Stereochemistry of family 52 glycosyl hydrolases: a β-xylosidase from *Bacillus stearothermophilus* T-6 is a retaining enzyme

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Abstract **A β-xylosidase from *Bacillus stearothermophilus* T-6 assigned to the uncharacterized glycosyl hydrolase family 52 was cloned, overexpressed in *Escherichia coli* and purified. The enzyme showed maximum activity at 65°C and pH 5.6–6.3. The stereochemistry of the hydrolysis of *p*-nitrophenyl β-D-xylopyranoside was followed by ^{1}H-nuclear magnetic resonance. Time dependent spectrum analysis showed that the configuration of the anomeric carbon was retained, indicating that a retaining mechanism prevails in family 52 glycosyl hydrolases. Sequence alignment and site-directed mutagenesis enabled the identification of functionally important amino acid residues of which Glu337 and Glu413 are likely to be the two key catalytic residues involved in enzyme catalysis.** © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: β-Xylosidase; Nuclear magnetic resonance; Retaining mechanism; Glycosyl hydrolase family 52; Catalytic residue; *Bacillus stearothermophilus*

1. Introduction

β-D-Xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) are hemicellulases that hydrolyze xylooligosaccharides (mainly xylobiose) to xylose and are essential for the complete utilization of xylan. This heteropolysaccharide is composed of β-1,4-linked xylopyranosyl units carrying side chain moieties such as arabinofuranose, methyl glucuronic acid and acetyl. The branching and variability of the xylan structure requires the concerted action of several hemicellulolytic enzymes [1,2] including endo-1,4-β-xylanases (EC 3.2.1.8) that hydrolyze the xylan backbone, and β-xylosidases that cleave the resulting xylooligomers into xylose monomers. Hemicellulases in general, and xylan-degrading enzymes in particular, have attracted much attention owing to their industrial potential in paper pulp biobleaching [3,4]. Hemicellulases are also used for the bioconversion of lignocellulose material to fermentative products, for the improvement of animal feedstock digestibility, and have recently been suggested as glycosynthases for oligosaccharide synthesis [5].

β-Xylosidases are currently classified into families 39, 43 and 52 of glycosyl hydrolases, based on their amino acid sequence similarities [6,7]. β-Xylosidases, as do all glycosidases, hydrolyze the glycosidic bond by one of two major mechanisms, giving rise to either an overall retention or an overall inversion of the configuration of the anomeric substrate carbon [8]. Inverting glycosidases use a direct displacement mechanism in which a water molecule directly displaces the aglycone trough the direct involvement of two carboxylic amino acids of the enzyme. In this general mechanism, one of these carboxylic side chain acts as a general base while the other carboxylic side chain acts as a general acid [9]. In a similar way, catalysis by retaining glycosidases proceeds via a two-step double-displacement mechanism involving a glycosyl-enzyme intermediate [9]. During the first step of this reaction (glycosylation), the acid–base catalyst protonates the glycosidic oxygen concomitantly with bond cleavage and the nucleophile attacks at the anomeric center of the substrate sugar to form a covalent glycosyl-enzyme intermediate. In the second step (deglycosylation), the acid–base catalytic group deprotonates the incoming water molecule, which then attacks at the anomeric center of the substrate while displacing the sugar [9].

Within a specific glycosyl hydrolase family these catalytic residues and the stereochemical course of reaction have shown to be completely conserved [10,11]. The β-xylosidase from *Butyrivibrio fibrisolvens*, a member of family 43, was shown to cleave the glycosidic bond with an inversion of the anomeric configuration [12], whereas the *Thermoanaerobacterium saccharolyticum* β-xylosidase of family 39 was shown to proceed via retention of the anomeric configuration [13].

We have recently cloned and sequenced a 23.5 kb chromosomal segment from *Bacillus stearothermophilus* T-6 containing a cluster of xylan utilization genes. This region includes a partial open reading frame of a β-xylosidase (*xynB2*) gene showing homology to family 52 glycosyl hydrolases [14]. To date, no information is available on the mechanism by which this family cleaves its target substrate, and the two corresponding catalytic residues have not been identified. In this paper, we described the cloning, expression, purification and biochemical characterization of the β-xylosidase (XynB2) from *B. stearothermophilus* T-6. We investigated its stereo-

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chemical course of hydrolysis and identified its key catalytic amino acids.

2. Materials and methods

2.1. Cloning of the xynB2 gene

The gene encoding XynB2 from *B. stearothermophilus* T-6 was cloned via PCR. The N-terminal primer (5′-GAGGAATTCACCATGGCAACCAATCTATTTTTCAACGCCCACC-3′) was designed based on the *xylA* DNA sequence from *B. stearothermophilus* 21 [15], and contained an ATG translational start codon inside a *Nco*I restriction site (CCATGG). The C-terminal primer (5′-CGATCTAGATCTTCATTCCCCCTCCTCCAACC-3′) was designed based on the partial sequence available for the carboxy-terminal segment of *xynB2* from *B. stearothermophilus* T-6 [14]. This primer also contained a stop codon (TAG) and a *Bgl*II restriction site (AGATCT) at the end of the gene. Following amplification, the PCR product was purified, digested with *Nco*I and *Bgl*II, and then cloned into the *Nco*I and *Bam*HI sites of the T7 polymerase expression vector pET9d (Novagen), resulting in a plasmid pET9d-*xynB2*.

2.2. Protein production and purification

Cultures (*Escherichia coli* BL21(DE3) (pET9d-*xynB2*)) were grown overnight on Terrific Broth [16] with kanamycin (25 μg ml^{-1}) without induction (500 ml in a 2 l shake flasks shaken at 230 rpm and 310 K). The overnight cultures (2 l) were harvested, resuspended in 60 ml of 50 mM Tris–HCl and 100 mM NaCl pH 7.0, and disrupted by two passages through a French press at room temperature. The cell extract was centrifuged, and the soluble fraction was then heat-treated (333 K, 30 min) and centrifuged again at room temperature. The enzyme was further purified by gel filtration at room temperature on a Superdex 200 26/10 column (Pharmacia), running at 2.5 ml min^{-1} with 50 mM Tris–HCl buffer pH 7.0, 100 mM NaCl and 0.02% NaN_3. Enzyme purity was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [17] and by gel filtration.

2.3. Kinetic studies

The substrate used for the kinetic studies was *p*-nitrophenyl β-D-xylopyranoside (PNPX) (Sigma). Steady state kinetic studies were performed by following the spectroscopic absorbance changes in the visible range, using a Biochrom 4060 spectrophotometer (Pharmacia) equipped with a temperature-stabilized water circulating bath. Initial hydrolysis rates were determined by incubating solutions with different substrate concentrations in 100 mM phosphate buffer (pH 7.0) at 40°C within the spectrophotometer until thermal equilibration was achieved. Reactions were initiated by the addition of appropriately diluted enzyme, and the release of the phenol product was monitored at 420 nm. The extinction coefficient of *p*-nitrophenyl was $\Delta\in = 7.61$ mM^{-1} cm^{-1}. Values of K_m and k_{cat} were determined by non-linear regression analysis using the program GraFit 3.0 [18]. In some cases, reactions were carried out under stopped assay conditions at 50°C, and terminated by the addition of Na_2CO_3 to a final concentration of 0.3 M. Under these conditions, the extinction coefficient of *p*-nitrophenyl was $\Delta\in = 18$ mM^{-1} cm^{-1}. For pH dependence studies, the buffers were citric acid–Na_2HPO_4 (pH 4.0–6.5) and phosphate buffer (pH 6.0–8.0). The effect of temperature on the reaction rate was determined by performing the standard reaction in phosphate buffer (pH 7.0) for 10 min at different temperatures ranging from 45 to 75°C. Thermoinactivation experiments were performed by incubating the enzyme (1.86 μg ml^{-1} in phosphate buffer, pH 7.0) at 65, 70 and 75°C, and removing samples at various times. The residual enzymatic activity in each tube was determined by the standard assay.

2.4. ^{1}H-nuclear magnetic resonance (NMR) experiment

The enzyme (3.5 mg) was dialyzed against a 5 mM triethanolamine hydrochloride buffer, pH 6.0 and 30 mM NaCl, lyophilized and resuspended in 1 ml D_2O. PNPX was dissolved in a deuterated buffer (5 mM triethanolamine hydrochloride and 30 mM NaCl, pH 6.0). ^{1}H-NMR spectra were recorded at an ambient temperature in a Bruker AM-400 spectrometer operated at 400 MHz and equipped with a 5 mm ^{1}H/^{13}C probe. After recording the spectrum of the substrate (0.56 ml of 2.8 mM), 40 μl of enzyme solution were added to the test tube and spectra were recorded at different time intervals. The ^{1}H-NMR spectrum of xylose was recorded under similar conditions at the mutarotation equilibrium. The assignment of important resonance peaks was done on the basis of published data [13].

2.5. Mutagenesis

Mutagenesis was performed following the method of Meza et al. [19] with slight modifications. This modified strategy employs two flanking primers (N-terminal and C-terminal primers that were used for cloning the *xynB2* gene) and an internal mutagenic primer. The mutagenic and C-terminal primers were used to amplify a fragment of 0.55–1.1 kb (depending on the mutation) that overlapped with a linearized pET9d-*xynB2* (digested with *Pau*I downstream to the locus of the target mutation). These overlapping fragments were then annealed and extended to produce the desired full-length *xynB2* gene. The flanking primers were then added to the reaction mixture, and the DNA was amplified by conventional PCR. Each mutagenic primer was designed to include the mutation and a restriction site that was then used for screening the appropriate mutant. The mutagenic primers were as follows (the mutated nucleotides are shown in bold and the restriction site is underlined): E335A (*Hpa*I), 5′-CCGATTTGGGTCGTTAACGCAGGCGAGTACCG-3′; E337A (*Hpa*I), 5′-GGAAAGCCGATTTGGGTCGTTAACGAAGGCGCGTACCGG-3′; E413A (*Mlu*I), 5′-ACGTTCTCACGCCCGCATTACTCGTCATACGCGTTATACGGGATCAGCGGCTGCTTTTCACAT-3′; E427A (*Spe*I), 5′-GGCTGCTTTTCACATATGACGCACGCACAACTAGTCAACTGGGTGCTTTGCGCAGCG-3′; E522A (*Nhe*I), 5′-CCTATGTGGCGCTAGCAAAGTTGTTC-3′. All the mutations were confirmed by DNA sequencing.

3. Results and discussion

3.1. Cloning and sequence analysis of XynB2

In the framework of studying the hemicellulolytic system in *B. stearothermophilus* T-6, we have recently cloned and sequenced a 23.5 kb chromosomal segment containing xylan degrading enzymes [14]. The segment contained a partial open reading frame of *xynB2* having a significantly high sequence homology to the *xylA* gene, which encodes a β-xylosidase from *B. stearothermophilus* 21[15]. This information was used to design PCR primers to amplify the entire *xynB2* gene from *B. stearothermophilus* T-6 chromosomal DNA and to clone it into the T7 polymerase expression vector, pET9d. The gene (AJ305327) was sequenced on both strands, and the confirmed full sequence indicated a protein consisting of 705 amino acids having a calculated molecular weight of 79 894 and a p*I* of 4.91. The amino acid sequence was scanned with the BLAST2 [20] program, and showed 94, 74, 60 and 51% identity to family 52 β-xylosidases from *B. stearothermophilus* 21, *Bacillus halodurans*, *Aeromonas caviae* and *B. stearothermophilus* 236, respectively (Fig. 1).

3.2. Overexpression and purification of XynB2

Expression of the *xynB2* gene was highly efficient even without induction [21] (Fig. 2). The overall purification procedure included two steps, heat treatment and gel filtration, and resulted in about 0.5 g of purified enzyme (>95% purity by inspection) from a 1 l culture with an overall yield of 60% (Table 1, Fig. 2). The simplicity and high yield of this purification procedure makes XynB2 an excellent representative target of family 52 glycosyl hydrolases for both structural and functional studies.

3.3. Biochemical characterization of XynB2

The molecular weight of the native enzyme was estimated by gel filtration using a Superdex 200 26/10 column (Pharmacia) with known M_r protein markers. The retention coefficients were: 0.230, 0.305, 0.361, 0.596, and 0.600 for α-arabi-

```
          1                                                                                                  80
{BsteT-6} .........m atnlFfNAHH sPvGafasFT LGfpGksGGl dLELarpprQ NvfIGvesph epglYhilPF aetagedEsk
 {Bste21} .........m ptnlFfNAHH sPvGafasFT LGfpGksGGl dLELarpprQ NvlIGveslh esglYhvlPF letaeedEsk
  {Bhalo} .......mft pkniFfNAHH sPvGafssFT LGfpGsgGGl dLELgrspkQ NvyIGles.e eigkYetlPF hlda.esEaa
   {Acav} .......mst fksiFyNAHH aPiGafasFT LGykGakGGl gLELgkpadQ NvyIGlqs.r dgenYqalPF ye.asqdEss
{Bste236} msveqevinl gkniFfNAHH sPmGcspvFT LGypGqsGGf dLELgsppnQ NiyIGlqddg q.erYrafPF f.gegldErd
Consensus ---------- ----F-NAHH -P-G----FT LG--G--GG- -LEL-----Q N--IG----- ----Y---PF -------E--

          81                                                                                                 160
{BsteT-6} RYdienpDpn .......pqk pniLi.pfak erIeReFrva tDtWkAGDLt ltiySPvkaV PdPetaseEe LklAlvPAVi
 {Bste21} RYdienpDpn .......pqk pniLi.pfak eeIqReFhva tDtWkAGDLt ftiySPvkaV PnPetadeEe LklAlvPAVi
  {Bhalo} RYdlenpDpn .......qnk pqiLf.pfgk deImRdFnag tDtWkAGDLk ftiySPvqsV PdPskaseEe LkaAivPAVl
   {Acav} RYdveklDng dtasitasqv pqpLitafrd edItReFtsg tDtWtAGDLt friySPvrpV PdPtngdrEe LmdAlvPAVl
{Bste236} RYttdesDsg sserhsvqqe esgLiipynr deIvRrFgaa iDeWqAGDLt frliSPfegV PdPetateEq LrfAlmPAVl
Consensus RY-----D-- ---------- ---L------ --I-R-F--- -D-W-AGDL- ----SP---V P-P-----E- L--A--PAV-

          161                                                                                                240
{BsteT-6} vEmTiDNtnG trtRrAfFGf egtDPytsmR ri.ddtcpql rGvGQGRilg Iaskdegvrs alhfsmedil tatleen..w
 {Bste21} vEmTiDNtnG traRrAfFGf egtDPytsmR ri.ddtcpql rGvGQGRils Ivskdegvrs alhfsmedil taqleen..w
  {Bhalo} aElTiDNsrG keqRrAfFGy egsDPyssmR ki.ddt.ssv aGvGQGRlts Ivsndpsvks alhfsmedil snpleen..w
   {Acav} vEmTiDNteG qqpRkAyFGy qgnDPysamR liggpeggsi tGvGQGRlta Imsaddglwp argftlekll qekhren..l
{Bste236} aEiTvDNtrG tstRkAfFGl qnnDPvsaiR rledvtggri cGiGQGRhta Iarairdyqr rifydgidsq akgsgkppvw
Consensus -E-T-DN--G ---R-A-FG- ---DP----R ---------- -G-GQGR--- I--------- ---------- ----------

          241                                                                                                320
{BsteT-6} tfglGkvgAl iadvpaGEkk TyqFavCFyR ggcvtagmda sYfYtrfFhn IeevglYALe qaevlkeqaf rsneliekew
 {Bste21} tfglGkvgAl ivdvpaGEkk TyqFavCFyR ggyvtagmda sYfYtrfFqn IeevglYALe qaevlkeqsf rsnkliekew
  {Bhalo} tfglGktgAl vvdvkpGEvk TvrFavCFfR ggvvtagldt eYlYtkfFkt IeevgeYALg qfdaiaakaa esdelvansk
   {Acav} afglGstaAl lmevpaGEkr TyqFavCFyR ggivttgmdt tYwYtryFsd IqaageYALq rfseltascg eieqrlgtaa
{Bste236} pws.Gr..Av lmdtpaGEka TyrFglCFyR pvrhnrnr.c sYyYsryFan IeevaeYALa qfnrklevar qadalvndak
Consensus ----G---A- ------GE-- T--F--CF-R ---------- -Y-Y---F-- I-----YAL- ---------- ----------

          321                                                                                                400
{BsteT-6} LsdDQkFmmA HaIrSYYgnT qLLeheGkPi WvVNEGEYRM MNTfDLtvDq LffELkmNPW TVkNvLdfyv eRYSYeDrVr
 {Bste21} LsdDQtFmmA HaIrSYYgnT qLLeheGkPi WvVNEGEYRM MNTfDLtvDq LffELklNPW TVkNvLdlyv eRYSYeDrVr
  {Bhalo} LsdDQkFmmA HaIrSYYgcT qLLeheGrPl WvVNEGEYRM MNTfDLtvDh LffELkmNPW TVkNvLdlfv dRYSYeDkVr
   {Acav} LteDQsFmlA HsIhSYYasT qLLdadGePl WvVNEGEYRM MNTlDLtaDq LyfELalNPW TVrNeLewfv kRYSYtDeVr
{Bste236} LseDQkFivA HaIrSYYgsT eLLehdGkPl WiVNEGEYRM MNTfDLlvDq LfyELkmNPW TVkNeLdlyt sRYSYrDtVd
Consensus L--DQ-F--A H-I-SYY--T -LL---G-P- W-VNEGEYRM MNT-DL--D- L--EL--NPW TV-N-L---- -RYSY-D-V-
                                             * *
          401                                                                                                480
{BsteT-6} FPgdeteYPg GIsFTHDmGV ANtFSrphyS sYELygisgC FshMthEqLV NWvlcAaVYi eQTkDwawrd rrltileqcl
 {Bste21} FPgeeteYPs GIsFTHDmGV ANtFSrphyS sYELygisgC FShMthEqLV NWvlcAaVYi eQTkDwawrd krlaileqcl
  {Bhalo} FPsndteYPg GIsFTHDmGV ANtFSrknyS sYELygldgC FShMtqEqLV NWllcAsVYv eQTgDkewkk srlpvfeqcf
   {Acav} FPgeeklYPg GItFTHDiGV ANvFSrpghS aYELvgiddC FSqMshEeLV NWlccAtVYi eQTqDqafvk nmlpiiqdcf
{Bste236} FPgdateYPg GIsFTHDmGV ANsFSlpgyS sYELhaiddC FShMthEqLV NWvlsAtVYv hQTnDrewle pqpadsgasl
Consensus FP-----YP- GI-FTHD-GV AN-FS----S -YEL-----C FS-M--E-LV NW---A-VY- -QT-D----- ----------
                                          *           *
          481                                                                                                560
{BsteT-6} eSMvrRDHPD pekRnGvMGL DStRTmGGae ittydsldvs lgqarnnlyl AGKcWAaYVA lEKlFrdvgk eelaalAreQ
 {Bste21} eSMvrRDHPD peqRnGvMGL DStRTmGGae ittydsldvs lgqarnnlyl AGKcWAaYVA lEKlFrdvgk eelaalAgeQ
  {Bhalo} eSMlnRDHPD pekRnGlMGL DStRTmGGae ittydsldvs lgqarnniyl AGKcWAaYVA lEKiFaeegl eelskaAgeQ
   {Acav} eSMlnRDHPD aehRnGlMGL DSsRTqGGae ittydsldvs lgqsrnniyl AGKcWAiYVA lEKlFatekl anlshqAglQ
{Bste236} dSMvnRDHPD psqRtGmMGL DSsRTmGGrk sppiqpgrlv gasaeqhlf. AGKsWAaYVA mEKlFrdngr ksaaalAgrQ
Consensus -SM--RDHPD ---R-G-MGL DS-RT-GG-- ---------- ---------- AGK-WA-YVA -EK-F----- ------A--Q
                                                                                 *
          561                                                                                                640
{BsteT-6} aekcAatIvs hvtedGyipa vmgegndski ipaiegLvfp Yftnchealr edgrfgdyir alrqhlqyvl regiclfpdg
 {Bste21} aekcAatIvs hvtddGyipa imgegndski ipaiegLvfp Yftnchеald engrfgayiq alrnhlqyvl regiclfpdg
  {Bhalo} aeraAktIcg nvtegGyvpa vigegnnski ipaiegLvfp Yftncreald rngrfgeyiq vlenhleavl eegiclfpng
   {Acav} adrcAasIaa qltdgGyipa viaenndsri ipaiegLvfp Yftgceaald vkgrfgpylk alqthlktvl vpgtclfedg
{Bste236} pelgAgtIts hllpgGifqp sfrrtmtprs slrsrvLssr Ytpdakkrwi qtadsvhilr rcpri..... ..........
Consensus ----A--I-- -----G---- ---------- ------L--- Y--------- ---------- ---------- ----------

          641                                                                                                721
{BsteT-6} gwkisstsnn swlskiylcq fiarrilgwe wdeqgkrada ahvawlthpt lsiwswsdqi iageisgsky yprgvtsilw
 {Bste21} gwkisstsnn swlskiylcq fiarhilgwe wdeqgkrada ahvawlthpt lsiwswsdqi iageitgsky yprgvtsilw
  {Bhalo} gwkisstsnn swlskiylsq fiarhiigle wdekgarada ahvewlthpt lsvwswsdqi isgeiagsky yprgvtsilw
   {Acav} gwklsstsnn swlskiylsq fiarelldve wdetgkaada ahvnwllhpe esywcwsdqi lsgvavgsky yprgvtsilw
{Bste236} .......... .......... .......... .......... .......... .......... .......... ..........
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------


          721               740
{BsteT-6} leege..... ..........
 {Bste21} leege..... ..........
  {Bhalo} leeit..... ..........
   {Acav} llegkgnrlt qiyaskeavq
{Bste236} .......... ..........
Consensus ---------- ----------
```

Fig. 1. Sequence alignment of the known enzymes comprising family 52 glycosyl hydrolases. The consensus sequence is shown at the bottom of the alignment. The abbreviations used and the GenPept accession numbers are as follows: {BsteT-6}-β-xylosidase from *B. stearothermophilus* T-6 (AAC98122); {Bste21}-β-xylosidase from *B. stearothermophilus* 21 (BAA05667); {Bste236}-β-xylosidase from *B. stearothermophilus* 236 (AAA50863); {Acav}-β-xylosidase from *A. caviae* (BAA74507); {Bhalo}-β-xylosidase from *B. halodurans* (BAB05833). Conserved residues are in capital bold letters. The asterisks indicate the conserved residues that were subjected to site-directed mutagenesis.

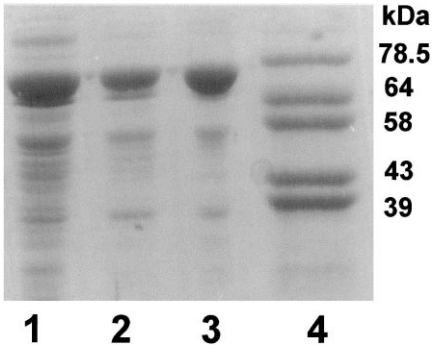

Fig. 2. SDS–PAGE of the different purification steps of XynB2. Lane (1): Crude extract. Lane (2): The soluble fraction after heat treatment (60°C for 30 min). Lane (3): After gel filtration. Lane (4): Molecular weight standards.

nofuranosidase T-6 [22] (M_r 228 864), XynB1 T-6 [23] (M_r 232 004), XynB2, XynA T-6 [24] (M_r 43 808) and XynA2 T-6 [25] (M_r 39 357), respectively, suggesting a M_r of about 157 000 for XynB2. Considering these results and the calculated M_r of a monomer of the enzyme (79 894), XynB2 is a dimer consisting of two identical subunits. The homologous β-xylosidase from *B. stearothermophilus* 21 is also a dimer [26].

XynB2 was shown to be most active at pH 5.6–6.3, and retained less than 40% of its activity at pH 5.0 and 7.3. The pH optima for the activity of other bacterial β-xylosidases are usually closer to pH 7.0, whereas fungal enzymes are active in more acidic conditions ($<$ pH 5.0) [27,28]. The optimal temperature for XynB2 activity in a 10 min reaction was 65°C and is similar to the optimal temperature observed for the β-xylosidases from *B. stearothermophilus* 21 [26], *Trichoderma harzianum* [29], *Phanerochaete chrysosporium* [30], *Clostridium stercorarium* [31] and *Trichoderma reesei* RUT c-30 [32]. A much higher temperature optimum, 98°C, was observed for the β-xylosidase from *Thermotoga* sp. strain FjSS3-B.1 [33]. Thermoinactivation experiments for XynB2 revealed half-lives of 2.05, 0.83 and 0.036 h at 65, 70 and 75°C, respectively.

3.4. Stereochemistry of the enzymatic hydrolysis

The stereochemical course of the hydrolysis reaction of PNPX as catalyzed by XynB2 was followed by ^{1}H-NMR spectroscopy. Fig. 3 shows the partial ^{1}H-NMR spectrum of the substrate, and several spectra recorded at several time intervals following the addition of the XynB2 enzyme.

In the spectrum of the PNPX substrate, the resonance at 5.09 ppm is assigned as the anomeric proton at the nitrophenylated carbon. 8 min after adding the enzyme, the reaction reached a completion as judged by the complete disappearance of this peak. Concomitantly, a new peak appeared at 4.43 ppm corresponding to the anomeric proton of β-xylose. After 45 min of the reaction, a new peak appears at 5.05 ppm, reflecting the anomeric proton of α-xylose that is formed due to mutarotation of the free sugar. The observed coupling constants, $^3J = 7.69$ Hz and $^3J = 4.64$ Hz, for the β-and α-anomers, respectively, provide further support for the assignment of these anomers. At this time, the ratio between the β-and the α-anomers was 1/0.17. After 22 h the reaction reached the equilibrium of free xylose with the anomeric ratio of 1/0.5. Since XynB2 initially produces β-xylose as the predominant anomer, this enzyme appears to operate with an overall retention of the anomeric configuration. Analysis of the stereochemical course of reactions catalyzed by a range of glycosyl hydrolases has shown that the mechanism of bond cleavage is conserved in enzymes belonging to the same glycosyl hydrolase family [11]. Thus, enzymes of family 52 act with retention of anomeric configuration.

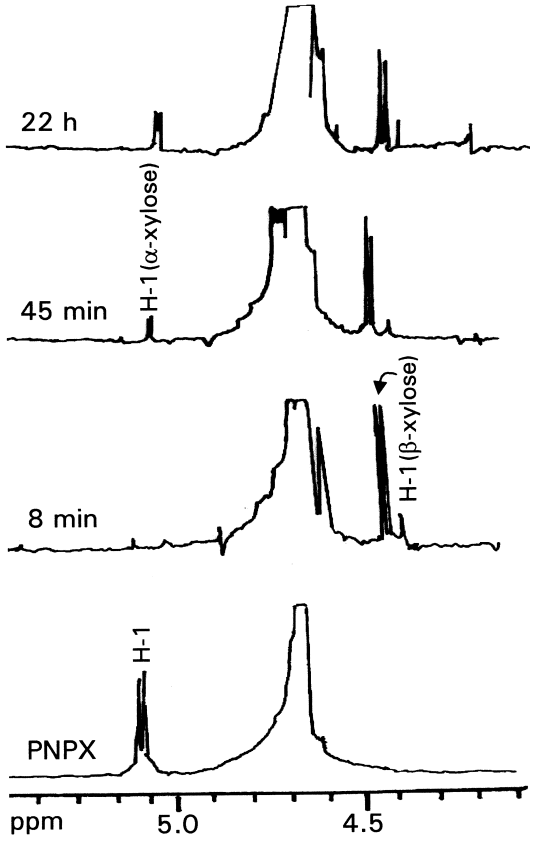

Fig. 3. ^{1}H-NMR spectra during the hydrolysis of PNPX by XynB2 after incubation for the times indicated. The assignments of the significant signals are indicated.

3.5. Identification of the key catalytic residues in glycosyl hydrolases family 52

The two key catalytic carboxylates in retaining glycosidases were previously shown to be invariant in enzymes from the same glycosyl hydrolase family [10]. To identify the corresponding catalytic pair for XynB2 (and hence for glycosyl hydrolases family 52), five conserved acidic residues (E335, E337, E413, E427, E522) were mutated to alanine and their kinetic constants were determined (Table 2).

Table 1
Purification of XynB2 from crude extract of recombinant *E. coli* BL21(DE3) pET9d-*xynB2*

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Cell free extract	80	8840	5700	1.6	100	1.0
Heat treatment	80	6955	3250	2.1	79	1.3
Gel filtration	125	5114	958	5.3	58	3.3

Table 2
Kinetic parameters for the hydrolysis of PNPX by wild-type and mutant forms of XynB2

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Wild-type	14.6	0.20	72.8
E335A	0.0047	0.86	0.0055
E337A	$<1\times10^{-5}$	ND[a]	ND
E413A	$<1\times10^{-5}$	ND	ND
E427A	0.79	11.9	0.07
E522A	14.1	0.18	78.3

[a]ND: not determined.

Of all of these mutations, only E522A retained significant activity, therefore it is unlikely that this residue is involved in catalysis and/or substrate binding. Elimination of E427 caused a relatively small reduction (18-fold) in k_{cat} but increased the K_m value, suggesting that this residue may be involved in substrate binding. The E335 residue was first suggested by us as the acid–base catalyst since it resides within a highly conserved region following an invariant asparagine [10]. Although the E335A mutation caused a large decrease in k_{cat} (over three orders of magnitude), surprisingly the K_m value increased (from 0.2 to 0.86 mM). In most reported studies, an acid–base mutation caused a decrease in K_m since the elimination of the acid–base should reduce the deglycosylation step [34]. In addition, the pH-activity profile of the E335A mutant was bell-shaped (results not shown), similar to that of the wild-type, suggesting that the two ionizable carboxylic acids remained intact. Thus, E335 does not appear to be the acid–base catalyst but is obviously important for activity. The E337A and E413A mutants caused the largest decrease in activity and were essentially inactive ($<1\times10^{-5}$). This magnitude of decrease in activity was also found in other catalytic mutants of retaining glycosyl hydrolases [34].

Several approaches may be employed to identify the catalytic residues in glycosyl hydrolases including: kinetic analysis of the catalytic mutants using substrates bearing different leaving groups, azide rescue of activity for these mutants and their pH dependence compared with that of the wild-type enzyme [34]. In addition, a definite identification of the catalytic nucleophile is possible by using fluorinated substrates functioning as mechanism-based inactivators [35]. Although it is likely that both E337 and E413 are the catalytic pair of XynB2, it should be noted that the E337A and E413A mutants did not exhibit significant rate enhancement in the presence of 1.8 M azide (results not shown), as should be the case with such catalytic mutants [34]. This puzzling experimental result should be further clarified and additional supporting experiments should be performed in order to fully determine the catalytic residues of this enzyme and their exact role in catalysis. A number of these experiments are now in progress.

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