constants at each inactivator concentration (k_{obs}) were determined by fitting each inactivation curve to a first-order rate equation [12]. The inactivation rate constant (k_i) and the dissociation constant for the inactivator (K_i) were determined by fitting to the equation:

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

Binding of the 2F-DNPX at the active site was proven by demonstrating protection against inactivation by a competitive inhibitor. Inactivation mixtures (60 μl) containing 0.68 mg/ml enzyme and 21.3 μM 2F-DNPX were incubated in the presence and absence of BTX (11.33 mM, $K_i = 5.3$ mM). At several time intervals, an aliquot (5 μl) was assayed for residual activity as above.

Turnover (reactivation) of the 2-fluoro-xylosyl–enzyme intermediate

2-Deoxy-2-fluoro-α-D-xylosyl (2F-xylosyl)–enzyme was generated using 213 μM 2F-DNPX. The trapped intermediate (450 μl, 1.2 mg/ml) was freed of excess inactivator by concentration of the inactivated enzyme using 30 kDa nominal molecular-mass cut-off centrifugal concentrators (Millipore Ultrafree-MC) followed by dilution of the concentrated enzyme stock (10–20 μl) to a final volume of 130 μl. Three-fold repetition of the process ensured at least a 99 % reduction in the free 2F-DNPX concentration. Aliquots (30 μl) of the inactivated enzyme freed from 2F-DNPX were then incubated at 37 °C in buffer A alone or with 45 mM xylobiose, 45 mM BTX or 45 mM xylose in a final total reaction volume of 110 μl. Reactivation was monitored by removing aliquots (5 or 10 μl) at several time intervals and assaying for activity as described above. Approx. 75 % of the activity was recovered relative to a control of native enzyme treated in an identical manner.

Labelling and proteolysis of *T. saccharolyticum* β-xylosidase

β-Xylosidase (15 μl, 3.4 mg/ml) was incubated with 2F-DNPX (3 μl, 2.13 mM) at 37 °C for 1 h in 50 mM sodium phosphate, pH 6.5. Complete inactivation (> 99 %) was confirmed by enzyme assay as above. This mixture was immediately subjected to peptic digestion: β-xylosidase (10 μl native or 12 μl 2F-xylosyl–enzyme, 3.4 mg/ml) was mixed with 150 mM sodium phosphate buffer, pH 2.0 (20 μl or 18 μl), and pepsin (10 μl, 0.4 mg/ml in 150 mM sodium phosphate buffer, pH 2.0). The mixture was incubated at room temperature for 20 min, frozen, and analysed immediately by LC/MS upon thawing.

Electrospray MS (ESMS)

Mass spectra were recorded on a PE-Sciex API 300 triple-quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an Ionspray ion source. Peptides were separated by reverse-phase HPLC on an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA, U.S.A.) directly interfaced with the mass spectrometer. In each of the MS experiments, the proteolytic digest was loaded onto a C_{18} column (Reliasil, 1 × 150 mm) equilibrated with solvent A [solvent A: 0.05 % trifluoroacetic acid (TFA)/2 % acetonitrile in water]. Elution of the peptides was accomplished using a gradient (0–60 %) of solvent B over 60 min followed by 100 % solvent B over 2 min (solvent B: 0.045 % TFA/80 % acetonitrile in water). Solvents were pumped at a constant flow rate of 50 μl/min. Spectra were obtained in the single-quadrupole scan mode (LC/MS), the tandem MS neutral-loss scan mode, or the tandem MS product-ion scan mode (MS/MS).

In the single-quadrupole mode (LC/MS), the quadrupole mass analyser was scanned over a mass to charge ratio (m/z) range of 400–1800 Da with a step size of 0.5 Da and a dwell time of 1.5 ms per step. The ion source voltage (ISV) was set at 5.5 kV and the orifice energy (OR) was 45 V. In the neutral-loss scanning mode, MS/MS spectra were obtained by searching for the mass loss of $m/z = 67.5$, corresponding to the loss of the 2F-xylose label from a peptide ion in the doubly charged state. In the tandem MS daughter-ion scan mode, the spectrum was obtained by selectively introducing the parent ion ($m/z = 1103$) from the first quadrupole (Q1) into the collision cell (Q2) and observing the product ions in the third quadrupole (Q3). Thus, Q1 was locked on $m/z = 1103$; Q3 scan range was 50–1120; the step size was 0.5; dwell time was 1 ms; ISV was 5 kV; OR was 45 V; Q0 $= -10$; IQ2 $= -48$.

Aminolysis of the labelled enzyme

Concentrated ammonium hydroxide (5 μl) was added to a sample of the inactivated 2F-xylosyl–enzyme (20 μl, 0.85 mg/ml). The mixture was incubated for 15 min at 50 °C, acidified with 50% TFA and analysed by ESMS as described above.

RESULTS AND DISCUSSION

Inactivation of the enzyme

Incubation of *T. saccharolyticum* β-xylosidase with 2F-DNPX resulted in pseudo-first-order inactivation of the enzyme in a time-dependent manner (Figure 1a). The enzyme-inactivation rate with 2F-DNPX was shown to be dependent on the inactivator concentration in a saturable manner, consistent with the inactivation scheme below where E = enzyme, DNP = 2,4-dinitrophenol, 2F-X = 2-deoxy-2-fluoro-D-xylose and R = reactivating sugar:

$$E + 2F\text{-}DNPX \underset{}{\overset{K_i}{\rightleftharpoons}} E \bullet 2F\text{-}DNPX \xrightarrow{k_i} E\text{-}2F\text{-}X + DNP$$

$$E\text{-}2F\text{-}X \xrightarrow{k_{re}} E + 2F\text{-}X$$

$$E\text{-}2F\text{-}X + R \rightleftharpoons E\text{-}2F\text{-}X \bullet R \xrightarrow{k_{trans}} E + 2F\text{-}X\text{-}R$$

Analysis of these data, as described in the Experimental section, allowed calculation of the inactivation rate constant ($k_i = 0.089 \pm 0.001$ min^{-1}) and of the dissociation constant ($K_i = 65 \pm 4$ μM) for the inactivator (Figure 1c). Incubation of the enzyme with 2F-DNPX (213 μM) in the presence of the competitive inhibitor BTX (11.33 mM, $K_i = 5.3$ mM) resulted in a decrease in the apparent inactivation rate constant from 0.060 min^{-1} in the absence of BTX to 0.047 min^{-1} in its presence (Figure 1b). This observed protection from inactivation in the presence of BTX is consistent with 2F-DNPX and BTX competing for the same site, strongly suggesting that inactivation is active-site directed [13]. These results suggest that inactivation is a consequence of accumulation of a stable covalent 2-F-xylosyl–enzyme intermediate [9]. This conclusion is supported by the mass spectral analysis of the inactivated enzyme (see below).

Catalytic competence

Further evidence supporting the existence of a covalent 2F-xylosyl–enzyme arises from demonstration of the catalytic competence of the trapped intermediate. After removal of excess inactivator from the labelled enzyme, the sample was incubated at 37 °C in the presence of sodium phosphate buffer alone, with xylose (45 mM), xylobiose (45 mM) or BTX (45 mM) and the recovery of activity associated with the regeneration of the free enzyme was monitored. The reactivation kinetics of the 2F-xylosyl–enzyme in buffer alone followed a first-order process with an apparent rate constant of $k_{re} = 1 \times 10^{-5}$ min^{-1} ($t_{\frac{1}{2}} =$ 67000 min) (Figure 2). Rate constants for reactivation by trans-

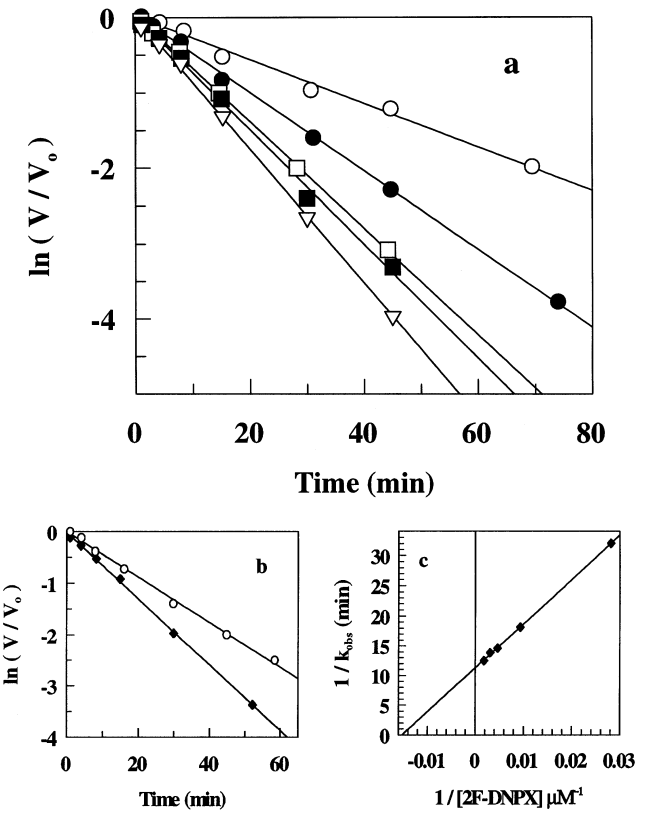

Figure 1 Inactivation of *T. saccharolyticum* β-xylosidase by 2F-DNPX

(**a**) Semilogarithmic plot of residual activity versus time at the indicated inactivator concentrations: (○) 36.5 μM, (●) 107 μM, (□) 213 μM, (■) 320 μM, and (▽) 532 μM. (**b**) Inactivation with 213 μM 2F-DNPX in the absence (◆) and presence (○) of 11.3 mM BTX. (**c**) Replot of the first-order rate constants from (**a**).

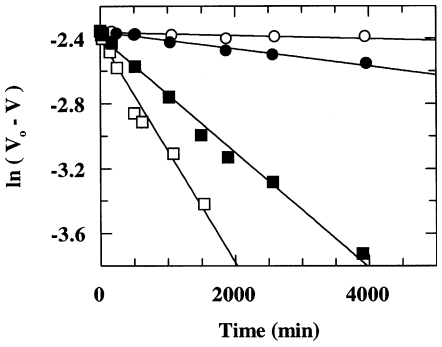

Figure 2 Reactivation of 2F-xylosyl xylosidase

Semilogarithmic plot of activity versus time in buffer alone (○), with 45 mM xylose (●), with 45 mM BTX (■), or with 45 mM xylobiose (□).

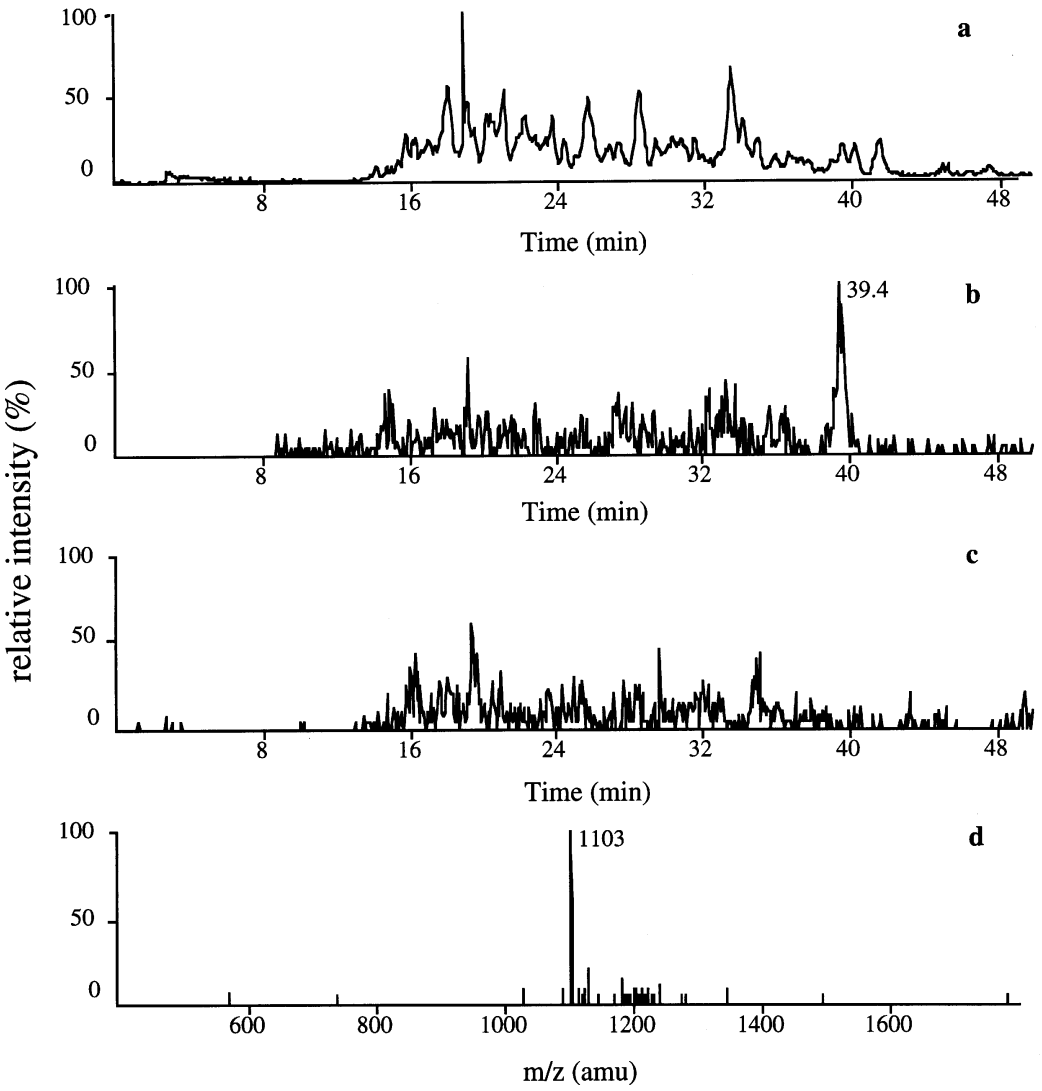

Figure 3 ESMS experiments on a peptic digest of *T. saccharolyticum* β-xylosidase

(**a**) Enzyme labelled with 2F-DNPX, TIC in normal MS mode, (**b**) enzyme labelled with 2F-DNPX, TIC in the neutral-loss mode, and (**c**) unlabelled enzyme, TIC in the neutral-loss mode. (**d**) Mass spectrum of peptide eluting at 39.4 min.

glycosylation (k_{trans}) were found to be 5×10^{-5} min^{-1} ($t\frac{1}{2} =$ 13000 min) with xylose, 8×10^{-4} min^{-1} ($t\frac{1}{2} = 900$ min) with xylobiose and 4×10^{-4} min^{-1} ($t\frac{1}{2} = 1800$ min) with BTX. The higher enzyme-reactivation rates observed in the presence of xylose, xylobiose or BTX (5, 75 and 37-fold higher than the spontaneous reactivation-rate respectively) suggest that reactivation is significantly accelerated by transglycosylation to an acceptor sugar. Furthermore, the variation in the reactivation rate seen for different acceptor sugars suggests that increased acceptor–enzyme interactions in the aglycone site promote reactivation by transglycosylation, presumably by increasing the effective concentration of the acceptor and stabilizing the transition state leading to glycosyl transfer. This process was fully analysed for the *Agrobacterium* sp. β-glucosidase [9], where it was shown that reactivation was accompanied by the formation of a 2-fluoroglycosyl glycoside (disaccharide) product. Such transglycosylations are a normal process, consistent with a catalytically competent intermediate.

Stoichiometry of incorporation of inactivator studied by ESMS

The mass of the native xylosidase was found by ESMS to be 58666 ± 6 Da. After inactivation with 2F-DNPX, only one species with a mass of 58800 ± 6 Da was observed. The mass difference between the native and inactivated enzymes is 134 Da, a value which is consistent, within error, with the addition of a single 2F-xylose label (135 Da).

Identification of the labelled active-site peptide by ESMS

Peptic hydrolysis of 2F-xylosyl–enzyme resulted in a mixture of peptides which were separated by reverse-phase HPLC using the ESMS as detector. When scanned in the normal LC/MS mode the total ion chromatogram (TIC) showed a large number of peaks, each corresponding to one or more peptides in the digest mixture (Figure 3a). The 2F-xylosyl–peptide was then located in a second experiment using a tandem mass spectrometer set up in the neutral-loss mode. This technique involves the limited

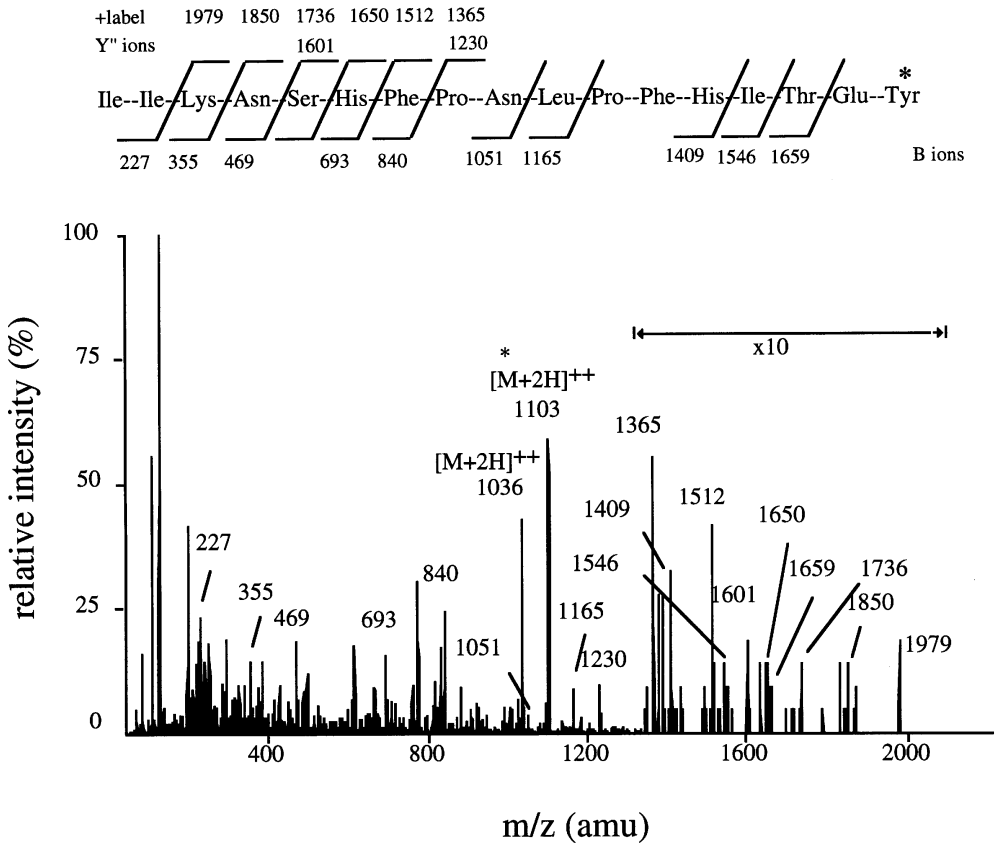

Figure 4 ESMS/MS daughter-ion spectrum of the 2F-xylosyl peptide (*m/z* 1103, in the doubly charged state)

Observed B and Y″ series fragments are shown below and above the peptide sequence respectively.

fragmentation of the ions by collision with an inert gas (N_2) in a collision chamber (Q2) between two quadrupoles (Q1 and Q3). Since the ester linkage between label and enzyme is one of the most labile linkages present, homolytic cleavage of this bond is expected to occur in the collision chamber. The resulting neutral loss of the label leaves the peptide with an unchanged charge state but a decrease in mass of 135 Da. The two quadrupoles were therefore scanned in a linked mode to permit only the passage of ions which lose a mass of 135 Da in the collision cell. No significant peaks were observed when scanned in this manner. However, when scanned in the neutral-loss tandem MS/MS mode searching for a mass loss of *m/z* 67.5, a single predominant peak was observed in the TIC (Figure 3b). No such peak was observed in the neutral-loss spectrum of the unlabelled xylosidase (Figure 3c). These results indicate that a doubly charged peptide bearing the 2F-xylose label is preferentially detected. This doubly charged labelled peptide was measured at *m/z* 1103 (Figure 3d), indicating that the mass of the peptide is 2204 Da [2(1103) − 2 H]. Since the mass of the label is 135 Da, the unlabelled peptide must have a mass of 2070 Da [2204 − 135 + 1 H]. Aminolysis of the isolated peptide resulted in a new single peak in the ion chromatogram of *m/z* 1035.5 (results not shown), whereas no peak corresponding to the labelled peptide was observed. The mass loss of 135 Da [(1103 − 1035.5) × 2] resulting from aminolysis of the labelled peptide is consistent with the expected mass loss from cleavage of the ester-linked 2F-xylose label. Candidate peptides of this mass (2070 Da) were identified by inspection of the enzyme amino acid sequence [10] and searching for all possible peptides of this mass. Eighteen possible peptides with a mass of 2070 ± 2.0 Da were identified. The peptide was then identified unambiguously by peptide sequencing using MS/MS.

Peptide sequencing

Information on the sequence was obtained by additional fragmentation of the peptide of interest (*m/z* 1103) in the daughter-ion scan mode (Figure 4). Both the labelled parent ion (*m/z* 1103) and the unlabelled intact peptide arising from the loss of the 2F-xylose label (*m/z* 1036) appear as doubly charged species. Peaks resulting from B ions correspond to fragments II (*m/z* 227), IIK (*m/z* 355), IIKN (*m/z* 469), IIKNSH (*m/z* 693), IIKNSHF (*m/z* 840), IIKNSHFPN (*m/z* 1051), IIKNSHFPNL (*m/z* 1165), IIKNSHFPNLPF (*m/z* 1409), IIKNSHFPNL-PFH (*m/z* 1546) and IIKNSHFPNLPFHI (*m/z* 1659). Interestingly, more labelled than unlabelled Y″ ions are observed in the spectrum. Peaks arising from Y″ ions bearing the label include KNSHFPNLPFHITEY (*m/z* 1979), NSHFPNLPFHITEY (*m/z* 1850), SHFPNLPFHITEY (*m/z* 1736), HFPNLPFHITEY (*m/z* 1650), FPNLPFHITEY (*m/z* 1512) and PNLPFHITEY (*m/z* 1365). Unlabelled Y″ ions include only SHFPNLPFHITEY (*m/z* 1601) and PNLPFHITEY (*m/z* 1230). Doubly charged fragments also appear in the spectrum, but aside from the labelled and unlabelled parent ions they are unassigned. This information, in conjunction with the mass of the labelled peptide and the primary sequence of the enzyme, permits identification of the peptide containing the active-site nucleophile as $_{261}$IIK-

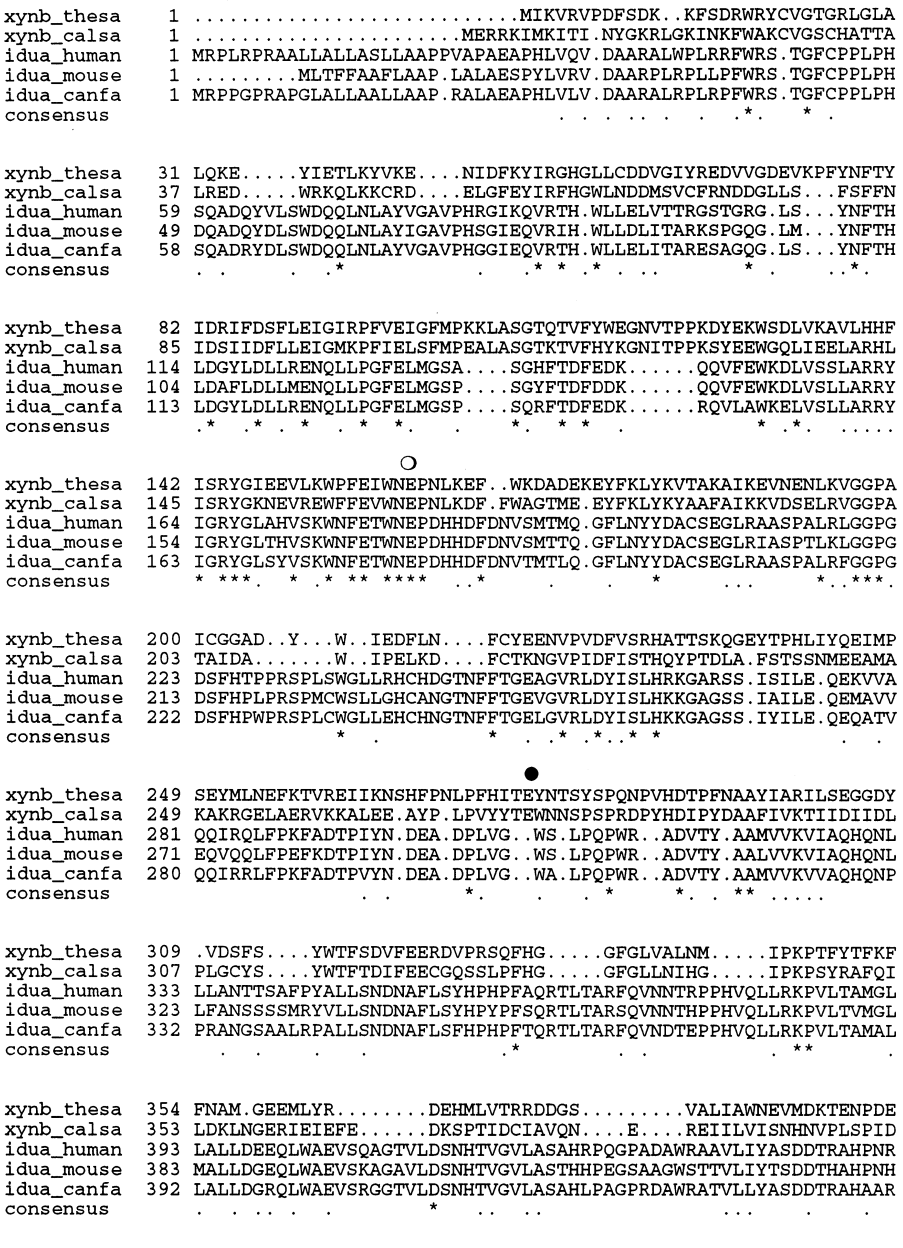

Figure 5 Partial multiple sequence alignment of the enzymes comprising family 39 of glycosyl hydrolases

The consensus sequence is shown at the bottom of the alignment, with (*) indicating fully conserved amino acid residues and (.) indicating similar residues. Numbers to the left denote residue positions. The abbreviations used, references to the published sequence and data bank accession numbers are as follows: xynb-thesa, β-xylosidase from *T. saccharolyticum* ([10], SwissProt identifier P36906); xynb-calsa, β-xylosidase from *Caldocellum saccharolyticum* ([17], SwissProt identifier P23552); idua-human, α-iduronidase from *Homo sapiens* ([18], SwissProt identifier P35475); idua-mouse, α-iduronidase from *Mus musculus* ([19], SwissProt identifier P48441); idua-canfa, α-iduronidase from *Canis familiaris* ([20], SwissProt identifier Q01634). The previously predicted acid/base catalytic residue is indicated by ○. The nucleophile in β-xylosidase identified in this paper (Glu-277) and the corresponding residue in *C. saccharolyticum* β-xylosidase (Glu-275) are indicated by ●.

$NSHFPNLPFHITEY_{278}$. Aminolysis, mentioned above, of the peptide, strongly suggests that the label is attached via a covalent ester linkage. By analogy to other glycosyl hydrolases, in which the nucleophile has been, without exception, an acidic residue [2,14], we can assign Glu-277 as the nucleophile residue since it is the only carboxylic acid present in the peptide.

Sequence conservation and alignment

Multiple sequence alignments of the members of family 39 glycosyl hydrolases revealed sequence similarities between the xylosidases and iduronidases of this family to be less than 16%. This overall sequence similarity is primarily accounted for by a few discrete regions of high similarity between family members. The sequence containing the nucleophile and flanking regions of *T. saccharolyticum* β-xylosidase showed similarity only to that of *Caldocellum saccharolyticum* β-xylosidase and little to those of other members of family 39 (Figure 5), suggesting that considerable differences exist between the active-site architecture of the β-xylosidases and α-iduronidases. Nonetheless, there are

regions that demonstrate significant sequence similarity across all family members, such as the segment approx. 100 residues before the catalytic nucleophile, which contains a glutamic acid in the sequence $_{159}$NEP$_{161}$. This sequence is reminiscent of the NEP sequence within which the acid/base catalytic residue is found in several other β-glycosidases, including family 1 *Agrobacterium* sp. β-glucosidase [15] and the family 10 xylanases [16]. These results suggest that Glu-160 is the acid/base catalyst, as suggested previously [3]. Furthermore, the local sequence around the nucleophile (ITE) also recalls the family 1 *Agrobacterium* sp. β-glucosidase (ITE) and the family 10 xylanases (ITE). Both enzyme classes, along with enzyme families 2, 5, 17, 26, 30, 35, 39, 42, 53, have been assigned as members of clan G-H A, a superfamily of glycosyl hydrolases with an α/β barrel fold [5]. Our results concur with this assignment and with the prediction of the active site as Glu-277 [5], but suggest there may be considerable differences between the fine structures of the active sites of β-xylosidases and α-iduronidases within this family.

Conclusion

This study has shown that inactivation of *T. saccharolyticum* β-xylosidase can be achieved by the accumulation of a stable 2F-xylosyl–enzyme intermediate and that this intermediate is catalytically competent. Labelling with 2F-DNPX in conjunction with tandem mass spectrometric techniques permitted the identification of the nucleophilic residue as Glu-277 in this biotechnologically interesting β-xylosidase. This assignment agrees with the earlier prediction of the catalytic nucleophile. Currently, family 39 of glycosyl hydrolases includes other bacterial β-D-xylosidases and eukaryotic α-L-iduronidases. The poor sequence alignment between β-D-xylosidases and α-L-iduronidases in the region surrounding the identified catalytic nucleophile of *T. saccharolyticum* β-xylosidase indicates that the active-site architecture of these two classes of enzymes differ markedly and may suggest the need to subdivide this family into two subfamilies containing either α-L-iduronidases or β-D-xylosidases.

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