Specific characterization of substrate and inhibitor binding sites of a glycosyl hydrolase family 11 xylanase from Aspergillus niger†

Tariq A. Tahir†, Jean-Guy Berrin‡§, Ruth Flatman†, Alain Roussel‖, Peter Roepstorff¶, Gary Williamson†‖, and Nathalie Juge†§,*

†This work was partially funded by the European Commission (GEMINI, QLK1-2000-00811) and the BBSRC. T.T. is the recipient of a BBSRC-case studentship (99/B2/D/05557).

‡Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom, §Institut Méditerranéen de Recherche en Nutrition, UMR INRA 1111, Faculté des Sciences de St Jérôme, Av. Escadrille Normandie-Niemen, Marseilles, F-13397 Cedex 20, France, ¶Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark, AFMB CNRS-UMR 6098, 31 ch. Joseph Aiguier, Marseilles, F-13402 Cedex 20, France

‖Present address: Nestlé Research Center, PO Box 44, CH-1000 Lausanne 26, Switzerland.

*To whom correspondence should be addressed. Nathalie Juge Tel. No. +44 1603 255068; Fax No. +44 1603 255038; E-mail: nathalie.juge@bbsrc.ac.uk.

Running Title: Mutational analysis of A. niger xylanase active site.
The importance of aromatic and charged residues at the surface of the active site of a family 11 xylanase from *Aspergillus niger* was evaluated using site-directed mutagenesis. Ten mutant proteins were heterologously produced in *Pichia pastoris*, and their biochemical properties and kinetic parameters determined. The specific activity of the Y6A, Y10A, Y89A, Y164A, and W172A mutant enzymes was drastically reduced. The low specific activity of Y6A and Y89A was entirely accounted for by a change in $k_{\text{cat}}$ and $K_M$, respectively, while the lower value of Y10A, Y164A and W172A was due to a combination of increased $K_M$ and decreased $k_{\text{cat}}$. Tyr6, Tyr10, Tyr89, Tyr164, and Trp172 are proposed as substrate-binding residues, a finding consistent with structural sequence alignments of family 11 xylanases and with the three-dimensional structure of the *A. niger* xylanase in complex with the modeled xylobiose. All other variants, D113A, D113N, N117A, E118A, and E118Q retained full wild-type activity. Only N117A lost its sensitivity to XIP-I, a protein inhibitor isolated from wheat, and this mutation did not affect the fold of the xylanase as revealed by circular dichroism. The N117A variant showed kinetics, pH stability, hydrolysis products pattern, substrate specificity and structural properties identical to that of the wild-type xylanase. The loss of inhibition, as measured in activity assays, was due to abolition of the interaction between XIP-I and the mutant enzyme, as demonstrated by surface plasmon resonance and electrophoretic titration. A close inspection of the three-dimensional structure of *A. niger* xylanase suggests that the binding site of XIP-I is located at the conserved “thumb” hairpin loop of family 11 xylanases.
INTRODUCTION

Endo-(1,4)-β-xylanases (EC 3.2.1.8) depolymerize the xylan backbone by cleaving the β-(1,4) glycosidic bonds between D-xylose residues in the main chain to produce short xylooligosaccharides (1,2). Based on amino acid sequence similarities, the endoxylanases have been grouped into two classes: family 10 and family 11 (3-5). The two families have different molecular structures, molecular weights, and catalytic properties (6-8). Family 11 xylanases hydrolyse xylosidic substrates with retention of anomeric configuration. This proceeds via a double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed in the glycosylation step and subsequently hydrolysed in the deglycosylation step via transition states including substrate distortion and electrophilic migration of C1 (9-11). According to this mechanism, the catalytic residues act as a nucleophile and an acid-base catalyst, respectively (12). The three-dimensional structures of 12 family 11 endoxylanases are available, from both bacteria (13-19) and fungi (20-27). All reported structures of family 11 endo-xylanases present one single (catalytic) domain with an all β-strand “sandwich-like” fold containing two β-sheets forming a large cleft that can accommodate the xylan polymers and the overall structure has the shape of a “right-hand” as described by Törrönen et al. (21). The active site contains two conserved glutamate residues located on either side of the extended open cleft, which have been identified as the nucleophilic and acid/base catalysts (28). Only a few ligand-enzyme complexes (13, 16, 17, 29, 30) have been crystallized. The subsites that bind the glycone or aglycone regions of the substrate are prefixed by – and +, respectively, and their number is related to the proximity to the site of bond cleavage (31). The cleavage by definition takes place between subsites –1 and +1. Subsites –3, –2 and –1 are well characterized by the inspection of the complex structures (13, 16, 17) but the characterization of the aglycone subsites is based only on modeling (22, 26). Aspergillus niger xylanase is a 20 kDa family 11 xylanase with a pI and a pH optimum of 3.5 (32, 33) for which an
X-ray crystal structure is available (24). The active site is located within a deep and long cleft, which is lined with many aromatic amino acid residues and is large enough to accommodate at least four xylose residues (24). The two conserved catalytic residues, Glu79 and Glu170, face each other on opposite sides (24). This fungal enzyme has been studied for its role as a bread improver (32, 34-36), in wheat processing (37) and as a supplement in animal feed (38, 39).

The presence of protein inhibitors of endo-1,4-β-D-xylanase in cereals was first reported in wheat flour protein extracts (40-42). To date, two types of endoxylanase inhibitors with different structures and specificities have been described. The first type are xylanase inhibitor protein (XIP)-like inhibitors and have been isolated from wheat (43, 44) and rye (45). They are monomeric glycosylated proteins with Mr’s of approx. 29 kDa and pI-values of 8.7-8.9. The second type are the Triticum aestivum L. xylanase inhibitor (TAXI)-like inhibitors (36). They are high pI, non-glycosylated proteins with Mr’s of approx. 40 kDa. At least two inhibitors of this type (TAXI I and TAXI II) with different pI values (8.8 and ~9.3, respectively) and varying specificities towards different endoxylanases have been identified in wheat (46). The N-terminal amino acid sequences of TAXI-I and TAXI-II showed a high degree of identity, but there was no similarity to XIP-I. The TAXI-like inhibitors are believed to be active against bacterial and fungal family 11 endoxylanases but not against family 10 endoxylanases (46, 47). XIP-I inhibited both family-10 and 11 fungal xylanases apart from the family 10 Aspergillus aculeatus xylanase with $K_i$ values ranging from 3.4 to 610 nM, but bacterial family 10 and 11 xylanases were not inhibited (48).

We have previously reported the production and characterization of the A. niger xylanase in Pichia pastoris and shown that the recombinant enzyme was similar to the native enzyme and was competitively inhibited by XIP-I with a $K_i = 350$ nM (33, 48). The chosen strategy for mutational analysis of A. niger xylanase took advantage of data available on the A. niger xylanase/XIP-I complex such as (a) kinetics of inhibition, (b) titration curves, (c) isothermal calorimetry data, and also the availability of (d) the three-dimensional structure of A. niger xylanase (protein data bank code: 1UKR) and (e) an efficient heterologous system for expression of A. niger xylanase. The
inhibition mechanism of XIP-I against family 11 fungal *A. niger* xylanase has been studied in detail (48). The inhibition is pH-dependent in the range 4-7, as determined by activity assays and titration curves, illustrating the importance of electrostatic interactions in the strength of the interaction. Moreover, isothermal titration calorimetry of the XIP-I/*A. niger* xylanase complex showed the formation of a complex with a stoichiometry of (1:1) and a heat capacity change of −1.38 kJ/mol, suggesting that the interaction was enthalpy-driven (48). Aromatic and charged amino acids are believed to play a pivotal role in protein-protein interactions, by forming hydrophobic stacking and electrostatic interactions, respectively with the target ligand. Since the inhibition is competitive, residues were selected near the active site. The three-dimensional structure of the *A. niger* xylanase revealed several aromatic and charged residues (Tyr6, Tyr10, Tyr89, Asp113, Asn117, Asp118, Tyr164 and Trp172) on the surface around the binding cleft. The structural analysis of the enzyme showed that these target residues are not part of the hydrogen bond network in the vicinity of the two catalytic residues (Glu79 and Glu170) (24). To investigate the importance of these residues in binding to XIP-I, appropriate mutations in the xylanase were constructed, and the biochemical properties of the mutated enzymes were evaluated in terms of kinetic properties and ability to interact with XIP-I.

Our results indicate that Asn117 is critical for the XIP-I-binding capacity of the enzyme. The mutational analysis also allowed a better understanding of the role of individual residues involved in a number of subsites in the active-site cleft of a family 11 glycosyl hydrolase.
Materials, plasmids and strains - The *Pichia pastoris* expression kit was obtained from Invitrogen (San Diego, CA, U.S.A.). The pCR®4-TOPO TA cloning vector was from Invitrogen (CA, USA). The pHIL-D2/*XylA* expression vector expressing wild-type *XylA* was from in house collection (33). Restriction endonucleases and DNA modifying enzymes were from Promega (Madison WI, USA) and used according to the manufacturer's recommendations. Pfu polymerase for polymerase chain reaction (PCR) was from Stratagene (UK). *E. coli* DH5 (*supE*44, *hsdR17, recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1) was used for DNA manipulation. *P. pastoris* strain (*his4*)/GS115 was from Invitrogen. Xylan birchwood, (1,4)-β-xylose and dinitrosalicylic acid (DNS) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Azo-Wheat-Arabinoylan and low viscosity wheat arabinoxylan were from Megazyme International Ireland Ltd (Co. Wicklow, Ireland), and birchwood (1,4)-β-xylan was from Fluka (Sigma-Aldrich). Oligonucleotides were synthesized by MWG Biotech (Germany) as High Purity Salt-Free oligos (HPSF). PCR cycling was performed in a Perkin Elmer GeneAmp PCR system 2400.

Cloning, mutagenesis, and protein expression - Site-directed mutagenesis was performed using the overlap extension PCR method previously described (49). The following internal mutagenic primers were designed to incorporate single mutations (in bold), Y6A forward 5’ ATT AAC GCC GTG CAA 3’; Y6A reverse 5’ TTG CAC GGC GTT AAT 3’; Y10A forward 5’ CAA AAC GCC AAC GGC 3’; Y10A reverse 5’ GCC GTT GGC GTT TTG 3’; Y89A forward 5’ GGT GAT GCC AAC CCT 3’; Y89A reverse 5’ AGG GTT GGC ATC ACC 3’; D113A forward 5’ TGC ACC GGC ACT CGA 3’; D113A reverse 5’ TCG AGT GGC GGT GCA 3’; D113N forward 5’ TGC ACC AAC ACT CGA 3’; D113N reverse 5’ TCG AGT GGC GGT GCA 3’; Y164A forward 5’ TGC ACC AAC ACT GCC GAA CCG 3’; N117A reverse 5’ CGG TTC GGC AGT TCG 3’; E118A forward 5’ ACT AAC CAA CCG TCC 3’; E118Q forward 5’ GGA CGG TGC GTT AGT 3’; Y164A forward
5’ TTC AAT GCT CAG GTC 3’; Y164A reverse 5’ GAC CTG AGC ATT GAA
3’; W172A forward 5’ GAA GCA GCG AGC GGT 3’ and W172A reverse
5’ ACC GCT CGC TGC TTC 3’.

Primers which hybridize to the extremities of the A. niger xylanase cDNA were as follows:
pHILD-2/XylAF 5’ TTT TTT GAA TTC ATG CTT TTG CAA GCC TTC C 3’ (forward primer);
pHILD-2/XylAR 5’ TTT TTT GAA TTC TTA AGA GGA GAT CGT GAC ACT GGC 3’ (reverse
primer). For the first round of PCR, 50 pmoles of the forward pHILD-2/XylAF or reverse pHILD-
2/XylAR primer were used along with equimolar amounts of reverse or forward mutagenic primer,
respectively. DNA amplification was carried out using 10 ng of template DNA (pHILD-2/XylA),
1.25 U of Pfu polymerase and 50 mM dNTP through 25 cycles of denaturation (1 min at 94 ºC),
annealing (2 min at 35-50 ºC [depending on the mutant]), and extension (2.5 min at 72 ºC). The
resulting PCR products were gel-purified using the Qiaquick PCR purification kit (Qiagen Inc.,
Chatsworth, CA). The final PCR was performed using 5 ng of each purified PCR products, along
with 1.25 U Pfu polymerase, 50 mM dNTP. After 5 cycles of denaturation (1 min at 94 ºC),
annealing (2 min at 42 ºC) and extension (3 min at 72 ºC) 50 pmole of the forward (pHILD-
2/XylAF) and reverse (pHILD-2/XylAR) primers were added and then subjected to 25 cycles of
denaturation (1 min at 94 ºC), annealing (2 min at 46 ºC) and extension (3 min at 72 ºC). After
completion of the amplification, 1 U of Taq polymerase was added to the reaction, and incubated at
72 ºC for 10 min in order to add adenines at the 3’ end of the PCR products, for subsequent TA
cloning. The PCR products were gel-purified and subcloned into a pCR®4-TOPO TA cloning
vector according to the manufacturers instructions. The insert in the TOPO vector was subjected to
DNA sequencing using the ABI prism Big Dye™ Terminator Cycle Sequencing kit to confirm the
presence of the mutation and that no errors were generated during the PCR. The EcoRI-cDNA insert
was gel-purified and cloned into pHILD-2 and the correct orientation checked by restriction
mapping. The transformation of the P. pastoris strain GS115 (his4) (50) was achieved using the
spheroplast method (51) as previously described (52) and the transformants screened for the best
expression performances using routine xylanase activity assay (33). A representative His+Mut6

Downloaded from http://www.jbc.org/ by guest on March 12, 2020
transformant for each mutant was selected for production of xylanase in shake-flask cultures (33). Large-scale expression of \textit{A. niger} xylanases in \textit{P. pastoris} was achieved in buffered minimal glycerol-complex medium (BMGY) as previously described (52). Cells grown in BMGY at 30 °C to \textit{A}$_{600}$ of 20-25 were harvested, resuspended in 200 mL buffered minimal methanol-complex medium (BMMY) pH 6.0 and incubated with shaking (200 rpm) in 250 mL flasks at 30 °C for 3 days.

\textit{Protein purification} - For purification of \textit{A. niger} xylanases (wild-type and variants), the culture supernatant was subjected to ammonium sulfate precipitation up to 70%, centrifuged at 10000 g for 30 min, and the pellet resuspended in McIlvaine’s buffer pH 6.5 (0.1 M citric acid, 0.2 M di-sodium hydrogen phosphate). The solution was then dialyzed against the same buffer at pH 6.5 overnight. The dialyzed sample was then loaded onto a McIlvaine’s buffer pre-equilibrated high-load 16/10 Q sepharose column (Amersham) and eluted, with a linear gradient of 0-1 M NaCl in McIlvaine’s buffer at a flow rate of 3 mL min$^{-1}$. Fractions containing activity were pooled and concentrated using a 200 ml stirred ultra filtration cell (model 202, Amicon, Gloucestershire, UK) and a PM 10 ultra filtration membrane (Millipore, Hertfordshire, U.K.).

The xylanase inhibitor was purified from 2 kg wheat flour (\textit{Triticum aestivum} var. Soisson) as described (43, 48).
Protein assays and protein sequencing - Total protein in supernatants and throughout the purification procedure was estimated using the method of Bradford (53). For purified xylanase mutants, total protein was calculated using an extinction coefficient at 280 nm determined from the amino acid composition that was derived from the primary structure (50 210 M\(^{-1}\) cm\(^{-1}\) for wild-type xylanase, D113A, D113N, N117A, E118A and E118Q variants; 48 930 M\(^{-1}\) cm\(^{-1}\) for Y6A, Y10A, Y89A and Y164A; 44 520 M\(^{-1}\) cm\(^{-1}\) for W172A). Protein sequencing was performed at the Protein Sequencing and Peptide Synthesis Facility, John Innes Centre, Norwich, using an ABI 491 Procise sequencer.

Gel electrophoresis and immunoblotting - SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 10% Bis-Tris pre-cast NuPAGE gels (Invitrogen) with biotinylated marker proteins (New England Biolabs). Proteins were transferred to nitrocellulose membranes by semi-dry blotting (BioRad). The blots were probed with a 5000-fold dilution of polyclonal antiserum raised in rabbits against \textit{A. niger} xylanase (a custom preparation from Unilever Laboratorium Research, The Netherlands). Immunoreactive proteins were visualized using a horseradish peroxidase anti-rabbit secondary antibody (Sigma, 1:2000) together with the chemiluminescent detection reagents (ECL Plus Detection Kit, Amersham Pharmacia Biotech, Uppsala, Sweden). Isoelectric focusing gels were run using the Phast system (Amersham Pharmacia Biotech).

Electrophoretic titration - Titration curves of the \textit{A. niger} xylanases (wild-type or N117A mutant), alone or in combination with XIP-I, were produced using the Phast system. Isoelectro focusing (IEF) 3-9 gels (Amersham Pharmacia Biotech) were used according to the manufacturer’s instructions. Briefly, the carrier ampholytes contained in the IEF gel were prefocused at 2000 V for 150 Vhr, to generate a stationary pH gradient (3 to 9). Prior to loading, XIP-I (2.7 mg mL\(^{-1}\), 1.5 μl) was preincubated with \textit{A. niger} xylanases (3.3 mg mL\(^{-1}\), 1.5 μl) in McIlvaine’s buffer pH 5.5 for 10 min at room temperature. The samples were then applied across the middle of the gel, perpendicular to the pH gradient, using a single well applicator. Electrophoresis perpendicular to the first dimension axis was run at 1000 V for 60 Vhr. For staining, the gels were fixed for 20 min in
trichloroacetic acid (TCA) (20%), rinsed in phosphoric acid solution (3%), followed by staining with SERVA Violet 17 stain (SERVA electrophoresis GmbH, Heidelberg, Germany) (1:1 ratio of 0.2% Serva Violet: 20% phosphoric acid) for 10 min. The gels were de-stained using phosphoric acid (3%).

**Xylanase activity assays** - Routine assays during screening for the highest xylanase producer was performed using a colorimetric assay from Megazyme as previously described (33). Purified xylanase activity was measured using the dinitrosalicylic acid assay (54) with 9 mg mL⁻¹ low viscosity arabinoxylan (Megazyme) in McIlvaine’s buffer pH 5.5 at 30 °C for 5 min. One unit of xylanase activity was defined as the amount of protein that released 1 μmol xylose per min at 30 °C and pH 5.5. For determination of Michaelis-Menten constants, the initial velocities of the enzymes were measured at 30 °C, 5 min in McIlvaine’s buffer pH 5.5 with low viscosity arabinoxylan ranging from 3 to 27 mg mL⁻¹. The kinetic parameters were calculated with the Grafit program (Biosoft Cambridge, U.K.). The IC₅₀ values were determined by adding increasing molar equivalents of XIP-I to the enzyme solution up to a molar ratio of 30:1, at a substrate concentration, where possible, at the $K_M$ (9 mg mL⁻¹) of the mutants (Y6A, D113A, D113N, N117A, E118A and E118Q) or at 18 mg mL⁻¹ for Y6A, Y10A, Y89A, Y164A, and W172A. Optimal pH for xylanase activity was estimated using the xylanase assay described above with low viscosity arabinoxylan (10 mg mL⁻¹) in McIlvaine’s buffer, in a range of 2.6 to 7.8. The difference in free energy ($\Delta \Delta G$) for the mutated enzymes was calculated from the equation $\Delta \Delta G = RT \ln \left( \frac{k_{cat}/K_M\text{ wild-type}}{k_{cat}/K_M\text{mutant}} \right)$.

**Matrix-assisted laser desorption ionization – mass spectrometry (MALDI-MS)** - MALDI mass spectra were obtained on a Voyager-DE-STR mass spectrometer (Perseptive Biosystems, Framingham, Ma) operated in linear mode. The samples were desalted and purified by application to a micro RP-column containing Poros R2 column material (Perseptive Biosystems, Framingham, MA) (55), washed with 0.1% trifluoroacetic acid and eluted directly with a solution of α-cyano-4-hydroxy cinnamic acid (HCCA) (Aldrich, Milwaukee, MI) in 70% acetonitrile/0.1% trifluoroacetic
acid onto a MALDI target precoated with a thin layer of HCCA. Mass calibration of the spectra was based on external calibration using appropriate protein standards.

*High pressure anion exchange chromatography (HPAEC) analysis of hydrolysis products* - 0.2 U per mg of substrate of *A. niger* xylanases (wild-type or N117A mutant) were incubated with low viscosity arabinoxylan at 1 mg mL\(^{-1}\) in McIlvaine’s buffer pH 5.5 at 30 °C. Aliquots were withdrawn at intervals 0, 1, 2, 18, 21 and 24 h. Samples were boiled for 10 min and then diluted with water (1/20) prior to injection (20 µl) on an HPAEC system consisting of a PA-1 column (250 x 4 mm), a Waters 626 pump and a Waters 717 auto sampler. Elution (1 mL min\(^{-1}\), 25 °C) was carried out using the following linear gradient program for solvent A (water), B (NaOAC 1M) and C (NaOH 0.5M): 0 min - 80:0:20 (% A:B:C), 5 min - 80:0:20, 20 min - 75:5:20, 30 min- 70:10:20, 40 min - 60:20:20, 41 min - 80:0:20, 60 min - 80:0:20. The effluent was monitored using a TSP EC 2000 electrochemical detector.

*Circular dichroism (CD) spectroscopy* - Solutions of 1 mg mL\(^{-1}\) *A. niger* xylanase (wild-type or N117A mutant) in 10 mM sodium phosphate buffer, pH 5.7, were analysed at 25 °C using a cell of 0.1 mm path length on an applied photophysics Pi-Star-180 CD-stop flow spectropolarimeter interfaced with Acorn Risc PC. Data were averaged from 20 acquisitions between 260-180 in 1 nm steps at 50 000 samples per nm wavelength. CD spectra of the xylanases were subtracted from CD spectra obtained from buffer alone.

*Surface Plasmon Resonance (SPR).* BIAcore X system, HBS buffer (10 mM HEPES pH 7.4 with 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P20), CM5 sensor chips and amine coupling kit were from BIAcore AB (Uppsala, Sweden). XIP-I (1 µM) in 10 mM sodium acetate buffer (pH 5.5) was immobilized using the amine coupling method as described (56) at a flow-rate of 10 µl/min using HBS buffer as running buffer. Briefly, equal volumes of *N*-hydroxysuccinimide (0.06 M in water) and *N*-ethyl-*N*-*'*(3-dithlyaminopropyl)carbodiimide (0.2 M in water) were mixed and injected onto a CM5 sensor chip to activate the carboxymethylated dextran surface. The volume used was adjusted to achieve immobilization levels of XIP-I giving 150 to 2000 resonance units.
(RU). After injection of XIP-I (40 μl), the residual NHS esters were deactivated by injection of 25 μl ethanolamine (1 M, pH 8.5). Flow cell 2 was used to immobilize XIP-I and control flow cell 1 was treated identically but without inhibitor. Increasing concentrations of *A. niger* xylanases (wild-type or N117A mutant) ranging from 0.3 to 13 μM in 10 mM sodium acetate pH 5.5 (40 μl) were injected at a flow rate of 30 μl/min using 10 mM sodium acetate (pH 5.5) as running buffer. The response, measured in resonance units (RU), directly correlates with the amount of protein interacting with the ligand (one RU is defined as 1 pg of bound protein per mm²). Sensorgrams are represented as a plot of the resonance units versus time (57).
RESULTS

*Production and structural properties of the xylanase variants* - All ten mutants were efficiently produced in *P. pastoris* with secretion yields ranging from 30 to 270 mg L\(^{-1}\), as judged by SDS-PAGE. Purified recombinant variants were obtained in yields ranging from 7 to 30 mg L\(^{-1}\) using a single chromatography step. The N-terminus, S-A-G-I-N, of the mutant xylanases was identical to that of the recombinant wild-type xylanase, indicating correct processing of the *A. niger* signal sequence. None of the mutations significantly modified the CD spectrum of the enzymes, indicating that the secondary structure content remained the same after mutation (data not shown). Therefore, loss of function and differences with the wild-type protein characteristics described hereafter for mutants Y6A, Y10A, Y89A, Y164A D113A, D113N, N117A, E118A, E118Q and W172A may only be due to minor local structural changes undetectable by UV CD.

*Enzymatic activity and inhibition of the xylanase variants* - To evaluate the consequences of the mutations on the enzyme, the specific activity of the xylanase variants was measured against wheat arabinoxylan (Table I). Five variants Y6A, Y10A, Y89A, Y164A, and W172A showed a significant decrease in activity. Particularly, the mutation at position Y10 dramatically reduced the specific activity by 98.3%. However these mutant proteins retained enough activity for accurate evaluation of their kinetic parameters (Table I). All variants exhibited normal Michaelis-Menten kinetics but the kinetic parameters varied with the nature of the mutation. The catalytic constant (\(k_{\text{cat}}\)) of the Y6A, Y10A, and Y164A variants was reduced by values similar to the specific activity (Table I). The \(K_M\) of the xylanase variants was increased for most of the mutations, indicating that these residues play a role in substrate binding. Only the Y6A mutation did not alter this parameter. The low specific activity of Y6A and Y89A was entirely accounted for by a change in \(k_{\text{cat}}\) and \(K_M\), respectively, while the lower value of Y10A, Y164A and W172A was due to a combination of increased \(K_M\) and decreased \(k_{\text{cat}}\). The results show that mutation of these aromatic residues caused a decrease of 92 – 75% in the specificity of the xylanase for xylan, which is reflected by losses in
apparent binding energy \([\Delta(\Delta G)]\) (58) ranging from \(-0.2\) to \(3.5\) kcal mol\(^{-1}\) (Table I). The D113A, D113N, N117A, E118A, and E118Q variants all exhibited normal Michaelis-Menten kinetics with \(K_M\) and \(k_{cat}\) (Table I) comparable to those of \(9.9 \pm 1.7\) mg mL\(^{-1}\) and \(129 \pm 11\) s\(^{-1}\), respectively, obtained for the wild-type enzyme. The \(k_{cat}/K_M\) value of the wild-type enzyme is \(13 \pm 1.1\) mL mg\(^{-1}\) s\(^{-1}\), also in good agreement with that obtained with the above variants.

XIP-I inhibited all active variants tested but a drastic loss of inhibition was observed for N117A (Table I); no inhibition was observed for the mutant up to an inhibitor:enzyme molar ratio of 5:1, as compared to 60% inhibition for the wild-type enzyme (not shown). An IC\(_{50}\) value could not be measured despite a 30-fold molar excess of XIP-I which corresponds to \(9\) \(\mu\)M (Table I). IC\(_{50}\) values for the D113A, D113N, E118A, and E118Q variant xylanases were measured at substrate concentrations corresponding to their \(K_M\) (9 mg mL\(^{-1}\)) and were comparable to that of \(1\) \(\mu\)M obtained with the wild-type enzyme. Due to the low activity of Y6A, Y89A, Y164A, and W172A variants, IC\(_{50}\) values were determined at higher substrate concentration (18 mg mL\(^{-1}\)) and ranged from \(0.65\) \(\mu\)M to \(1.5\) \(\mu\)M (Table I). No IC\(_{50}\) could be measured for the Y10A variant due to the very low enzymatic activity of the mutant.

**Detailed structural and enzymatic characterization of N117A variant** - To determine if the loss of inhibition observed with the N117A variant was solely due to the one amino acid change affecting the protein-protein interaction and was not a consequence of potential minor structural change at the active site, the enzyme was fully characterized in terms of enzymatic and structural properties and compared to the wild-type xylanase.

The purified N117A xylanase migrated as a single band on SDS-PAGE identical to that of the recombinant wild-type enzyme (Fig. 1A), and no contaminating band was seen after silver-staining; this 20 kDa protein reacted with polyclonal antibodies raised against *A. niger* xylanase (Fig. 1B), and IEF revealed that N117A xylanase consisted of one single molecular form of approx. pI 3.5 which is similar to that of the wild-type (Fig. 1C). The N-terminus, S-A-G-I-N-Y-V, of N117A mutant xylanase was identical to that of the recombinant wild-type xylanase, indicating correct
processing of the \textit{A. niger} signal sequence. The molecular mass of wild-type and N117A xylanases as determined by MALDI-MS was 19872.45 and 19834.88 Da, respectively, in agreement with expected difference for the Asn$\leftrightarrow$Ala mutation. Moreover both spectra showed the presence of a partial glycosylation with the presence of a small peak of molecular mass 20022.44 and 19991.82 Da for the wild-type and variant enzyme, respectively (spectra not shown). The CD spectrum of N117A was similar to that of the wild-type (Fig. 2), indicating that this amino acid substitution did not significantly alter the secondary structure of the mutated enzyme.

The specific activities of wild-type and N117A xylanases on wheat low viscosity arabinoxylan were $172 \pm 10 \text{ U mg}^{-1}$ and $162 \pm 5 \text{ U mg}^{-1}$ and kinetic parameters were similar (Table I). High-performance liquid chromatographic analysis of the hydrolysis products of wheat arabinoxylan showed that mutation of this residue did not alter the profile of products (data not shown). The xylanase activity of the recombinant N117A mutant as well as the wild-type enzyme was further determined on a range of different substrates (Table II). Both xylanases displayed comparable kinetic parameters on Birchwood xylan and oat spelt xylan substrates (Table II).

The effect of pH on N117A xylanase mutant was compared with the wild-type xylanase. The pH activity curves of both enzymes were identical, with the optimum pH at 3.5. Substantial amount of activity was found at pH 2.0 while a drastic decline in enzyme activity was detected at pH values above the pH optimum (not shown).

\textit{Interaction of wild-type and N117A xylanases with XIP-I} - In order to determine if the loss of inhibition, as observed using activity assays, was correlated with a loss of interaction between XIP-I and N117A variant, the two protein partners were tested in binding assays in the absence of substrate.

The relative affinities and pH dependencies of the interaction of XIP-I with \textit{A. niger} xylanases were studied using titration curves (Fig. 3). The pI value of the wild-type and mutant \textit{A. niger} xylanase is 3.5 (Fig. 1C), whereas that of XIP-I is 8.7-8.9 (43), in agreement with the titration curves of the individual xylanases (Fig. 3A,B) and inhibitor (Fig. 3C). \textit{A. niger} wild-type xylanase
formed a complex on the IEF titration gel across a pH range of approximately 4-7 (Fig. 3D) while no complex could be detected in the case of the N117A variant (Fig. 3E), in agreement with data from the activity assays.

The interaction between XIP-I and wild-type or N117A \textit{A. niger} xylanases was studied by using a biosensor based on surface plasmon resonance (SPR) (59, 60). XIP-I was immobilized as a ligand on the sensor surface, while the \textit{A. niger} xylanase was passed in solution as an analyte over the surface. The results of the analyses of the interaction of XIP-I with increasing amounts of the wild-type or N117A xylanases are reported in Fig. 4. In Fig. 4A, the increase in RU (resonance units) from the initial baseline represents the binding of the wild-type xylanase to the surface-bound XIP-I. The plateau line represents the steady-state phase of the xylanase-XIP-I interaction while the decrease in RU from the plateau represents the dissociation phase. The SPR data showed that mutation N117A strongly affects the interaction (Fig. 4B). The kinetic constants could not be calculated due to the undetectable interaction between the mutated protein and the enzyme. The $K_D$ is therefore higher than 1 mM, which is the limit of detection of the BIAcore, as compared to 5.68 $\mu$M calculated for the wild-type xylanase (48).
DISCUSSION

The active site of glycosyl hydrolases often contain aromatic residues, such as tyrosine and tryptophan, which hydrophobically stack against sugar rings, as well as side chains which hydrogen bind to hydroxyl groups of the substrate. The replacement of the aromatic amino acid residues, Tyr6, Tyr10, Tyr89, Tyr164, and Trp172 with Ala significantly reduced the enzyme activity of \textit{A. niger} glycosyl hydrolase family 11 xylanase. These mutations did not affect the fold of the xylanase as revealed by circular dichroism spectra of these variants. Although these residues are not essential for the hydrolytic reaction per se (24), the present findings indicate that they play an important role in ligand binding and catalysis.

In family 11 xylanases, the resolution of a limited number of structures of enzyme-ligand complexes has revealed several active site residues which have the potential to play key roles in the interaction of subsites with xylose moieties. The structure of a catalytically inactive \textit{Bacillus circulans} xylanase (BCX) mutant incubated with xylotetraose has been characterized, but only a portion of the ligand (a xylobiose moiety) could be observed at the active site (subsites –1 and –2) (13) and recently the structure of the glycosyl-enzyme intermediate has been obtained providing more insight into the –1 subsite (30). In order to evaluate the number of subsites in the active site of \textit{Trichoderma reesei} xylanases, different xylo oligomer models were docked to the active site of both XYNI and XYNII (22). XYNI probably has space for three subsites (-2, -1, +1) while the active site of XYNII is longer and may have five subsites (-2, -1, +1, +2, +3). Structures of covalently attached epoxyalkyl glycosides to the active site residues of XYNII are also available, but these include only one xylose residue (29). Modeling studies with a xyloheptaose in the active site of \textit{Thermomyces lanuginosus} xylanase (Xyna) identify residues involved in subsite –2. (26). Recently the structure of an inactive mutant of the Xyn11 from \textit{Bacillus agaradhaerens} in complex with xylotriose has been obtained (17). The interactions are very similar to that described at subsites –2 and -1 for the covalent intermediate complex (16), with the addition of the interactions for the –3 subsite.
The active site residues in the \textit{A. niger} xylanase correspond remarkably closely in both position and orientation with the residues that contact the sugar rings in these complexes. In Fig. 5, structural alignment of the above xylanases with that of \textit{A. niger} enzyme showed that four of the \textit{A. niger} xylanase variants (Y10A, Y89A, Y164, and W172A) have been mutated at positions corresponding to residues which have been involved in discrete subsites of other family 11 xylanases. Tyr10 corresponds to Trp9_{BCX}, Tyr9_{XYNI}, Trp18_{XYNI}, Trp18_{Xyna}, and Trp19_{Xyn11}, all involved in stacking interaction with the xylose ring in substite –2 of these xylanases (13, 16, 17, 22, 26). Tyr89 corresponds to Tyr96_{XYNI} thought to determine subsite +3 of the \textit{T. reseei} enzyme (22). Tyr164 is equivalent to Tyr166_{BCX} and Tyr172_{Xyna}, both shown to form hydrogen bonds with the substrate in subsite –2 of the xylanases (13, 26). Trp172 corresponds to Trp166_{XYNI} and Tyr179_{XYNI} which are thought to determine subsites +1 and +2 of XYNI and XYNII enzyme, respectively (22). Moreover the oxygen atom OH of residue Tyr6 is in the same position as atom Glu17_{Xyn11} OE1 (not shown) which, in \textit{B. agaradhaerens}, is involved in one of the solvent-mediated hydrogen bonds associating the -3 substite sugar (17). These findings are consistent with the substantial decrease in the activity of the Y6A, Y10A, Y89A, Y164, and W172A mutants against xylan, although these residues do not necessarily play equivalent roles in different family 11 enzymes. In Fig. 6, the two xylose rings found at substites –1 and –2 of the BCX mutant complexed with a substrate (13) are superimposed to the \textit{A. niger} xylanase three-dimensional structure. In this view Tyr6, Tyr10 and Tyr164 are in close contact with the ligand, also suggesting their involvement in substites of the \textit{A. niger} enzyme. Mutational analyses of active site residues have been carried on other family 11 xylanases but, to our knowledge, none of the present mutations were reported apart for Tyr166_{BCX} (corresponding to Tyr164 in \textit{A. niger} xylanase), which, replaced by Phe in \textit{B. circulans} xylanase, led to a small decrease of enzyme activity (13). The substrate binding cleft of \textit{A. niger} xylanase contains at least four xylose-binding subsites (24). Our data clearly show that Tyr10, Tyr164 are likely to play an important role in ligand binding in the glycone region of the substrate binding cleft (probably at subsite –2) while Trp172 and Tyr89 are involved in the aglycone subsites (probably at subsites +1
and +2, respectively) of *A. niger* xylanase. Interestingly, the finding that Tyr6 played a role in enzyme activity together with its position in the 3D structure might indicate the presence of another subsite (-3) in the substrate binding cleft of *A. niger* xylanase.

Interestingly, all the fungal xylanases tested so far are inhibited by XIP-I apart from the family 10 *A. aculeatus* xylanase, whereas none of the bacterial enzymes are (48). The specific inhibition/recognition of fungal xylanases was not due to the binding of XIP-I to glycosylation on the fungal enzymes, since both the native and *E. coli*-expressed recombinant forms of *P. funiculosum* xylanase were inhibited to the same extent (48). In the same way, the loss of XIP-I inhibition towards the N117A xylanase mutant was not due to a potential requirement for protein glycosylation on Asn117 residue since the wild-type and mutant enzymes showed the same glycosylation content (approx. 150 Da), probably corresponding to a single hexose or a hexosamine.

Alignment of the *A. niger* xylanase with other family 11 xylanases from fungal and bacterial origin showed that the Asn117 residue was conserved in most sequences (8), indicating that the environment of this amino acid in the three-dimensional structure is more likely to be responsible for the difference in binding. The *A. niger* xylanase three-dimensional structure comprises a single domain containing one α-helix and 13 β-strands, which are arranged in two mostly antiparallel β-sheets A and B (Fig. 7A). The data obtained from titration curves and surface plasmon resonance clearly showed that the Asn→Ala mutation at the solvent-exposed position 117 abolished the capacity of the molecule to interact with XIP-I. This residue is present on the β-strand 8 of the larger, eight-stranded β-sheet (sheet B) which twists around the catalytic cleft (Fig. 7A). The *A. niger* xylanase has been compared to the shape of a right hand with the “fingers” at the top, the “palm” at the bottom and the “thumb” at the right hand side of the molecule as represented in Fig. 7A. Asn117 is located at the end of the β-bend positioned at the tip of the “thumb” (Fig. 7A/B). In all other family 11 xylanases with known structure, this loop is significantly longer and includes parts of β-strands B7 and B8 of the *A. niger* xylanase (Fig. 5). However, these differences in secondary structures do not influence the general shape of the “thumb”, which points back towards
the bottom of the cleft (Fig.7A). It is stabilized in this position by hydrophobic interactions as well as by several hydrogen bonds (8) but also able to move and thus regulate the width of the active site cleft (29). The interaction of XIP-I with the thumb region could thus be responsible for the inhibition, preventing access by the substrate to the catalytic cleft (Fig. 7A). In the thumb region of _A. niger_ xylanase a number of residues, Thr114, Thr116, Asn117, Thr124, Thr126 and Thr128, have their side-chains highly solvent exposed, and could interact with XIP-I (Fig.7B). The bacterial xylanase from _Bacillus agaradhaerens_ is not inhibited by XIP-I (48) and the superimposition of the “thumb” to that of the _A. niger_ xylanase shows that, although Asn117 is conserved both in position and orientation, four threonines present in the vicinity of Asn117 in the _A. niger_ enzyme are replaced by hydrophobic or basic residues in _B. agaradhaerens_ xylanase (Fig. 7B). Interestingly, among the xylanases from _A. niger_, one xylanase has a threonine instead of Asn117 at this position (61). Taken together these findings suggest that the “thumb” region, rather than a single residue, is involved in XIP-I binding to _A. niger_ xylanase. This region is overall conserved among the family 11 fungal enzymes and is proposed as the binding site for the interaction of XIP-I with family 11 xylanases.

In summary, this is the first report investigating the molecular interactions of the proteinaceous inhibitor XIP-I with a target xylanase. Characterization of the xylanase N117A variant of _A. niger_ xylanase and the analysis of the three-dimensional structure of the wild-type xylanase converge towards an essential role of the “thumb” hairpin loop in binding to the inhibitor. This mutational analysis of _A. niger_ xylanase also allowed identification of key residues that play an important role in ligand binding at the various subsites.
References

1. Biely, P. Microbial xylanolytic systems (1985) Trends Biotechnol. 3, 286-290
2. Biely, P. (1993) in Hemicellulose and hemicellulases (Coughlan M. P., Hazlewood G. P., eds), pp. 29-51, Portland Press, London
3. Henrissat, B. (1991) Biochem. J. 280, 309-316
4. Henrissat, B., and Bairoch, A. (1993) Biochem. J. 293, 781-788
5. Henrissat, B., and Bairoch, A. (1996) Biochem. J. 316, 695-696
6. Jeffries, T. W. (1996) Curr. Opin. Biotechnol. 7, 337-342
7. Biely, P., Vrsansaka, M., Tenkanen, M., and Kluepfel, D. (1997) J. Biotechnol. 57, 151-166
8. Sapag, A., Wouters, J., Lambert, C., de Ioannes, P., Eyzaguirre, J., and Depiereux, E. (2002) J. Biotechnol. 95, 109-131
9. McCarter, J. D., and Withers, S. G. (1994) Curr. Opin. Struct. Biol. 4, 885-892
10. Davies, G., and Henrissat, B. (1995) Structure 3, 853-859
11. Vocadlo, D. J., Davies, G. J., Laine, R., and Withers, S. G. (2001) Nature 412, 835-838
12. Withers, S. G. (1995) in Carbohydrate Engineering (Petersen, S. B., Svensson, S., and Pedersen, S., eds) pp 97-113, Elsevier Science Publishers B. V., Amsterdam
13. Wakarchuk, W. W., Campbell, R. L., Sung, W. L., Davoodi, J., and Yaguchi, M. (1994) Protein Sci. 3, 467-475
14. Joshi, M. D., Sidhu, G., Nielsen, J. E., Brayer, G. D., Withers, S. G., and McIntosh, L. P. (2001) Biochemistry 40, 10115-10139
15. Harris, G. W., Pickersgill, R. W., Connerton, I., Debeire, P., Touzel, J. P., Breton, C., and Perez, S. (1997) Proteins 29, 77-86
16. Sabini, E., Sulzenbacher, G., Dauter, M., Dauter, Z., Jorgensen, P. L., Schulein, M., Dupont, C., Davies, G. J., and Wilson, K. S. (1999) Chem. Biol. 6, 483-492
17. Sabini, E., Wilson, K. S., Danielsen, S., Schulein, M., and Davies, G. J. (2001) *Acta Crystallogr. D* **57**, 1344-1347

18. McCarthy, A. A., Morris, D. D., Bergquist, P. L., and Baker, E. N. (2000) *Acta Crystallogr. D* **56**, 1367-1375

19. Wouters, J., Georis, J., Engher, D., Vandenhaute, J., Dusart, J., Frere, J. M., Depiereux, E., and Charlier, P. (2001) *Acta Crystallogr. D* **57**, 1813-1819

20. Campbell, R., Rose, D., Wakarchuk, W., To, R., Sung, W., and Yaguchi, M. (1993) *Proceedings of the 2nd TRICEL symposium on Trichoderma reesei cellulases and other hydrolases*, Foundation for Biotechnical and Industrial Fermentation Research, Helsinki

21. Törrönen, A., Harkki A., and Rouvinen, J. (1994) *EMBO J.* **13**, 2493-2501

22. Törrönen, A., and Rouvinen, J. (1995) *Biochemistry* **34**, 847-856

23. Muilu, J., Törrönen, A., Perakyla, M., and Rouvinen, J. (1998) *Proteins* **31**, 434-444

24. Krengle, U., and Dijkstra, B. W. (1996) *J. Mol. Biol.* **263**, 70-78

25. Fushinobu, S., Ito, K., Konno, M., Wakagi, T., and Matsuzawa, H. (1998) *Protein Eng.* **11**, 1121-1128

26. Gruber, K., Klintschar, G., Hayn, M., Schlacher, A., Steiner, W., and Kratky, C. (1998) *Biochemistry* **37**, 13475-13485

27. Kumar, P. R., Eswaramoorthy, S., Vithayathil, P. J., and Viswamitra, M. A. (2000) *J. Mol. Biol.* **295**, 581-593

28. Törrönen, A., and Rouvinen, J. (1997) *J. Biotechnol.* **57**, 137-149

29. Havukainen, R., Törrönen, A., Laitinen, T., and Rouvinen, J. (1996) *Biochemistry* **35**, 9617-9624

30. Sidhu, G., Withers, S. G., Nguyen, N. T., McIntosh, L. P., Ziser, L., and Brayer, G. D. (1999) *Biochemistry* **38**, 5346-5354

31. Davies, G. J., Wilson, K., and Henrissat, B. (1997) *Biochem. J.* **321**, 557-559
32. Hessing, J. G. M., van Rotterdam, C., Verbakel, J. M. A., Roza, M., Maat, J., van Gorcom, R. F. M., and van den Hondel, C. A. M. J. J. (1994) *Curr. Genet.* **26**, 228-232

33. Berrin, J. G., Williamson, G., Puigserver, A., Chaix, J. C., McLauchlan, W. R., and Juge, N. (2000) *Prot. Exp. Purif.* **19**, 179-187

34. van Gorcom, R. F. M., Hessing, J. G. M., Maat, J., Roza, M., and Verbakel, J. M. A. (1991) PCT International Patent application, WO/91/19872

35. Maat, J., Roza, M., Verbakel, J., Stam, H., Santos da Silva, M. J., Bosse, M., Egmond, M. R., Hagemans, M. L. D., Gorcom, R. F. M. V., Hessing, J. G. M., van der Hondel, C. A. M. J. J., and Rotterdam, C. V. (1992) in *Xylans and Xylanases* (Visser, J., Beldman, G., Kusters-van Somerson, M. A., and Voragen, A. G. J., eds.) pp. 349-360, Elsevier Science Publishers B. V., Amsterdam

36. Debyser, W., Peumans, W. J., Van Damme, E. J. M., and Delcour, J. A. (1999) *J. Cereal Sci.* **30**, 39-43

37. Christophersen, C., Andersen, E., Jacobsen, T. S., and Wagner P. (1997) *Starch/Stärke* **1**, 5-12

38. Bedford, M. R., and Classen, H. L. (1992) in *Xylan and Xylanases* (Visser, J., Beldman, G., Kusters-van Somerson, M. A., and Voragen, A. G. J., eds.) pp. 361-370, Elsevier Science Publishers B. V., Amsterdam

39. Graham, H. and Inborr, J. (1992) in *Xylans and Xylanases* (Visser J., Beldman G., Kusters-van Someren M. A., and Voragen A. G. J. eds.), pp. 535-538, Elsevier Science Publishers B. V., Amsterdam

40. Debyser, W., Derdeelinckx, G., and Delcour, J. A. (1997) *J. Am. Soc. Brew. Chem.* **55**, 153-156

41. Debyser, W., and Delcour, J.A. (1997) European patent application WO98/49278

42. Rouau, X., and Surget, A. (1998) *J. Cereal Sci.* **28**, 63-70

43. McLauchlan, W. R., Garcia-Conesa, M. T., Williamson, G., Roza, M., Ravenstein, P. and Maat, J. (1999) *Biochem. J.* **338**, 441-446

44. Hessing, M., and Happe, R. P. (2000) European Patent EP 0979830 A1
45. McLauchlan, W. R., Flatman, R. H., Sancho, A. I., Kakuta, J., Faulds, C. B., Elliot, G. O., Kroon, P. A., Furniss, C. S. M., Juge, N., Ravestein, P., and Williamson, G. (2000) The Second European Symposium on Enzymes in Grain Processing, VTT Technical Research Centre, Finland

46. Gebruers, K., Debyser, W., Goesaert, H., Proost, P., Van Damme, J., and Delcour, J.A. (2001) *Biochem. J.* **353**, 239-244

47. Goesaert, H., Debyser, W., Gebruers, K., Proost, P., Van Damme, J., and Delcour, J. A. (2001) *Cereal Chem.* **78**, 453-457

48. Flatman, R., McLauchlan, W. R., Juge, N., Furniss, C. S., Berrin, J. G., Hughes, R. K., Manzanares, P., Ladbury, J. E., O'Brien, R., and Williamson, G. (2002) *Biochem. J.* **365**, 773-781

49. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77**, 51-59

50. Cregg, J. M., Barringer, J., Hessler, A. Y., and Madden, K. R. (1985) *Mol. Cell Biol.* **5**, 3376-3385

51. Hinnen, A., Hicks, J. B., and Fink, G. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1929-1933

52. Juge, N., Williamson, G., Puigserver, A., Cummings, N., J., Connerton, I., F., and Faulds, C. B. (2001) *FEMS Yeast Res.* **1**, 127-132

53. Bradford, M.M., (1976) *Anal. Biochem.* **72**, 248-254

54. Bailey, M. J., Biely, P., and Poutanen, K. (1992) *J. Biotechnol.* **23**, 257-270

55. Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R., Roepstorff, P. (1999) *J. Mass Spectrom.* **34**, 105-116

56. Jonsson, U., Fagerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Lofas, S., Persson, B., Roos, H., and Ronnberg, I. (1991) *Biotechniques* **11**, 620-627

57. Johnsson, B., Lofas, S., and Lindquist, G. (1991) *Anal. Biochem.* **198**, 268-277

58. Wilkinson, A. J., Fersht, A. R., Blow, D. M., and Winter, G. (1983) *Biochemistry* **22**, 3581-3586
59. Granzow, R., and Reed, R. (1992) *Biotechnology* **10**, 390-393

60. Schuster, S. C., Swanson, R. V., Alex, L. A., Bourret, R. B., and Simon, M. I. (1993) *Nature* **365**, 343-347

61. Luttig, M., Pretorius, I. S., and van Zyl, W. H. (1997) *Biotechnol. Lett.* **19**, 411-415

62. Christopher, J. A. (1998) Center for Macromolecular Design, Texas A&M University, College Station, TX

63. Roussel, A., and Cambillau, C. (1991) in *Silicon Graphics Geometry Partners Directory*, pp. 86, Silicon Graphics, Mountain View, CA, USA

**Footnotes**

*Acknowledgements* - We thank Luc Saulnier (INRA, Nantes, France) for HPAEC analysis and Andrew Leach (UEA, Norwich, UK) for CDs spectroscopy analysis. Françoise Payan (CNRS, Marseille, France), and John Jenkins (IFR, Norwich, UK) are acknowledged for their help with structural analysis.

Abbreviations used: BCX, *Bacillus circulans* xylanase; BMGY, buffered minimal glycerol-complex medium; BMMY, buffered minimal methanol-complex medium; CD, circular dichroism; DNS, dinitrosalicylic acid; HCCA, α-cyano-4-hydroxy cinnamic acid; HPAEC, high pressure anion exchange chromatography; HPSF, high purity salt free; IC$_{50}$, inhibitor concentration for 50% inhibition; IEF, isoelectro focusing; MALDI-MS, matrix-assisted laser desorption ionization - mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RU, resonance units; SPR, surface plasmon resonance; TAXI, *Triticum aestivum* L. xylanase inhibitor; TCA, trichloroacetic acid; wt, wild-type; XIP-I, xylanase inhibitor protein I; xylanase, endo-1,4-β-D xylanase; XylA, *A. niger* xylanase; XynA, *Thermomyces lanuginosus* xylanase; XYNI, *Trichoderma reesei* xylanase I, XYNII, *Trichoderma reesei* xylanase II; Xyn11, *Bacillus agaradhaerens* xylanase.
**Figure legends**

**FIG. 1. Electrophoretic separation of purified xylanases.** Panel A, SDS-PAGE analysis. Lane 1, prestained molecular weight standards (Bio-Rad), lane 2, 1 µg of *A. niger* wild-type xylanase, lane 3, 1 µg of the purified N117A xylanase mutant. Panel B, Western blot of lane 1, 0.3 µg of *A. niger* wild-type xylanase, lane 2, 0.3 µg of the purified N117A xylanase mutant. Panel C, Analytical electrofocusing of lane 1, 1 µg of *A. niger* wild-type xylanase, lane 2, 1 µg of the purified N117A xylanase mutant, lane 3, Pharmacia pl marker proteins.

**FIG. 2. Circular dichroism spectra (650-180 nm) of *A. niger* wild-type xylanase (full line) and *A. niger* xylanase N117A mutant (broken line).** The spectra are normalized to the protein concentration and expressed as molar ellipticity [θ] (deg cm$^2$d$mol^{-1}$).

**FIG. 3. Electrophoretic titration curves of *A. niger* xylanases and XIP-I.** Panel A, *A. niger* wild-type xylanase (4 µg); Panel B, *A. niger* xylanase N117A mutant (4 µg); Panel C, XIP-I (4 µg); Panel D, a mixture containing *A. niger* wild-type xylanase (5 µg) and XIP-I (4 µg); Panel E, a mixture containing *A. niger* xylanase N117A mutant (5 µg) and XIP-I (4 µg). The pH-mobility curves of the proteins, free or in complex, are indicated with an arrow.

**FIG. 4. Surface plasmon resonance sensorgrams showing the interaction between XIP-I and *A. niger* xylanase** Panel A, overlay plots of *A. niger* wild-type xylanase binding to XIP-I immobilized on a carboxymethyl dextran surface. Panel B, overlay plots of *A. niger* xylanase N117A mutant binding to immobilized XIP-I. In both panels, the superimposed curves are derived from experiments using 0.32, 1.3, 2.6, 5.4 and 13 µM (from bottom to top curve) of *A. niger* xylanases. For both graphs, the signal is indicated in resonance units (RU).
FIG. 5. Multiple structural alignment of family 11 xylanases from *Aspergillus niger* (1ukr), *Bacillus circulans* (1xn), *Trichoderma reesei* I (1xyn), *Aspergillus kawachii* (1bk1), *Trichoderma reesei* II (1enx), *Trichoderma harzianum* (1xnd), *Thermomyces lanuginosus* (1yna), *Paecilomyces variotii* bainier (1pvx), *Dictyoglomus thermophilum* (1f5j), *Streptomyces sp.* S38 (1hix), *Bacillus subtilis* B230 (1igo) and *Bacillus agaradhaerens* (1h4g). Secondary-structure elements are highlighted and given at the top of the alignments. Numbering of the β-strands was done according to Törrönen et al. (21). In addition the position of the “thumb” is shown. Residues that are identical in all enzymes are marked (*). The two catalytic Glu residues are marked in bold. The arrows indicate the position of the mutated residues in *A. niger* xylanase.

FIG. 6. Molecular surface representation of the *A. niger* xylanase active cleft.

Two sugar residues have been modeled into the active site by superimposing the structure of *B. circulans* xylanase complexed with xylobiose (PDB code 1BCX) on top of the *A. niger* xylanase. The molecular surface of the protein is shown in grey, and the oligosaccharide is represented as a wire model in light green. The target residues for site-directed mutagenesis are highlighted (the aromatic residues Y6A, Y10, Y89, Y164, and W172 are in yellow, N117, D113, and E118 are in turquoise). The nucleophile (E79) and acid/base catalyst (E170) (in purple) are located on opposite sides of the active site cleft. The “thumb” region (in dark green) points back towards the bottom of the cleft. The drawing was generated with SPOCK (62) and one-letter amino acid codes are used.

FIG. 7. Predicted binding site of XIP-I in *A. niger* xylanase. Panel A shows a ribbon diagram of *A. niger* xylanase showing the thumb region in grey. The molecule is rotated about 90 degrees along the horizontal axis compared to the orientation of Fig.6. The overall fold shows the two β-sheets in blue (sheet A) and red (sheet B), with the β-strands and other structural features labeled as in Törrönen et al. (21). The drawing was generated with SPOCK (62). Panel B shows a close-up view of the B8-B7 hairpin loop ("thumb") of *A. niger* xylanase in the same orientation as in panel A.
“thumb” region is shown in grey with the residues from *A. niger* xylanase in atom type color (carbon in yellow, oxygen in red, nitrogen in blue) and the corresponding residues in *B. agaradhaerens* xylanase in blue. The drawing was generated with Turbo-Frodo (63).
### TABLE I

Specific activity, kinetic parameters and IC$_{50}$ value of the wild-type and mutated xylanases determined with wheat arabinoxylan as substrate

| Enzyme | Specific Activity (U mg$^{-1}$) | $K_M$ (mg mL$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ (mL mg$^{-1}$ s$^{-1}$) | $\Delta\Delta G^a$ (kcal mol$^{-1}$) | IC$_{50}^b$ (µM) |
|---------|--------------------------------|---------------------|---------------------|-------------------------------------|-----------------------------------|------------------|
| wild-type | 172 ± 10 | 9.9 ± 1.7 | 129 ± 11 | 13.0 | - | 0.98 |
| Y6A | 38 ± 3 | 9.9 ± 1.8 | 32 ± 2 | 3.2 | 0.9 | 0.86 |
| Y10A | 1 ± 0.03 | 51.7 ± 6.4 | 2 ± 0.2 | 0.041 | 3.5 | N/d |
| Y89A | 79 ± 5 | 35.2 ± 9.8 | 115 ± 21 | 3.3 | 0.9 | 0.8 |
| D113A | 204 ± 7 | 9.5 ± 1.6 | 158 ± 13 | 17 | -0.2 | 0.9 |
| D113N | 191± 9 | 15.7 ± 2.2 | 279 ± 26 | 18 | -0.2 | 0.7 |
| N117A | 162 ± 5 | 9.9 ± 1.8 | 104 ± 9 | 10 | 0.2 | >9 |
| E118A | 257 ± 7 | 12.9 ± 3.1 | 187 ± 25 | 14 | -0.04 | 0.85 |
| E118Q | 201 ± 2 | 11.2 ± 2.3 | 217 ± 24 | 19 | -0.2 | 1.5 |
| Y164A | 35 ± 1 | 23.8 ± 6.3 | 37 ± 5 | 2 | 1.1 | 0.6 |
| W172A | 26 ± 1 | 71.9 ± 26.2 | 82 ± 72 | 1 | 1.6 | 1.8 |

N/d not determined

$^a \Delta\Delta G = R T \ln [(k_{cat}/K_M)_{wild-type} / (k_{cat}/K_M)_{mutant}]$

$^b$IC$_{50}$ values were determined using molar ratios of XIP-I: Xylanase ranging from 3:1 to 30:1
Table II

Substrate specificity of wild-type and mutant N117A xylanases

| Substrate          | Xylanases | wild-type | N117A |
|--------------------|-----------|-----------|-------|
|                    |           | $K_M$     | $k_{cat}$ | $k_{cat}/K_M$ | $K_M$    | $k_{cat}$ | $k_{cat}/K_M$ |
|                    |           | (mg mL$^{-1}$) | (s$^{-1}$) | (mL mg$^{-1}$ s$^{-1}$) | (mg mL$^{-1}$) | (s$^{-1}$) | (mL mg$^{-1}$ s$^{-1}$) |
| Wheat arabinoxylan |           | 9.9 ± 1.7 | 129 ± 11 | 13 ± 1.1 | 9.9 ± 1.8 | 104 ± 9 | 10 ± 0.9 |
| Birchwood xylan    |           | 12 ± 1    | 105 ± 5  | 9 ± 0.4  | 11 ± 1  | 120 ± 5 | 11 ± 0.4 |
| Oat spelt xylan    |           | 5 ± 0.5   | 99 ± 3   | 20 ± 0.5 | 10 ± 1  | 134 ± 7 | 13 ± 0.7 |
Specific characterization of substrate and inhibitor binding sites of a glycosyl hydrolase family 11 xylanase from Aspergillus niger
Tariq A. Tahir, Jean-Guy Berrin, Ruth Flatman, Alain Roussel, Peter Roepstorff, Gary Williamson and Nathalie Juge

J. Biol. Chem. published online August 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205657200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts