Identification of Glutamic Acid 78 as the Active Site Nucleophile in *Bacillus subtilis* Xylanase Using Electrospray Tandem Mass Spectrometry†

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Received March 11, 1994; Revised Manuscript Received April 22, 1994®

ABSTRACT: A new mechanism-based inactivator of β-1,4-xylanases, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside, has been synthesized and used to trap the covalent intermediate formed during catalysis by *Bacillus subtilis* xylanase. Electrospray mass spectrometry confirmed the 1:1 stoichiometry of the incorporation of inactivator into the enzyme. Inactivation of xylanase followed the expected pseudo-first-order kinetic behavior, and kinetic parameters were determined. The intermediate trapped was relatively stable toward hydrolytic turnover ($t_{1/2}$ = 350 min). However, turnover could be facilitated by transglycosylation following the addition of the acceptor benzyl thio-β-xylobioside, thus demonstrating the catalytic competence of the trapped intermediate. Reactivation kinetic parameters for this process of k_{re} = 0.03 min^{-1} and K_{re} = 46 mM were determined. The nucleophilic amino acid was identified as Glu78 by a tandem mass spectrometric technique which does not require the use of radiolabels. The peptic digest of the labeled enzyme was separated by high-performance liquid chromatography and the eluent fed into a tandem mass spectrometer *via* an electrospray ionization device. The labeled peptide was identified as one of m/z = 826 (doubly charged) which fragmented in the collision chamber between the mass analyzers with loss of the mass of a 2-fluoroxylobiosyl unit. Confirmation of the peptide identity was obtained both by tandem mass spectrometric sequencing and by Edman degradation of the purified peptide. Glu78 is completely conserved in all members of this xylanase family and indeed is shown to be located in the active site in the recently determined X-ray crystal structure.

Xylan is a major component of plant cell walls and comprises a β-1,4-linked polymer of xylose substituted with other sugars such as L-arabinose or D-glucuronic acid (Joselau *et al.*, 1992). There is considerable biotechnological interest in enzymes which degrade xylan since a number of applications are envisaged, including the saccharification of biomass for the production of feedstocks (Linko *et al.*, 1989) and biobleaching in paper manufacture (Senior *et al.*, 1990). Complete degradation of xylan requires a combination of enzymes, the most important being the endoxylanase which cleaves the linkage between the internal β-1,4-xylose residues.

The endo-β-1,4-xylanase from *Bacillus subtilis* is a member of a sequence-related family of xylanases, family G (Gilkes *et al.*, 1991; Oku *et al.*, 1993) or family 11 in the classification of Henrissat and Barrioch (1993), currently containing at least 15 members. It has been shown to catalyze hydrolysis with retention of anomeric configuration (Gebler *et al.*, 1992a), thus suggesting a double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolyzed *via* oxocarbonium ion-like transition states. Such a mechanism for "retaining" glycosidases was originally formulated by Koshland (1953) and has since been substantiated for a number of enzymes [see Sinnott (1990) for a recent review]. On the basis of analogies with lysozyme, and experience with other glycosidases, this mechanism likely involves carboxyl-containing amino acids both as an acid/base catalyst and as the nucleophile responsible for formation of the glycosyl-enzyme intermediate (Sinnott, 1990). Indeed, chemical modification studies with another family G xylanase from *Schizophyllum commune* have suggested that such acidic residues are involved in catalysis (Bray & Clarke, 1990). *B. subtilis* xylanase contains 7 aspartic acid and 2 glutamic acid residues, of which only the 2 glutamic acids are completely conserved in all members of family G.

The three-dimensional structures of three of these xylanases have been determined. The structure of the enzyme from *Bacillus pumilus* has been solved to 2.2-Å resolution but not yet published [see Ko *et al.*) (1992) for an α-carbon backbone drawing]. The structure of the *Bacillus circulans* enzyme, which differs from *B. subtilis* xylanase only in the replacement of the surface Ser147 by Thr, has been determined to the slightly better resolution of 1.49 Å (Campbell *et al.*, 1993; Wakarchuk *et al.*, 1993). The structure of the *Trichoderma harzianum* enzyme has been determined to a similar resolution (Campbell *et al.*, 1993). These structures reveal that Asp11 (*B. subtilis* numbering) and both the conserved glutamic acid residues are located in the putative active site region. Site-directed mutagenesis studies on both the *B. pumilus* (Ko *et al.*, 1992) and the *B. subtilis* enzymes (Wakarchuk *et al.*, 1992) confirm the importance of the two glutamic acid residues and indicate that Asp11 plays a less crucial enzymatic role. These studies have not, however, allowed assignment of specific roles to the individual amino acids.

Identification of the key nucleophilic residue in glycosidases has been achieved in a number of cases by use of activated 2-deoxy-2-fluoroglycosides as mechanism-based inactivators which function by forming stabilized glycosyl-enzyme inter-

† We thank the Natural Sciences and Engineering Research Council of Canada, the Medical Research Council of Canada, and Industry Science and Technology, Canada, as well as the Protein Engineering Network of Centres of Excellence, 1 of 15 Networks of Centres of Excellence established by the Government of Canada, for financial assistance.

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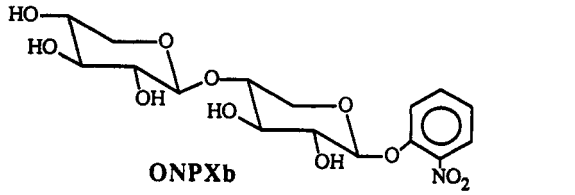
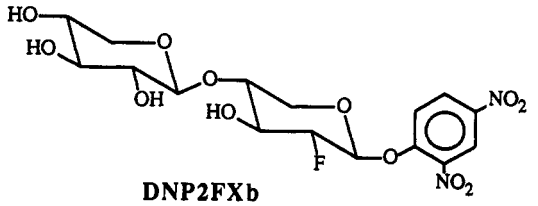
® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

0006-2960/94/0433-7027$04.50/0

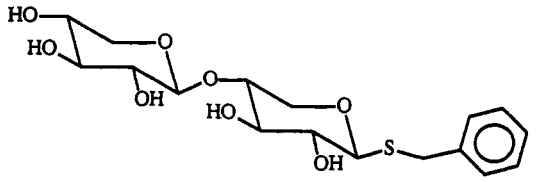
Chart 1



ONPXb



DNP2FXb



BTXb

mediates. This has been investigated for *Agrobacterium* β-glucosidases (Withers *et al.*, 1990), *Cellulomonas fimi* exoglycanase (Tull *et al.*, 1991), *Escherichia coli* β-galactosidase (Gebler *et al.*, 1992b), and *Clostridium thermocellum* endoglucanase (Wang *et al.*, 1993). The fluorine at the 2-position destabilizes the oxocarbonium ion-like transition states for formation and hydrolysis of these intermediates. The presence of a good leaving group ensures that the formation of the intermediate is faster than hydrolysis, thus leading to its accumulation (Street *et al.*, 1992). Identification of the labeled nucleophilic residue was achieved by standard methods involving proteolysis of enzyme inactivated with radiolabeled fluorosugar substrates, followed by HPLC separation and Edman sequencing of the purified labeled peptide.

The paper describes the inactivation of *B. subtilis* xylanase by 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside (Chart 1) and use of this mechanism-based inactivator, coupled with a tandem mass spectrometric technique (Busch & Cooks, 1983), to identify the key nucleophile at the active site of *B. subtilis* xylanase.

EXPERIMENTAL PROCEDURES

General. B. subtilis xylanase was a generous gift from Dr. W. Wakarchuk and was prepared as described previously (Sung *et al.*, 1993). Substrate *o*-nitrophenyl β-xylobioside (ONPXb),[1] competitive inhibitor benzyl thio-β-xylobioside (BTXb), and inactivator 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside (DNP2FXb) were synthesized from xylose *via* a route which will be published elsewhere L. Ziser, L. MacKenzie, and S. G. Withers, unpublished results). All buffer chemicals and other reagents were obtained from Sigma Chemical Co. unless otherwise noted.

Mass spectra were recorded on a PE-Sciex API *III* triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an ionspray ion source (Bruins *et al.*, 1987). Protein or peptide samples were separated by reverse-phase HPLC on an ultrafast microprotein analyzer (Michrom BioResources Inc., Pleasanton, CA) directly interfaced with the mass spectrometer. A post-column flow splitter was used to introduce 15% of the HPLC eluate into the mass spectrometer, while 85% was recovered, as described (Hess *et al.*, 1993).

Kinetics. All kinetic studies were performed at 40 °C, pH 6.0, in a buffer containing 20 mM MES, 50 mM sodium chloride, and 0.1% bovine serum albumin. Enzyme assays involved monitoring the hydrolysis of substrate ONPXb by spectrophotometric measurement of the rate of *o*-nitrophenolate release (λ = 400 nm, $\Delta\epsilon$ = 1024 M^{-1} cm^{-1}).

The inactivation of xylanase by DNP2FXb was monitored by incubation of the enzyme (0.71 mg·mL^{-1}) under the above conditions in the presence of different concentrations (0, 0.42, 0.63, 1.05, 1.69 mM) of DNP2FXb. Residual enzyme activity was determined at appropriate time intervals by removal of an aliquot (5 μL) of the inactivation mixture, addition to a solution of ONPXb (2.4 mM) in the above buffer, and measurement of nitrophenolate release. Pseudo-first-order rate constants at each inactivator concentration (k_{obs}) were determined by fitting the residual activity *versus* time data directly to the expression for a first-order reaction using the program GraFit (Leatherbarrow, 1990). Approximate values for the inactivation rate constant (k_i) and the equilibrium dissociation constant for the inactivator (K_i) were determined by fitting (also using GraFit) the observed rate constants (k_{obs}) to the equation (I = DNP2FXb):

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

Values obtained were somewhat inaccurate since the relative insolubility of the inactivator precluded study at concentrations of DNP2FXb even approaching its K_i value. However, the value of k_i/K_i, which can be determined from the slope of a reciprocal plot, is accurate. Protection against inactivation was determined by following the rate of inactivation with 0.42 mM DNP2FXb in the presence and absence of 26 mM BTXb, a competitive inhibitor of xylanase.

Reactivation of the enzyme was studied as follows. A sample of xylanase was first inactivated by extended treatment with DNP2FXb and then dialyzed against the same MES buffer for 12 h at 4 °C to remove excess inactivator. Samples of the inactivated enzyme (50 μL, 0.94 mg·mL^{-1}) were then incubated under the standard conditions in the presence of a range of concentrations (0, 26, 52, 103, 181 mM) of BTXb as transglycosylation acceptor. Reactivation was monitored by the removal of aliquots (5 μL) at appropriate time intervals and assay of these samples as above. First-order rate constants for reactivation at each acceptor concentration ($k_{re,obs}$) were determined by direct fit of the activity *versus* time data to a first-order equation. A reactivation rate constant (k_{re}) and an acceptor dissociation constant (K_d) were calculated by using GraFit to fit values of $k_{re,obs}$ to the expression (A = BTXb):

$$k_{re,obs} = k_{re}[A]/(K_d + [A])$$

Stoichiometry of Inactivation. Xylanase (10 μg, native or 2FXb labeled) was introduced into the mass spectrometer through a microbore PLRP column (1 × 50 mm) on the Michrom HPLC system. The quadrupole mass analyzer (in the single quadrupole mode) was scanned over a m/z range of 300–2400 Da with a step size of 0.5 Da and a dwell time of 1 ms per step. The ion source voltage (ISV) was set at 5 kV and the orifice energy (OR) was 80 V. The molecular weight of xylanase was determined from these data by using the deconvolution software supplied by Sciex.

[1] Abbreviations: ONPXb, *o*-nitrophenyl β-xylobioside; DNP2FXb, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside; BTXb, benzyl thio-β-xylobioside.

Peptic Digestion of Xylanase. Both native and 2FXb-inactivated xylanase resisted pepsin digestion; thus heat denaturation (boiling for 2 min) was employed to denature xylanase before proteolysis. Denatured xylanase (native or 2FXb labeled, 5 μL at 5 mg/mL) was mixed with 30 μL of phosphate buffer (pH 2) and 5 μL of pepsin (in pH 2 buffer, 0.1 mg/mL), yielding a final pH of 2.5–3.0. The mixture was incubated at room temperature for 3 h until the solution became clear. ESMS analysis confirmed that xylanase was completely digested.

ESMS Analysis of the Proteolytic Digest. In each of the MS experiments, the xylanase proteolytic digest was loaded onto a C18 column (Reliasil, 1 × 150 mm) and then eluted with a gradient of 0–60% solvent B over 20 min followed by 100% solvent B over 2 min at a flow rate of 50 μL/min (solvent A = 0.05% trifluoroacetic acid and 2% acetonitrile in water; solvent B = 0.045% trifluoroacetic acid and 80% acetonitrile in water).

The single-quadrupole mode (normal LC/MS) MS conditions used were identical to those used for analysis of the intact protein. The neutral loss MS/MS spectra were obtained in the triple-quadrupole neutral loss scan mode searching for the mass loss of m/z 133.5, corresponding to the loss of 2FXb from a peptide ion in the doubly charged state. Thus, scan range = m/z 300–1200; step size = 0.5; dwell time = 1 ms/step; ion source voltage (ISV) = 5 kV; orifice energy (OR) = 80; RE1 = 115; DM1 = 0.16; R1 = 0 V; R2 = −50 V; RE3 = 115; DM3 = 0.16; collision gas thickness (CGT) = (3.2–3.6) × 10^{14} molecules/cm^2 (CGT = 320–360). To maximize the sensitivity of neutral loss detection, normally the resolution (RE and DM) is compromised without generating artifact neutral loss peaks. The MS/MS daughter ion spectrum was obtained in the triple-quadrupole daughter scan mode by selectively introducing the m/z 826 peptide from Q1 into the collision cell (Q2) and observing the daughter ions in Q3. Thus, Q1 was locked on m/z 826; Q3 scan range = 300–1800; step size = 1.0; dwell time = 1 ms; ion source voltage (ISV) = 5 kV; orifice energy (OR) = 80; RE1 = 112; DM1 = 0.18; R1 = 0 V; R2 = −50 V; RE3 = 112; DM3 = 0.18; collision gas thickness = 4.5 × 10^{14} molecules/cm^2 (CGT = 450).

Aminolysis of the 2FXb-Labeled Xylanase Digest. To a 10-μL peptic digest of the 2FXb-labeled xylanase (1 mg/mL) was added 5 μL of concentrated amonium hydroxide. The mixture was incubated at 50 °C for 15 min, acidified with 50% TFA, and analyzed by ESMS.

Chemical Sequencing. The peptic digest of the 2FXb-labeled xylanase was separated by HPLC as described above, and fractions containing the 2FXb-labeled peptide were collected *via* the post-column flow splitter. The amino acid sequence of the 2FXb-labeled peptide was determined using standard pulsed liquid-phase protocols and instrumentation on a model 477A sequencer and model 120A PTH analyzer (Applied Biosystems, Foster City, CA) as described previously (Wang *et al.*, 1993).

RESULTS AND DISCUSSION

Inactivation Kinetics. Incubation of *B. subtilis* xylanase with DNP2FXb resulted in time-dependent inactivation of the enzyme according to pseudo-first-order kinetics, as shown in Figure 1a. Unfortunately, due to the poor solubility of the inactivator and the high estimated K_i value, it was not possible to obtain accurate values of the maximal inactivation rate constant, k_i, or the inactivator dissociation constant, K_i, as can be seen in the reciprocal replot in Figure 1c. Nonetheless, estimates of these values of k_i = 2.2 (±0.6) min^{-1} and K_i = 6.4 (±2.1) mM were obtained by a direct fit of the data as noted in Experimental Procedures (values listed in parentheses represent standard errors). The value of k_i/K_i = 0.34 min^{-1} mM^{-1} is, however, a reasonably accurate number describing the second-order rate constant for reaction of inhibitor and enzyme. Incubation of xylanase with DNP2FXb (0.42 mM) in the presence of the competitive inhibitor BTXb (K_i = 26 mM) reduced the apparent rate constant for inactivation from 0.15 to 0.06 min^{-1} (Figure 1b), as would be expected if the two ligands compete for the same site. These results indicate that the inactivator is binding at the enzyme active site and, by analogy with observations on β-glucosidase (Withers & Street, 1988; Withers *et al.*, 1990), that inactivation is a consequence of accumulation of a relatively stable 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate. Additional evidence that this inactivation arises through stabilization and trapping of the normal intermediate in catalysis was obtained by demonstrating its catalytic competence as described below.

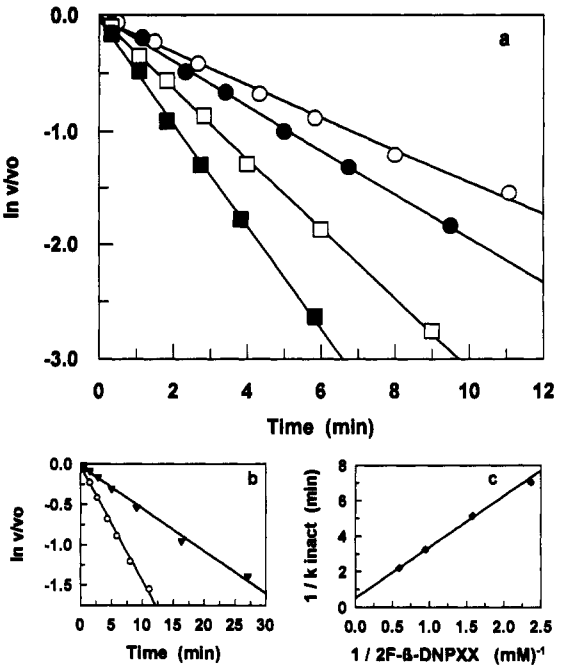


FIGURE 1: Inactivation of *B. subtilis* xylanase by DNP2FXb. (a) Semilogarithmic plot of residual activity *vs* time at the indicated inactivator concentrations: (○) 0.42 mM; (●) 0.63 mM; (□) 1.05 mM; (■) 1.69 mM. (b) Inactivation with 0.42 mM DNP2FXb in the (○) absence and (▼) presence of 26 mM BTXb. (c) Replot of first-order rate constants from (a).

Reactivation of Inactivated Xylanase. After removal of excess inactivator from a labeled sample of enzyme, the material was incubated in buffer at 40 °C, and the return of activity associated with regeneration of the free enzyme was monitored. The return of activity followed a first-order process, allowing a spontaneous reactivation rate constant of 0.0021 min^{-1} to be calculated. This corresponds to a half-life of $t_{1/2}$ = 330 min. The inclusion of xylobiose derivatives in this reactivation mixture increased the rate of activity regain considerably. This suggested that, as with other enzymes (Withers *et al.*, 1990; Street *et al.*, 1992; McCarter *et al.*, 1992; Wang *et al.*, 1993), reactivation could be significiantly accelerated by transglycosylation to an added sugar acceptor. This process of transglycosylation was investigated in more detail by determining reactivation rate constants at a series of acceptor (BTXb) concentrations. As can be seen in Figure 2a, the reactivation rate was dependent upon the BTXb concentration in a saturable manner, allowing kinetic parameters for the reactivation process of k_{re} = 0.03 min^{-1} and

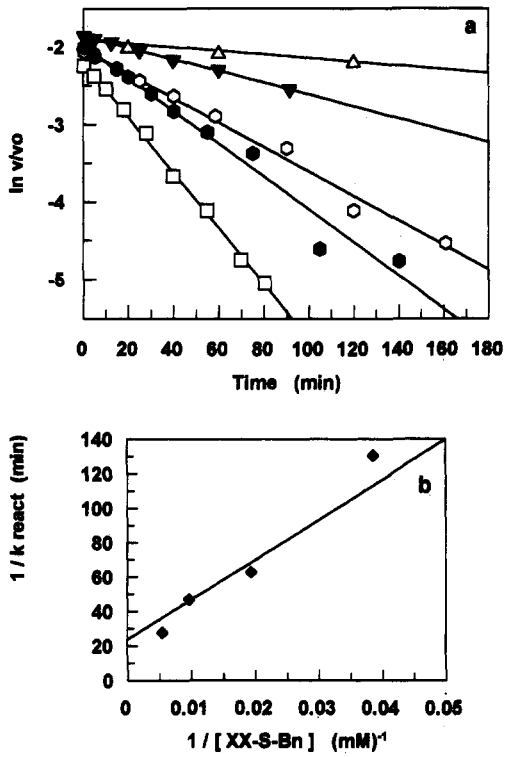


FIGURE 2: Reactivation of 2-deoxy-2-fluoroxylobiosyl xylanase by BTXb. (a) Semilogarithmic plot of activity *vs* time for (△) 0 mM, (▼) 26 mM, (○) 52 mM, (●) 103 mM, and (□) 181 mM BTXb. (b) Double-reciprocal replot of the first-order rate constants from (a) *vs* BTXb concentration.

K_{re} = 46 mM to be determined by direct fit to the suitable expression. This is illustrated in Figure 2b using a reciprocal replot. Transglycosylation therefore occurs some 14-fold faster than simple hydrolysis, pointing out the importance of binding interactions developed between the aglycon sugar moiety and the protein in stabilizing the transition state for glycosyl transfer. The dissociation constant measured is that of the acceptor ligand binding to the "aglycon" site and is quite comparable to those determined for acceptors in other systems (Street *et al.*, 1992; McCarter *et al.*, Wang *et al.*, 1993).

Stoichiometry of Incorporation of Inactivator by ESMS. The mass of the native xylanase (the *B. circulans* xylanase, which differs only in replacement of Ser147 by Thr, and is kinetically indistinguishable, was used for this experiment) obtained from ESMS was 20 398 ± 3 Da (expected 20 396 Da). After inactivation with 2FDNPXb a new peak was observed at 20 666 ± 2 Da with no peak from the underivatized enzyme. The molecular weight difference between the labeled and unlabeled xylanase is therefore 268 ± 5 Da, equal, within experimental error, to that of the 2FXb label (267 Da). Therefore, only one 2-deoxy-2-fluoroxylobiosyl unit is incorporated into each molecule of xylanase.

Identification of the Labeled Active Site Peptide by ESMS. Peptic hydrolysis of 2FXb-labeled xylanase resulted in a mixture of peptides which was separated by reverse-phase HPLC using the ESMS as detector. When the spectrometer was scanned in the normal LC/MS mode, the total ion chromatogram (TIC) of the 2FXb-labeled xylanase digest displayed a large number of peaks, which arise from every peptide in the mixture (Figure 3A). The peptide bearing the 2-fluoroxylobiosyl label was then identified in a second run by using the tandem mass spectrometer set up in the neutral loss mode. In this technique the ions are subjected to limited fragmentation by collisions with an inert gas (Ar) in a collision

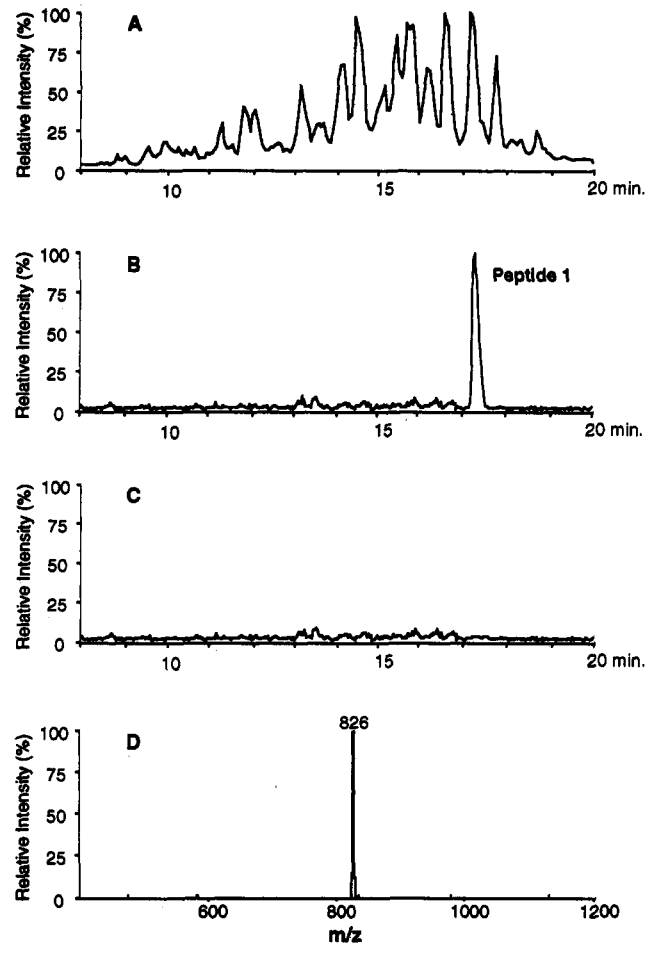


FIGURE 3: ESMS experiments on xylanase proteolytic digests (A) labeled with 2FXb, TIC in normal MS mode, (B) labeled with 2FXb, TIC in neutral loss mode, and (C) unlabeled, in neutral loss mode. (D) Mass spectrum of peptide 1 in panel B.

cell. Since the ester linkage between the sugar inhibitor and the peptide is one of the more labile linkages present, it is anticipated that homolytic cleavage of this bond will occur. This will result in the loss of a neutral sugar residue of known mass (267), leaving the peptide moiety with its original charge. The two quadrupoles were then scanned in a linked mode such that only ions *differing* in mass by the mass of the lost sugar moiety (267) could pass through both quadrupoles and be detected. Since the peptide may bear more than one charge, it may be necessary to look for m/z differences of one-half, or one-third, of the mass of the neutral species if the peptide has two, or three, charges, respectively. When the spectrometer was scanned in the neutral loss tandem MS/MS mode searching for the mass loss m/z 267, corresponding to the loss of the 2FXb label (MW = 267) from the labeled active site peptide in the singly charged state, no signal was detected. However, when the spectrometer was scanned for the mass loss m/z 133.5, a single peak was observed in the total ion chromatogram (Figure 3B). No such peak was detected in the neutral loss spectrum of the unlabeled xylanase digest (Figure 3C). These results therefore indicate that a doubly charged peptide corresponding to the covalently modified active site peptide is being selectively detected. This doubly charged labeled peptide was measured at m/z 826 (Figure 3D); thus its molecular weight is 650 [(826 × 2) − 2H]. Since the mass of 2FXb is 267, the unlabeled peptide must have a molecular weight of 1384 (1650 − 267 + 1 H).

Candidate peptides were then identified by inspection of the amino acid sequence of the enzyme (Oku *et al.*, 1993) and

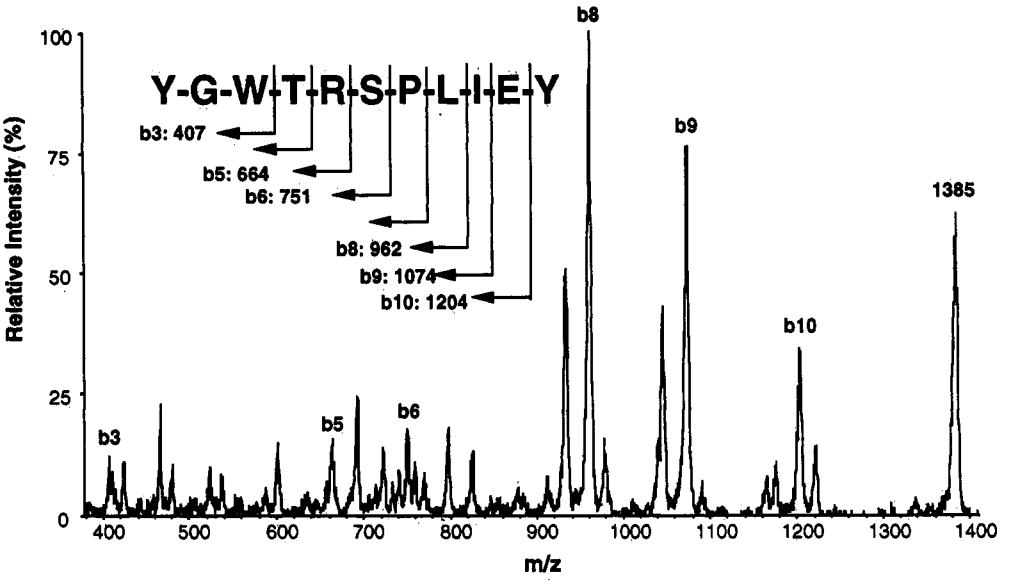


FIGURE 4: Tandem MS/MS daughter ion spectrum of the 2FXb-labeled active site peptide (m/z 826, in the doubly charged state).

searching for all possible peptides with this mass. Only three peptides with a mass 1384 ± 1 could be identified, namely, YGWTRSPLIEY (69–79), GWTRSPLIEYY (70–80), and PLIEYYVVDSW (75–85). They all contain the same Glu78 residue, and the third peptide also contains an aspartate (Asp83). Since an acidic residue is expected for the nucleophile of xylanase, Glu78 and Asp83 seemed the most likely candidates for the catalytic nucleophile in xylanase. Of these, Glu78 is the more likely since Asp83 is not conserved. The complete amino acid sequence of the labeled active site peptide was then determined by a combination of ESMS/MS and chemical sequencing.

Sequencing of Peptide. Sequence information on the labeled peptide was first obtained, without a need for further purification, by additional fragmentation of the peptide of interest in the daughter ion scan mode according to established protocols. The parent ion of m/z 826 was selected in the first quadrupole and subjected to collision-induced fragmentation in a collision cell in the second quadrupole; then the masses of the daughter ions produced were detected in the third quadrupole. The family of daughter ions produced is shown in Figure 4. The daughter ion at m/z 1385 arises from the loss of the 2FXb label plus a proton from the parent ion, resulting in the unlabeled peptide ion in the singly charged state (MH^+). Further fragmentations observed represent loss of fragments from the C-terminus. The N-terminal fragments are not observed in this mode since the loss of the charged N-terminal amino acid produces neutral peptides which are not detected. The peak at m/z 1204 (b10) is attributed to the loss of C-terminal tyrosine (m/z 181) from the parent ion peak at m/z 1385. The other peaks (b9, b8, b6, b5, and b3) result from the respective losses of EY, IEY, PLIEY, SPLIEY, RSPLIEY, and TRSPLIEY fragments from the C-terminus. A parallel "a" series of ions is also observed. Although low ion transmission was observed in the region below m/z 900, this sequence information, combined with the xylanase sequence and the mass of the intact peptide, is sufficient to identify the labeled peptide as YGWTRSPLIEY (69–79). Within this sequence, the only likely candidate for the nucleophile, based on chemical precedent (Sinnott, 1990) and on the relatively facile fragmentation in the mass spectrometer, is Glu78. Covalent attachment of 2FXb to Glu78 through an ester linkage was confirmed by aminolysis of the 2FXb-labeled xylanase digest. Thus after treatment with ammonium hydroxide, the labeled peptide with a molecular weight of 1650 [m/z 1651 (MH^{1+}, 826 (MH^{2+})] was replaced by a new peptide with a molecular weight of 1383 [m/z 1384 (MH^{1+}), 692.5 (MH^{2+})]. This indicates that the 2FXb-labeled Glu78, the only acidic residue in this peptide, has been replaced with a Gln, thereby indicating that the catalytic nucleophile in *B. subtilis* xylanase is Glu78.

Table 1: Edman Degradation of Active Site Peptide

cycle	PTH derivative	yield (pmol)	cDNA sequence
1	Y	56	Y
2	G	43	G
3	W	27	W
4	T	19	T
5	R	14	R
6	S	11	S
7	P	16	P
8	L	15	L
9	I	15	I
10	E	5	E
11	Y	7	Y

As additional confirmation of the sequence identity, a sample of the labeled peptide was obtained from the collected fractions split off prior to entry into the ESMS. This sample proved (by analytical HPLC) to be of sufficient purity for sequence determination. Subjection of this sample to Edman degradation resulted in the sequence information shown in Table 1. Once again these data confirm that YGWTRSPLIEY (69–79) is the peptide labeled with the 2FXb residue; thus Glu78 is the essential active site nucleophile in *B. subtilis* xylanase.

Conclusions. This study has shown that inactivation of *B. subtilis* xylanase can be achieved by the accumulation of a stable 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate and that this intermediate is catalytically competent. By use of this reagent in combination with a new tandem mass spectrometric technique for peptide identification, Glu78 has been identified as the functional nucleophile in the active site of this enzyme. This residue is one of two glutamic acids which are completely conserved in this family of enzymes. Furthermore, these two residues are convincingly located in a crevice believed to be the active site, with their two carboxyl groups facing each other at a separation of 7 Å. Mutation of Glu78 to Asp has been shown to reduce activity 2300-fold, while mutation to Gln abolishes activity (Wakarchuk *et al.*,

1993), exactly as has been seen for the catalytic nucleophiles in several other enzymes (Withers *et al.*, 1992; Gebler *et al.*, 1992b). Additionally, a recent publication (Bray & Clark, 1994) identified the same glutamic acid in the *S. commune* xylanase as the catalytically essential nucleophile by chemical modification studies using carbodiimides. Although carbodiimides are not as specific as our 2-fluoro-β-xylobioside for labeling the catalytic nucleophile, these data do combine to confirm the identify of Glu78 as the catalytic nucleophile in this biotechnologically interesting enzyme.

ACKNOWLEDGMENT

We thank Karen Rupitz for technical assistance, Dr. Heinz Nika and Ted Bures for chemical sequencing, and Lloyd MacKenzie for synthesis of the benzyl thioxylobioside. We also thank Drs. Warren Wakarchuk and Robert Campbell for many helpful discussions.

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