Functional importance of Asp37 from a family 11 xylanase in the binding to two proteinaceous xylanase inhibitors from wheat

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Abstract

Aspergillus niger xylanase is a target enzyme of the two wheat proteinaceous inhibitors, XIP-I and TAXI-I. We previously suggested that the xylanase “thumb” region was XIP-I binding site. Here, we expressed the Asp37Ala mutant in Pichia pastoris and showed that the mutation abolished the enzyme capacity to interact with both inhibitors, suggesting a direct contact at the active site. The mutant pH profile was altered, confirming the key role of Asp37 in determining the pH optima of glycoside hydrolase family 11. The results are consistent with a competitive inhibition mode and underline the strategic importance of Asp37 in the inhibition mechanism.

Keywords: Proteinaceous inhibitors; Enzyme inhibition; Aspergillus niger xylanase; Family 11 glycoside hydrolase; Site-directed mutagenesis; Pichia pastoris

1. Introduction

Endo-(1,4)-β-xylanases (EC 3.2.1.8) depolymerize the xylan backbone by cleaving β-(1,4) glycosidic bonds between D-xylose residues in the main chain producing short xylooligosaccharides [1]. According to the sequence-based glycoside hydrolase (GH) classification [2], most xylanases belong to GH10 and GH11 families (CAZY website http://www.afmb.cnrs-mrs.fr/CAZY/). These families have different molecular structures, molecular weights, and catalytic properties [3]. GH11 xylanases hydrolyse xylosidic substrates with retention of anomeric configuration with two catalytic residues acting as nucleophile and acid–base catalyst, respectively [4]. The Aspergillus niger xylanase used in this study is a 20 kDa GH11 xylanase with a pl and a pH optimum of 3.5 [5,6]. The X-ray structure shows a β-jelly roll fold [7] typical for GH11 xylanases which have been compared to the shape of a right hand with a two-β-strand “thumb” forming a lid over the active site [8].
The active site is located within a deep, long cleft, lined with numerous aromatic amino acids and is large enough to accommodate at least four xylose residues [7,9]. The two conserved catalytic residues, Glu79 and Glu170, face each other on opposite sides [7]. This fungal enzyme has been studied for its role as a bread improver [5] in wheat processing [10] and as a supplement in animal feed [11,12]. However, the in vitro degradation of arabinoxylans by xylanases does not always match the effect of these enzymes in the actual processes and applications. This could be partially explained by the presence of proteinaceous inhibitors of xylanase in cereals [13,14].

To date, two distinct types of xylanase inhibitors with different structures and specificities have been described. The xylanase inhibitor protein (XIP)-like inhibitors are monomeric glycosylated proteins with Mr's of ≈29 kDa and pI-values of 8.7–8.9 [15,16], whereas the *Triticum aestivum* xylanase inhibitor (TAXI)-like inhibitors are high pI, non-glycosylated proteins with Mr's of ≈40 kDa [17,18]. Of the latter type at least two inhibitors (TAXI-I and TAXI-II) with different pI values (8.8 and ~9.3, respectively) and varying specificities towards different xylanases have been identified in wheat [19]. XIP-I can inhibit both GH10 and GH11 xylanases from fungal origin in a competitive manner [20] whereas TAXI-I and TAXI-II can only inhibit GH11 xylanases, but from both bacterial and fungal origins [19]. XIP-I and TAXI-I inhibit *A. niger* xylanase in a competitive manner with a *K*ₐ of 317 nM [20] and 20.1 nM (Gebruers, K., unpublished results), respectively. The inhibition mechanism of XIP-I against *A. niger* xylanase has been studied using kinetics, biophysical methods and site-directed mutagenesis [9,20]. The inhibition is pH-dependent in the range 4–7, illustrating the importance of electrostatic interactions in the strength of the interaction. We previously suggested that the “thumb” region of GH11 xylanases could be responsible for the inhibition, preventing access by the substrate to the catalytic cleft. In contrast, no information is available on the forces that drive the interaction between TAXI-I and the *A. niger* xylanase.

In the present work, site-directed mutagenesis was used to investigate the importance of electrostatic interactions in the binding of XIP-I and TAXI-I to *A. niger* GH11 xylanase.

2. Materials and methods

2.1. Materials, plasmids and strains

The *Pichia pastoris* expression kit (including *P. pastoris* strain (his4)/GS115) and the pCR®4-TOPO TA cloning vector were from Invitrogen (San Diego, CA, USA). The pHIL-D2/XylA vector expressing wt XylA was from in house collection [6]. Restriction and DNA modifying enzymes were from Promega (Madison WI, USA). *Pfu* polymerase for polymerase chain reaction (PCR) was from Stratagene (UK). *E. coli* DH5α (supE 44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1) was used for DNA manipulation. Xylan birchwood, (1,4)-β-xylose and dinitrosalicylic acid (DNS) were from Sigma Chemical Co (St. Louis, MO, USA), azo-wheat-arabinoxylan and low viscosity wheat arabinoxylan were from Megazyme International Ireland Ltd (Co. Wicklow, Ireland). Oligonucleotides were synthesized by MWG Biotech (Germany) as High Purity Salt-Free oligos.

2.2. Cloning, mutagenesis, and protein expression

Mutations were introduced into the pHIL-D2/xylA plasmid using the QuickChange® XL site-directed mutagenesis kit (Stratagene Europe, Amsterdam Zuidoost, The Netherlands). Two overlapping complementary oligonucleotides for each mutation were designed to contain the corresponding nucleotide changes (in bold), D37A forward 5′-GAA GAT GGA GTG TCT TCG GCC TTT TGC GTT GGT GGC-3′; D37A reverse 5′-GCC CAG ACC AAC GAC AAA GGC-3′. Each primer (125 ng) was annealed to pHILD-2/xylA (15 ng) and both strands of the plasmid amplified using 1 U *Pfu* polymerase in a linear extension reaction carried out by PCR under the following conditions: 1 min denaturation at 95 °C, and 18 cycles of 1 min denaturation at 95 °C, 1 min annealing at 60 °C and 18 min extension at 68 °C. PCR cycling was performed in a Perkin–Elmer GeneAmp PCR system 2400. The reactions were cooled to room temperature and treated with *DpnI* restriction enzyme for 1 h at 37 °C to hydrolyse the methylated parental plasmid strands. *DpnI*-treated DNA (1 μl) was used to transform XL10-Gold® ultra-competent cells (50 μl, Stratagene) and transformed bacteria were grown in NZY Plus broth (0.5 ml) for 1 h at 37 °C. Transformants were selected on LB medium plates containing 50 μg mL⁻¹ ampicillin. Recombinant plasmids were isolated using Qiagen columns (Mini-Prep kit), and checked for integrity by restriction mapping. The insert in the PHIL-D2 vector was subjected to DNA sequencing using the ABI prism Big Dye™ Terminator Cycle Sequencing kit to confirm the presence of the single mutation and ensure that no unintended errors were generated during PCR.

Transformation of *P. pastoris* strain GS115 (his4) was achieved using the spheroplast method as previously described [21] and transformants screened for best expression performances using routine xylanase activity assay [6]. A representative His⁺Mut⁺ transformant for each mutant was selected for production of xylanase in shake-flask cultures [6]. Large-scale expression of *A. ni-
ger xylanases in P. pastoris was achieved in buffered minimal glycerol-complex medium (BMGY) as previously described [21]. Cells grown in BMGY at 30 °C to \(A_{600}\) of 20–25 were harvested, resuspended in 200 mL buffered minimal methanol-complex medium (BMMY) pH 6.0 and incubated with shaking (200 rpm) at 30 °C for five days.

2.6. Xylanase activity assays

Routine assays during screening was performed using a colorimetric assay from Megazyme as previously described [6]. The activity of purified xylanase was measured using the dinitrosalicylic acid assay [23] with 9 mgmL\(^{-1}\) low viscosity arabinoxylan (Megazyme) in McIlvaine's buffer pH 5.5 at 30 °C for 5 min. One unit of xylanase activity is defined as the amount of protein that releases 1 μmol xylose per min at 30 °C and pH 5.5. For inhibition assays, increasing molar equivalents of inhibitors were added to the enzyme solution up to a molar ratio of 30:1 using the activity assay described above. 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 mgmL\(^{-1}\). The kinetic parameters were calculated with the Grafit program (Biosoft Cambridge, UK). Optimal pH for xylanase activity was estimated using the xylanase assay described above with low viscosity arabinoxylan (10 mgmL\(^{-1}\)) in McIlvaine's buffer, in a range of 2.6–7.8.

2.7. Surface plasmon resonance

Surface plasmon resonance (SPR) was carried out using the 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 from BIAcore AB (Uppsala, Sweden), as previously described [9,20]. XIP-I or TAXI-I (1 μM) in 10 mM sodium acetate buffer pH 5.5 was immobilized using the amine coupling method at a flow-rate of 10 μl/min using HBS buffer as running buffer. Equal volumes of N-hydroxysuccinimide (0.06 M in water) and N-ethyl-N’-(3-diethlyaminopropyl)carbodimide (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 150–2000 resonance units (RU). After injection of inhibitor (40 μl), the residual NHS (N-hydroxysuccinimide) esters were deactivated by injection of 25 μl ethanolamine (1 M, pH 8.5). Flow cell 2 was used to immobilize XIP-I or TAXI-I and control flow cell 1 was treated identically but without inhibitor. Increasing concentrations of A. niger xylanases ranging from 0.3 to 13 μM in 10 mM sodium acetate buffer pH 5.5 (40 μl) were injected at a flow rate of 30 μl/min using the same buffer as running buffer.

2.8. Circular dichroism spectroscopy

Solutions of 1 mgmL\(^{-1}\) xylanase in 10 mM sodium phosphate buffer pH 5.7 were analysed at 25 °C using a cell of 0.1 mm path length on a Jasco (Japan) Ltd J-710 circular dichroism (CD) spectropolarimeter. Data were averaged from 20 acquisitions between 260 and 180 nm in 1 nm steps at 50,000 samples per nm wavelength.
spectra of the xylanases were subtracted from CD spectra obtained from buffer alone.

3. Results and discussion

3.1. Enzymic and structural properties of the Asp37Ala xylanase variant

The Asp37Ala mutant was efficiently produced in *P. pastoris* at a secretion yield of 60 mgL$^{-1}$ and purified using a single chromatography step. The N-terminus of the mutant, SAGINY, was identical to that of the wt enzyme, demonstrating correct processing of the signal peptide. The Asp37Ala mutation did not significantly alter the secondary structure, as shown by the CD spectra (Fig. 1). The mutant consisted of one single molecular form of pl 3.7 (as determined by IEF) and of 19,893 Da molecular mass (as determined by ES-MS).

To evaluate the consequence of the Asp37Ala mutation on enzyme activity, the specific activity, pH optimum and Michaelis–Menten kinetics were determined using wheat arabinoxylan as substrate. The mutant showed a significant decrease in activity (5.5 Umg$^{-1}$) compared to a value of 172 Umg$^{-1}$ for the wt xylanase. However, the Asp37Ala mutant exhibited normal Michaelis–Menten kinetics with a $K_M$ of 5.7 mgmL$^{-1}$, similar to that of the wt enzyme (9.9 mgmL$^{-1}$) and a $k_{cat}$ of 3.1 s$^{-1}$, thus drastically reduced compared to 129 s$^{-1}$ for the wt xylanase. The wt enzyme showed a pH optimum of 3.5 followed by a sharp decrease in activity with only 1.2% activity remaining at pH 7.6 (Fig. 2(a)) whereas the activity of the mutant, albeit very low, remained relatively constant from pH 3–7.6 (Fig. 2(b)).

GH11 xylanases exhibit pH optima spanning a remarkable range from approximately 2–11. The mutation of Asp37 to Ala significantly altered both the catalysis and pH profile of the *A. niger* xylanase. However, the mutation did not affect the substrate binding, indicating that the integrity of the active site was retained in the mutated enzyme. Asp37 in *A. niger* xylanase is located in the active site (Fig. 3) at hydrogen-bonding distance (2.8 Å) of the acid–base catalyst, Glu170 [7], as also reported for other xylanases with low pH optima [24,25]. Structural alignment of GH11 xylanases showed that there is a strong correlation between the nature of the residue hydrogen bonded to the general acid/base catalyst and the pH optimum of these enzymes; it is Asn in the so-called “alkaline” xylanases (>5) and Asp in those with a more acidic pH optimum (<5) [25,26]. Furthermore, the single substitution of Asp for Asn at this key position in *A. kawachii* xylanase dramatically elevated its pH optimum from 2 to 5, with an ~85% reduction in xylanolytic activity [27], whereas the inverse mutation of Asn to Asp in the alkaline *Bacillus circulans* xylanase [28] shifted the pH optimum from 5.7 to 4.6, with an ~20% increase in activity [25] and a doubling of overall activity when Asn was replaced by Ala [29]. The importance of this residue in acidophilic adaptation of family 11 xylanases was also recently confirmed by the Asn to Asp mutation of Xyl1 from *Streptomyces* sp. S38, lowering its optimum pH (6.0–5.0) but at a cost of a 50% decrease in specific activity [30]. Taken together these studies suggest that “acidic” GH11 xylanases function using a reverse protonation mechanism [25].

![Fig. 1. CD spectra (280–200 nm) of *A. niger* wt xylanase (solid line) and Asp37Ala mutant xylanase (dotted line).](image)

![Fig. 2. Effect of pH on the xylanase activity of (a), *A. niger* wt xylanase and (b), Asp37Ala mutant. The activity assays were performed as described in Section 2 using wheat arabinoxylan as substrate. Activity (U) is expressed as μmolmin$^{-1}$mg$^{-1}$.](image)
3.2. Interaction between the Asp37Ala xylanase variant and the inhibitors XIP-I and TAXI-I

The xylanase activity of the Asp37Ala mutant remained unaffected by the addition of either XIP-I or TAXI-I up to an inhibitor:enzyme molar ratio of 5:1, as compared with 60% inhibition for the wt xylanase. The kinetics parameters of the inhibition could not be measured due to the very low enzymatic activity of the mutant; instead, the interaction between the variant xylanase and the inhibitors was investigated using two biophysical approaches. The relative affinities and pH dependencies of the interaction of XIP-I and TAXI-I with \textit{A. niger} xylanases were first studied using electrophoretic titration curves (Fig. 4). The wt \textit{A. niger} xylanase formed a complex with TAXI-I across the whole pH range (Fig. 4(a)) whereas the complex with XIP-I was seen in the pH range 4–7, as previously observed ([20]; Fig. 4(b)). The higher affinity of \textit{A. niger} xylanase for TAXI-I \((K_i = 20.1\ \text{nM}, \text{Gebruers, K., unpublished result})\) compared to XIP-I \((K_i = 317\ \text{nM}, [20])\) is further indicated by the fact that all concentration-limited components form the complex. There was no complex formation between TAXI-I and the Asp37Ala mutant (Fig. 4(c)) and no complex was observed between this mutant and XIP-I (Fig. 4(d)). SPR analysis was previously used to study the complex of \textit{A. niger} xylanase-XIP-I \([9, 20]\). However, using the same technique (see Section 2), no binding of the Asp37Ala mutant to surface-bound XIP-I and TAXI-I was detected using increasing amounts of xylanase (not shown). The \(K_D\) is therefore higher than 1 mM, which is the limit of detection of the BIAcore and is much higher than the 5.68 μM value calculated for the wt xylanase and XIP-I [20].

Taken together, the results show that the mutation of Asp37 to Ala in the enzyme active site results in the abolition of the interaction of the \textit{A. niger} xylanase with both TAXI-I and XIP-I. We recently demonstrated the importance of the “thumb” region (Fig. 3), in particular Asn117, in the interaction of the \textit{A. niger} xylanase with XIP-I [9], suggesting that the inhibition occurred by steric hindrance, i.e. blocking access to the active site. Since Asp37 is essential for the pH dependence of enzymatic catalysis, the loss of interaction of Asp37Ala
mutant with XIP-I is likely to be the result of a disruption of the hydrogen bonding network in the vicinity of the active site. The three-dimensional structure of XIP-I has recently been solved and showed the presence of a flexible loop region which could play a role in the interaction with fungal xylanases [31]. Considering the effects of both Asn117Ala and Asp37Ala mutants with regards to the binding to XIP-I, the inhibition could be the result of a sequence of events in which XIP-I interacts with Asn117 and later to the whole thumb region, which would in turn direct the loop of XIP-I to the active site where it would make a strong contact with the acid–base catalysts and/or other active site residues. Interestingly, the Asp37Ala xylanase is not inhibited by TAXI-I, suggesting that the mechanism of inhibition by TAXI-I also involves direct interactions at the active site, in agreement with the competitive mode of inhibition reported with this enzyme [19]. This characteristic has implications for the binding of the wheat protein inhibitors towards low pH optimum GH11 xylanases. Knowledge on the molecular requirements responsible for the specificity of the inhibition can be exploited to design novel industrial enzymes with improved properties.

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References


