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Targeted molecular engineering of a family 11 endoxylanase to decrease its sensitivity towards *Triticum aestivum* endoxylanase inhibitor types

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Abstract

The *Bacillus subtilis* endoxylanase XynA (BSXY) is frequently used to improve the functionality of arabinoxylan-containing material in cereal based industries. The presence of endogenous *Triticum aestivum* xylanase inhibitors (TAXI-I and TAXI-II) in wheat is a real concern as they have a direct negative impact on the efficiency of this enzyme. Here, we used the recently determined structure of the complex between TAXI-I and an endoxylanase of *Aspergillus niger* to develop inhibitor-insensitive BSXY variants by site-directed mutagenesis of strategically chosen amino acids. We either induced steric hindrance to reject the inhibitors or interrupted key interactions with the inhibitors in the endoxylanase substrate-binding groove. The first strategy was successfully applied to position G12 where G12W combined inhibition insensitivity with unharmed catalytic performance. Variants from the second strategy showed altered inhibitor sensitivities concomitant with changes in enzyme activities and allowed to gain insight in the binding-mode of both TAXI-I and TAXI-II with BSXY.

Keywords: *Bacillus subtilis* endoxylanase; *Triticum aestivum* xylanase inhibitor; Inhibition sensitivity; Site-directed mutagenesis

1. Introduction

Endo-β-1,4-xylanases (E.C.3.2.1.8; further referred to as endoxylanases) are key enzymes in the degradation of xylan, the most abundant natural cell wall polysaccharide after cellulose, and more specifically arabinoxylan (AX), an important quality determining non-starch polysaccharide in cereals. Most endoxylanases belong to the structurally unrelated glycoside hydrolases families (GH) 10 and 11. GH 11 endoxylanases have a β-jelly roll structure, which resembles a partly closed right hand, with well-known characterized active site geometry (Törrönen and Rouvinen, 1997). They use a conserved configuration of two catalytic glutamic acids to hydrolyze xylan. Both active site glutamic acids are involved in a network of hydrogen bonds with neighbouring residues. The amino acid adjacent to the acid/base catalyst determines the pH optimum of enzyme activity. Hence, 'acidic endoxylanases' like *Aspergillus niger* endoxylanase ExlA (ANXY) have a conserved Asp ($Asp37_{ANXY}$) (Tahir et al., 2004), while 'alkaline endoxylanases' like *Bacillus subtilis* endoxylanase XynA (BSXY) display an Asn ($Asn35_{BSXY}$) (Joshi et al., 2000). Within the substrate binding cleft, each region that accommodates a xylose moiety is known as a subsite. Subsites are given a negative or positive number depending on whether they bind the glycone or aglycone regions of the substrate, respectively. Glycosidic bond cleaving thus occurs between the −1 and +1 subsite (Davies et al., 1997).

Abbreviations: ANXY, *Aspergillus niger* endoxylanase ExlA; AX, arabinoxylan; BSA, bovine serum albumin; BSXY, *Bacillus subtilis* endoxylanase XynA; GH, glycoside hydrolase family; GPC, gel permeation chromatography; IU, inhibition unit; rBSXY, recombinant BSXY; rTAXI-I, recombinant TAXI-I; rTAXI-II, recombinant TAXI-II; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TAXI, *Triticum aestivum* xylanase inhibitor; U, enzyme unit; WT, wild-type; XIP, xylanase inhibitor protein

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Microbial endoxylanases, which can belong to GH 10 or GH 11, are frequently used to modify the AX functionality in biotechnological processes such as bread-making (Rouau, 1993; Courtin et al., 1999; Courtin and Delcour, 2002; Sørensen, 2003), gluten-starch separation (Christophersen et al., 1997; Frederix et al., 2003; Van Der Borght et al., 2005), and animal feed preparations (Bedford, 2003).

An important development is the discovery of cereal proteins that inhibit endoxylanases and may affect the functionality of the endoxylanases in the above cited processes. In literature, two structurally different cereal inhibitors have been described, i.e. the *Triticum aestivum* L. endoxylanase inhibitor (TAXI)-type (Debyser and Delcour, 1997) and the xylanase inhibitor proteins (XIP)-type (McLauchlan et al., 1999). These two types of inhibitors have distinct inhibition specificities towards endoxylanases. TAXI-type xylanase inhibitors inhibit GH 11 endoxylanase of bacterial and fungal origin, but show no activity towards GH 10 endoxylanases (Gebruers et al., 2001). XIP-type inhibitors mainly inhibit fungal endoxylanases of GH 10 and 11 (Flatman et al., 2002). The first characterized wheat xylanase inhibitor, TAXI, was found to be a mixture of two different inhibitors: TAXI-I and TAXI-II (Gebruers et al., 2001). Both are high p*I* proteins (p*I* TAXI-I: 8.8; TAXI-II: $\geq$9.3) with similar N-terminal amino acid sequences and similar positions of disulfide bonds (Gebruers et al., 2001). They occur in two forms, i.e. a 40 kDa single polypeptide chain and a processed form of two disulfide-linked subunits of 30 and 10 kDa. Despite their structural similarity, TAXI-I and TAXI-II can be clearly distinguished by assaying their inhibition activity towards 'acidic' and 'alkaline' endoxylanases. 'Acidic' endoxylanases are not or only weakly inhibited by TAXI-II and strongly affected by TAXI-I, while 'alkaline' endoxylanases are strongly inhibited by both TAXIs (Gebruers et al., 2004).

The molecular interaction between ANXY and TAXI-I has recently been described by Sansen et al. (2004). The three dimensional structure of the complex revealed a direct interaction of the inhibitor with the active site region of the enzyme and further substrate-mimicking contacts with binding subsites filling the whole substrate docking region. Molecular engineering of both TAXI-I and TAXI-II has further characterized two key residues in endoxylanase inhibition (Fierens et al., 2004; Raedschelders et al., 2005). First of all $His374_{TAXI-I}$ is the key residue for inhibition of ANXY, as its imidazole ring is positioned in the active site of the endoxylanase and interacts with both catalytic glutamic acid residues as well as with the pH-determining $Asp37_{ANXY}$. Secondly, $Leu292_{TAXI-I}$ or the equivalent $Pro294_{TAXI-II}$ accomplish a perfect substrate mimic in the −2 glycon subsite. Additional interactions between ANXY and TAXI-I are located in the His374-containing carboxyterminal loop of TAXI-I, but these have not yet been fully examined. More particularly, main chain atoms from $Phe375_{TAXI-I}$ and $Thr376_{TAXI-I}$ are hydrogen-bonded to $Gln129_{ANXY}$ and $Arg115_{ANXY}$ in the endoxylanase glycon subsite −1. In addition, on the aglycon site, there is a clear hydrogen bond interaction between $Gln187_{TAXI-I}$ and $Trp172_{ANXY}$, which interferes directly with subsites +1 and +2.

There are only a few reports on molecular engineered endoxylanases with altered inhibition sensitivity towards TAXI-type inhibitors. Tahir et al. (2004) constructed a D37A mutant from which the importance of $Asp37_{ANXY}$, in inhibition of ANXY by TAXI-I as well as XIP could be derived. Recently, site-directed mutagenesis of 22 surface residues surrounding the active site cleft of BSXY, has indicated that residues on "finger IV", more specifically $Asp11_{BSXY}$, play an important role in the interaction with TAXI-I (Sibbesen and Sørensen, 2001; Sørensen and Sibbesen, 2006). Mutations at $Asp11_{BSXY}$ into different amino acids completely abolished the interaction between BSXY and the inhibitor. Although this concomitantly resulted in a serious reduction (74–86%) of the specific activity of the enzyme, the finding indicated that steric hindrance may well be used as a strategy to decrease the sensitivity of GH 11 endoxylanases for TAXIs.

The functionality and the performance of an endoxylanase in bread making are importantly influenced by the inhibition sensitivity of the endoxylanase (Trogh et al., 2004). Therefore, the aim of the present study was to generate variants of BSXY, a widely used endoxylanase and only inhibited by TAXIs, with a decreased sensitivity towards both TAXI-type inhibitors and an unaffected specific activity. To this end, we used a targeted approach based on the knowledge of the molecular interactions between GH 11 endoxylanase and TAXI (pdb accession 1T6G). This allowed two strategies to be followed: (i) creation of steric hindrance to prevent docking of inhibitor into enzyme and (ii) impeding molecular interactions between inhibitor and major residues of the active site and the substrate-binding region. In addition, our results provided further insight in the importance of particular endoxylanase residues in the molecular interaction with TAXI.

2. Materials and methods

2.1. Materials

Primers were purchased from Proligo Primers and Probes (Paris, France). Restriction enzymes were from Roche Diagnostics GmbH (Mannheim, Germany) and *Pfu* DNA polymerase for polymerase chain reaction (PCR) was from Fermentas GmbH (St. Leon-Rot, Germany). *Escherichia coli* TOP10F' (Invitrogen, Carlsbad, CA, USA), was used as host strain for transformation of DNA constructs, except in case of pCR4-TOPO vector (Invitrogen), for which *E. coli* TOP10 (Invitrogen) was used. *E. coli* WK6 (Δ(*lac-pro*AB), *gal*E, *str*A, nal^R, {F' *lac*ZΔM15 *pro*AB^+}) was used as host strain for heterologous expression with expression vector pQE-Ec. This vector is principally the same as the pQE-En vector described by Hertveldt et al. (2003), apart form the E-tag which is C-terminally fused to the protein instead of N-terminally. The EasySelect *Pichia* Expression Kit was from Invitrogen. Vector pPICZαC, carrying the Zeocin resistance gene for selection in *E. coli* as well as *P. pastoris*, was used for recombinant protein secretion in *P. pastoris* strain X33 (Invitrogen).