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Molecular determinants of substrate and inhibitor specificities of the *Penicillium griseofulvum* family 11 xylanases

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ABSTRACT

Penicillium griseofulvum possesses two endo-(1,4)-β-xylanase genes, *PgXynA* and *PgXynB*, belonging to family 11 glycoside hydrolases. The enzymes share 69% identity, a similar hydrolysis profile i.e. the predominant production of xylobiose and xylotriose as end products from wheat arabinoxylan and a specificity region of six potential xylose subsites, but differ in terms of catalytic efficiency which can be explained by subtle structural differences in the positioning of xylohexaose in the PgXynB model. Site-directed mutagenesis of the "thumb" region revealed structural basis of PgXynB substrate and inhibitor specificities. We produced variants displaying increased catalytic efficiency towards wheat arabinoxylan and xylo-oligosaccharides and identified specific determinants in PgXynB "thumb" region responsible for resistance to the wheat xylanase inhibitor XIP-I. Based on kinetic analysis and homology modeling, we suggested that $Pro130_{PgXynB}$, $Lys131_{PgXynB}$ and $Lys132_{PgXynB}$ hamper flexibility of the loop forming the "thumb" and interfere by steric hindrance with the inhibitor.

1. Introduction

β-1,4-Xylan, the most abundant component of hemicelluloses in the cell walls of monocots and hard woods, is a heteropolysaccharide consisting of a backbone of β-1,4-linked D-xylopyranosyl residues substituted with L-arabinosyl, 4-*O*-methyl-glucuronosyl, and acetyl side chains [1,2]. As xylan varies in structure between different plant species, complete hydrolysis requires a large variety of cooperatively acting enzymes such as xylanases, xylosidases, arabinofuranosidases, glucuronidases, acetylxylan esterases, ferulic acid esterases, and *p*-coumaric acid esterases [3–5]. All of these enzymes act cooperatively to convert xylan into its constituent sugars. Of these, endo-(1,4)-β-xylanases (xylanases; EC 3.2.1.8) are of particular significance since they can hydrolyse the internal 1,4-β-D-xylosidic linkages within the xylan backbone to produce short-chain xylo-oligosaccharides of varying length [5]. Many xylanolytic microorganisms secrete multiple isoforms of xylanases that can cooperate to enhance the hydrolysis of the complex xylan [6], fungi being the most potent producers [7,8]. Xylanolytic enzymes of microbial origin have received great attention due to their biotechnological utility and potential application in a range of industrial processes but the observed effects vary depending on xylanase specificities [9]. In the past few years, the potential applications of xylanases for bioconversion of lignocellulosic feedstocks to fuel-grade ethanol have been of particular interest to researchers.

Based on amino acid sequence similarities, xylanases are mainly clustered into families 10 and 11 of glycoside hydrolases (GH) (www.cazy.org [10]). The two families have different molecular structures, molecular weights, and catalytic properties (see [5] for a review). Family 11 consists of 20–30 kDa molecular weight proteins with 21 structures folded as a β-jelly roll available from both bacteria and fungi. GH10 xylanases exhibit higher affinity for shorter linear β-1,4-xylo-oligosaccharides than GH11 xylanases [11]. GH10 xylanases have a $(\beta/\alpha)_8$-barrel as a catalytic domain and typically contain one or more carbohydrate-binding domains, which increase the effective concentration of the catalytic domain on polymeric substrates [12]. The structure of GH11 xylanases has been described as a partially closed right hand [13]. It consists of a single domain folding into two β-

Abbreviations: A/X, arabinose to xylose ratio; DNS, Dinitrosalicylic acid; GH, Glycoside hydrolase; GST, glutathione-*S*-transferase; HPAEC-PAD, High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection; LVAX, Low viscosity wheat arabinoxylan; MALDI-MS, Matrix-Assisted Laser Desorption Ionization/Mass Spectrometry; PfXynC, xylanase C from *Penicillium funiculosum*; PgXynA, xylanase A from *Penicillium griseofulvum*; PgXynB, xylanase B from *Penicillium griseofulvum*; TAXI, *Triticum aestivum* xylanase inhibitor; TLXI, thaumatin-like xylanase inhibitor; XIP-I, xylanase inhibitor protein I; X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentose; X6, xylohexaose

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sheets, which are packed against each other, and one α-helix. The two β-sheets are strongly twisted and form a cleft on one side of the protein in which the active site is situated. This cleft is covered by a long loop region, called the "thumb" region and partly closed on one side by the cord, a long irregular loop with a well-defined structure. A consequence of the difference in structure is their difference in substrate specificity. The active site of GH10 xylanases is a shallow groove, which is reflected in their specificity towards a lower number of unsubstituted consecutive xylose units. In contrast, GH11 xylanases show higher affinity towards a larger number of unsubstituted consecutive xylose units because of their cleft-shaped active site [11]. Multiple xylanases also occur within each category of GH families 10 and 11 [6]. Typically many introns are present in GH10 xylanase genes from filamentous fungi [14] whereas GH11 xylanase genes contain one or two introns [15].

Microbial GH10 and GH11 xylanases are target of proteinaceous xylanase inhibitors present in cereals, *Triticum aestivum* xylanase inhibitor (TAXI), xylanase inhibitor protein (XIP), and thaumatin-like xylanase inhibitor (TLXI) (for a review, see [16]). The specificity of these inhibitors is dependent on the source and nature of the xylanase tested: TAXI and TLXI inhibitors can inhibit GH11 xylanases from both fungal and bacterial origin but not those of GH10 whereas XIP-type inhibitors typically inhibit GH10 and GH11 endoxylanases from fungal sources (for a review, see [9]). Several lines of evidence point towards a role of these inhibitor proteins in plant defense: (i) their lack of inhibition activity against endogenous xylanases (ii) their ability to inhibit xylanases from pathogens *Fusarium graminearum* and *Botrytis cinerea* (iii) the fact that some TAXI and XIP genes are wound and pathogen inducible and (iv) the homologies of TLXI and XIP with pathogenesis-related (PR) proteins of family 5 (PR-5) and 8 (PR-8), respectively [16].

The *Penicillia* are mostly saprophytic in nature and numerous species have been exploited in the production of xylanolytic enzymes (reviewed in [17]). The presence of family 10 and family 11 xylanases has been reported for *Penicillium purpurogenum* [18], *Penicillium citrinum* [15,19], *Penicillium funiculosum* [20,21] and *Penicillium capsulatum* [22,23]. A xylanase from *Penicillium griseofulvum* (PgXynA) was isolated using a screening method based on the ability to digest wheat flour arabinoxylan and the corresponding gene was cloned and expressed in *Aspergillus oryzae* [24]. We have previously characterised PgXynA heterologously expressed in *Escherichia coli* [25]. Computational design methods was applied to guide structure-based site-directed mutagenesis analyses of PgXynA enzymes with changed specificity, altered pH profile, and improved overall catalytic activity [26]. In the present study, we report the production and enzymatic, biochemical and structural characterisation of PgXynB, a second xylanase from *P. griseofulvum* with 69% identity to PgXynA [24]. The enzyme was characterised in terms of kinetics towards arabinoxylan, xylo-oligosaccharides products formation and inhibition sensitivity. We engineered PgXynB variants displaying increased catalytic efficiency towards wheat arabinoxylan and xylo-oligosaccharides and identified unique PgXynB determinants in the "thumb" region responsible for resistance to the wheat xylanase inhibitor XIP-I. The PgXynA and PgXynB xylanases constitute an ideal system for the study of structure–function relationships within family 11 xylanases.

2. Materials and methods

2.1. Materials, plasmids, and strains

E. coli DH5α strain was used for DNA manipulation and BL21 strain (DE3) *pLys* for protein expression (Novagen, Merck Biosciences, Fontenay, France). High purity salt-free oligonucleotides, pDONrZeo and pDEST17 plasmids were from Invitrogen (Cergy Pontoise, France), restriction endonucleases and DNA modifying enzymes were from Promega (Madison, WI, USA), pGEX-1λT expression vector, GST glutathione agarose beads, and thrombin protease from Amersham Biosciences (GE healthcare, Orsay, France), BugBuster and benzonase from Novagen, lysosyme, β-D-xylose, and dinitrosalicylic acid from Sigma (Lyon, France), and low viscosity wheat arabinoxylan (LVAX) and 1,4-β-D-xylo-oligosaccharides (xylose X1, xylobiose X2, xylotriose X3, xylotetraose X4, xylopentose X5, and xylohexaose X6) from Megazyme International (Wicklow, Ireland). Purified wheat arabinoxylan substrates with different arabinose to xylose ratio (A/X) were kindly provided by C. Courtin (Katholieke University Leuven, Belgium).

2.2. PgXynB cloning and site-directed mutagenesis

The intron present in *PgXynB* genomic sequence [27] was eliminated by overlap extension method [28] using primers hybridizing with each exon of *PgXynB*. Two independent PCR reactions (PCRI and PCRII) were performed using *PgXynB* as template (10 ng), together with 1.25 U of DNA polymerase (Prime STAR DNA polymerase TAKARA) and 0.2 mM dNTP. The primers used in this study are given in Table 1. In a first PCR run, PCRI and PCRII reactions were carried out using PGB1F and PGB2R primers (20 pmol) for PCRI and PGB3F and PGB4R primers (20 pmol) for PCRII in 50 μL volume and for 25 cycles of denaturation (1 min 98 °C), annealing (2 min at 48 °C) and extension (1.5 min at 72 °C) in a Mastercycler® gradient thermocycler (Eppendorf, Hambourg, Germany). The resulting PCRI (195 bp) and PCRII (426 bp) products were gel-purified using the QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). The final PCR run was carried out with 150 ng of purified PCRI and PCRII products, 1.25 U of DNA polymerase and 0.2 mM dNTP. After five cycles of denaturation (1 min at 94 °C), annealing (2 min at 42 °C) and extension (3 min at 72 °C), either PGBHIF and PGBEIR or PGBGWF and PGBGWR primers (20 pmol) were added and the reaction was subjected to 25 cycles of denaturation (1 min at 94 °C), annealing (2 min at 53 °C) and extension (1.5 min at 72 °C).

The final PCR product (597 bp) obtained with PGBHIF and PGBEIR primers was gel-purified, digested by BamHI and EcoRI, and ligated into the pGEX-1λT plasmid. After transformation in *E. coli* DH5α strain, the recombinant vector pGEX-1λT/*PgXynB* was checked by restriction mapping and double stranded DNA sequencing (Genome Express, Meylan, France).

The final PCR product (627 bp) amplified with the help of the *att* tagged primer pair, PGBGWF and PGBGWR, was combined with the donor vector (pDONrZeo) following manufacturer's instructions (Gateway® Cloning, Invitrogen) resulting in the formation of an entry clone (pDONrZeo/*PgXynB*). After sequencing, the *PgXynB* coding sequence was further transferred by a recombination reaction from the Gateway® entry clone to the pDEST17 destination vector, allowing expression of *PgXynB* with a poly-histidine tagged $(His)_6$ at the N-terminal to produce $(His)_6$PgXynB.

Table 1
Oligonucleotide sequences used for elimination of the intron sequence and construction of pGEX1λT/*PgXynB* and pDEST17/*PgXynB*

Name	Oligonucleotide sequences (5′→3′)
PGB1F	GCT CTC TTT ACA AGC CAA ACT GGA G
PGB2R	ATA GTG GAT GTT CCG GGC ACT ACC AGT GCT CCA GCC CTT GCC
PGB3F	GGT AGT GCC CGG AAC ATC CAC TAT GCT GGA GAT TTC AAA CCC
PGB4R	TAA CCA AAC AGA CAT GTC AGC GTA TCC
PGBHIF	TTT TTT **GGA TCC** GCT CTC TTT ACA AGC CAA ACT GGA G
PGBEIR	TTT TTT **GAA TTC** TCA CCA AAC AGA CAT GTC AGC GTA TCC
PGBGWF	**GGG ACA AGT TTG TAC AAA AAA GCA GGC TTA** GCT CTC TTT ACA AGC CAA ACT GGA G
PGBGWR	**GGG CCA CTT TGT ACA AGA AAG CTG GGT** TTA CCA AAC AGA CAT GTG AGC CTA TCC

Restriction sites and *att*-flanked sites are shown in bold and bold underlined, respectively.