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Protein engineering of GH10 family xylanases for gaining a resistance to cereal proteinaceous inhibitors

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ABSTRACT

Proteinaceous inhibitors from cereals reduce the efficiency of xylanase applications as a component of animal feeds, in bread making and other technologies based on processing of cereal grains. The inhibitors of XIP-type inhibit most of fungal xylanases from the GH10 and GH11 families of glycoside hydrolases, while they have no affect on bacterial xylanases. In order to gain a resistance to XIP-type proteins, an insertion of 5 amino acid residues was introduced into the peptide loop forming the active site cleft of two GH10 xylanases (PcXylA from *Penicillium canescens* and TrXyn3 from *Trichoderma reesei*) using a protein engineering technique. Genes encoding the wild-type and engineered forms of PcXylA and TrXyn3 were cloned and expressed in *Penicillium verruculosum* B1-537 and *P. canescens* RN3-11-7 auxotrophic recipient strains, respectively. The secreted recombinant xylanases were purified for characterization. Using a method based on measuring the enzyme effect on viscosity of the native extract of rye flour and that subjected to boiling in order to inactivate its inhibitory proteins, it was shown that the 5 aa insertion leads to relieving the inhibition of PcXylA and TrXyn3 by the proteinaceous inhibitors from rye.

1. Introduction

Xylanases (endo-β-l,4-xylanases, EC 3.2.1.8) are the major enzymes of the extracellular hemicellulolytic systems of filamentous fungi that carry out the biodegradation of plant biomass in nature (Collins et al., 2005). Most of known microbial xylanases are classified into families 10 and 11 (GH10 and GH11) of glycoside hydrolases (http://www.cazy.org/). Xylanases from both families found applications in different industries and agriculture, including chemical production, biobleaching of pulps, bread making, brewing, and manufacturing of animal feeds (Collins et al., 2005; Juturu and Wu, 2012).

Quite recently, in the end of the last century, proteinaceous inhibitors of xylanases have been discovered in the grains of cereals (Debyser et al., 1997; Goesaert et al., 2004). The inhibitors are a negative factor reducing the enzyme efficiency on using as a component of animal feeds based on wheat, rye, barley, etc., in bread making and other cereal-based technologies. There are three major types of cereal proteinaceous inhibitors of xylanases: those of TAXI-, XIP- and TLXI-type (Goesaert et al., 2004; Gusakov, 2010; Dornez et al., 2010). The TAXI- and TLXI-type proteins specifically inhibit fungal and bacterial GH11 family xylanases but do not affect those of the GH10 family. The XIP-type proteins inhibit fungal xylanases belonging to both GH10 and GH11 families, while they have no affect on bacterial xylanases from these families. However, exceptions to these rules exist, in particular, a few fungal GH10 xylanases are known, for which a resistance to XIP-type inhibitors has been reported (Flatman et al., 2002; Gusakov and Ustinov, 2009; Denisenko et al., 2015). Analysis of the crystal structure of XIP-I from wheat in complex with the GH10 xylanase XlnC from *Aspergillus nidulans* showed that Lys234 of the XIP-I makes a direct hydrogen bond with the acid-base catalytic residue Glu131, while the XIP-I α7-helix interacts with four loops forming the XlnC active site groove (Payan et al., 2004). The authors of the cited report hypothesized that the amino acid insertions in loops β4-α4 and β8-α8 of the bacterial Xyn10A, for which the absence of inhibition had been demonstrated, may be responsible for the enzyme resistance to XIP-type inhibitors. This assumption was in agreement with the finding that similar insertions are also present in xylanase II from *A. aculeatus*, one of the fungal exceptions, for which the lack of the inhibition by XIP has been demonstrated (Flatman et al., 2002). However, no experimental studies based on enzyme bioengineering approach have been carried

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out in order to test the hypothesis on how the length of the loops adjacent to the GH10 family xylanase active site cleft affects the enzyme susceptibility to the inhibitors from cereals.

In this paper, using a protein engineering technique, we introduced an insertion of five amino acid residues into the loop forming the active site cleft of two GH10 family xylanases, thus demonstrating that the elongation of this loop leads to gaining a resistance of the enzymes to proteinaceous inhibitors from rye extract. One of the xylanases under study (PcXylA) represented a major xylanase secreted by *Penicillium canescens* fungus (Sinitsyna et al., 2003), while the other was xylanase III (TrXyn3) from *Trichoderma reesei* (Ogasawara et al., 2006). It should be noted that the amino acid sequence of PcXylA is 100% identical to that of xylanase A from *P. simplicissimum* (Serebryanyi et al., 2002), for which a few crystal structures are available (Schmidt et al., 1998, 1999).

2. Materials and methods

2.1. Protein engineering of PcXylA and TrXyn3

Cloning and expression of the *P. canescens xylA* gene (GenBank accession number **AY756109**), encoding the wild-type PcXylA, into *P. verruculosum* B1-537 (*niaD-*) auxotrophic strain under the control of the *cbh1* gene promoter was described previously (Denisenko et al., 2017). The genetic engineering manipulations for cloning and expression of the *T. reesei xyn3* gene (**AB036796**), encoding the wild-type TrXyn3, into *P. canescens* RN3-11-7 (*niaD-*) host strain under the control of the *xylA* gene promoter were very similar to those described elsewhere (Volkov et al., 2014). In order to introduce the GQGGA insertion into certain positions of PcXylA and TrXyn3 amino acid sequences (see section 3.1 and Fig. 1 below), the pairs of oligonucleotide primers were synthesized and purified (Table 1). Primers 1–2 and 5–6 contained additional 15 nucleotides encoding the GQGGA insertion in TrXyn3 and PcXylA, respectively. Primers 3–4 and 7–8 had Ligation Independent Sites on 5′-ends to place the mutated genes into the pXEG or pCBHI vector, respectively, by Ligation Independent Cloning method developed by Aslanidis and De Jong (1990) and applied by us elsewhere (Volkov et al., 2014; Dotsenko et al., 2015; Denisenko et al., 2017). The target GQGGA insertion in TrXyn3 and PcXylA was confirmed by DNA sequencing of full-size expression plasmids. Then, using the previously developed protocols (Aleksenko et al., 1995; Volkov et al., 2014; Denisenko et al., 2017), the plasmid DNAs were transformed into *P. canescens* RN3-11-7 or *P. verruculosum* B1-537 host strains to express the TrXyn3 or PcXylA, respectively. The resulting recombinant strains were supplied for flask fermentations, allowing screening for the highest enzyme productivity. The selected clones were cultured in 3-L laboratory fermenters as described elsewhere (Volkov et al., 2014; Denisenko et al., 2017).

2.2. Purification and identification of xylanases

The wild-type and engineered forms of PcXylA and TrXyn3 were isolated and purified as described elsewhere (Denisenko et al., 2015, 2017). Briefly, after crude protein precipitation from a culture liquid with $(NH_4)_2SO_4$ followed by desalting on a Bio-Gel P-2 column (Bio-Rad Laboratories, Hercules, CA, USA), two-stage chromatographic purification of the enzymes was carried out. This included anion-exchange chromatography on a Source 15Q column followed by hydrophobic-interaction chromatography on a Source 15ISO column (GE Healthcare, Uppsala, Sweden). Enzyme purity was characterized by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out in 12% gel using a Mini Protean II equipment (Bio-Rad Laboratories, Hercules, CA, USA).

For the enzyme identification, pieces of protein bands were cut from the electrophoretic gels, digested with trypsin according to a standard protocol (Smith, 1997), and the resulting peptide mixture was analyzed by MALDI-TOF mass spectrometry (MS) on an UltrafleXtreme mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The MS data were analyzed using the online MASCOT service (http://www.matrixscience.com/). Peptides with the 5 aa insertion in PcXylA and TrXyn3 were identified using the online PeptideMass tool (https://web.expasy.org/peptide_mass/), and their structures were confirmed by tandem MS/MS.

2.3. Xylanase activity and thermostability

Xylanase activity was assayed against 0.5% glucuronoxylan from birch wood (Sigma, St. Louis, MO, USA) and arabinoxylan from wheat (Megazyme, Boronia, Australia) by analyzing reducing sugars released after 10 min of the enzymatic reaction at 50 °C and pH 5.0 (0.1 M Na-acetate buffer) using the Nelson–Somogyi method (Nelson, 1944) as described elsewhere (Sinitsyna et al., 2003). The assays were carried out in triplicates.

The enzyme thermostability was assayed as described elsewhere (Denisenko et al., 2017).

2.4. Assaying xylanase susceptibility to proteinaceous inhibitors from rye

A modified method (Gusakov and Ustinov, 2009) based on measuring changes in viscosity of water extracts of rye flour (a native extract and that subjected to boiling for 15 min in a water bath) was used to assay the susceptibility of xylanases to the inhibition by proteinaceous inhibitors from rye. The rye extracts were prepared as described elsewhere (Gusakov and Ustinov, 2009), however the viscosity measurements were carried out using an A&D Vibro Viscometer SV-10 (A&D Company, Tokyo, Japan) instead of the Ostwald viscometer used in our previous work (Gusakov and Ustinov, 2009). The measurements of changes in viscosity of the rye extracts were carried out in a 50-mL container at pH 5.0 and 40 °C according to the instruction manual of the instrument.

3. Results

3.1. Alignment and structural features of fungal GH10 family xylanases

As already mentioned in the Introduction, most of GH10 family fungal xylanases are inhibited by XIP-type proteins from cereals, while a few exceptions are known, for which a resistance to cereal proteinaceous inhibitors have been demonstrated (Flatman et al., 2002; Gusakov and Ustinov, 2009; Denisenko et al., 2015). The alignment of amino acid sequences of seven fungal xylanases from the GH10 family is shown in Fig. 1. The upper three enzymes are susceptible to such kind of inhibition, while the four xylanases at the bottom of the alignment, including xylanase II from *A. aculeatus* (AaXyl2), xylanase E from *P. canescens* (PcXylE) and two xylanases (MtXyl3 and MtXyl4) from *Myceliophthora thermophila* (formerly *Chrysosporium lucknowense*) displayed the resistance to the inhibition by XIP-type proteins (Flatman et al., 2002; Payan et al., 2004; Gusakov and Ustinov, 2009; Denisenko et al., 2015). A common feature of the enzymes resistant to the inhibition is the presence of two insertions (10–12 aa and 5 aa residues, respectively) in their amino acid sequences, shown in bold in Fig. 1. The superposition of crystal structures of PcXylA, TrXyn3 and PcXylE (Fig. 2) showed that these insertions form two loops adjacent to the enzyme active site cleft. Since the 5 aa insertion (GQGGA in the case of PcXylE) is located closer to the enzyme active site and, in particular, closer to the Glu catalytic residue that directly interacts with the XIP (Payan et al., 2004), this insertion is more likely responsible for the lack of the inhibition in four xylanases shown in Fig. 1. Therefore, in order to test this hypothesis, we decided to implement the GQGGA insertion between the relevant Pro and Leu residues in PcXylA and TrXyn3 (shown with an arrow in Fig. 1).

```
TrXyn3  LPTETIHLDPELAALRANLTERTADLWDRQASQSIDQLIKRKGKLYFGTATDRGL-LQRE 59
PcXylA  -----------------------------QASVSIDAKFKAHGKKYLGTIGDQYTLTKNT 31
AnXlnC  ------------------------------QSASLNDLFVAAGKSYFGTCSDQAL-LQNS 29
PcXylE  -----------------------APHLPGNKDIDLNKLAQRRGKHWFGTAADIPGTAETT 37
MtXyl4  ----------------------KGKGHGHGPHTGLHTLAKQAGLKYFGSATDSPGQRERA 38
AaXyl2  --------------------------------VGLDQAAVAKGLQYFGTATDNPEL-TDI 27
MtXyl3  ---------------------------------QLNDLAVRAGLKYFGTALSESVINSDT 27
                                          :.      *  ::*:  .

TrXyn3  -------K--NAAIIQADLGQVTPENSMKWQSLENNQGQLNWGDADYLVNFAQQNGKSIR 110
PcXylA  -------K--NPAIIKADFGQLTPENSMKWDATEPNRGQFTFSGSDYLVNFAQSNGKLIR 82
AnXlnC  -------Q--NEAIVASQFGVITPENSMKWDALEPSQGNFGWSGADYLVDYATQHNKKVR 80
PcXylE  -------DAAYLKVLKQNFGEITPANAMKFMYTETEQNVFNFTEGEQFLEVAERFGSKVR 90
MtXyl4  GYEDKYAQYDQIMWKSGEFGLTTPTNGQKWLFTEPERGVFNFTEGDIVTNLARKHGFMQR 98
AaXyl2  -------PYVTQLNNTADFGQITPGNSMKWDATEPSQGTFTFTKGDVIADLAEGNGQYLR 80
MtXyl3  -------RYAAILSDKSMFGQLVPENGMKWDATEPSRGQFNYASGDITANTAKKNGQGMR 80
                          :*  .* *. *:   * .:. : :  .:   : *   .   *

TrXyn3  GHTLIWHSQLPAWVNNIN-NADTLRQVIRTHVSTVVGRYKGKIRAWDVVNEIFNEDG-TL 168
PcXylA  GHTLVWHSQLPGWVSSIT-DKNTLISVLKNHITTVMTRYKGKIYAWDVLNEIFNEDG-SL 140
AnXlnC  GHTLVWHSQLPSWVSSIG-DANTLRSVMTNHINEVVGRYKGKIMHWDVVNEIFNEDG-TF 138
PcXylE  CHNLVWASQVSDFVTSKTWTAKELTAVMKNHIFKTVQHFGRRCYSWDVVNEALNGDG-TF 149
MtXyl4  CHALVWHSQLAPWVESTEWTPEELRQVIVNHITHVAGYYKGKCYAWDVVNEALNEDG-TY 157
AaXyl2  CHTLVWYNQLPSWVTSGTWTNATLTAALKNHITNVVSHYKGKCLHWDVVNEALNDDG-TY 139
MtXyl3  CHTMVWYSQLPSWVSSGSWTRDSLTSVIETHMNNVMGHYKGQCYAWDVINEAINDDGNSW 140
         * ::* .*:  :* .       *  .: .*:  .   :  :   ***:** :* ** :

TrXyn3  RSSVFSRLLGEEFVSIAFRAARD-----ADPSARLYINDYNLDRANYGKVNGLKTYVSKW 223
PcXylA  RNSVFYNVIGEDYVRIAFETARS-----VDPNAKLYINDYNLDSAGYSKVNGMVSHVKKW 195
AnXlnC  RNSVFYNLLGEDFVRIAFETARA-----ADPDAKLYINDYNLDSASYAKTQAMASYVKKW 193
PcXylE  SSSVWYDTIGEEYFYLAFKYAQEALAQIGANDVKLYYNDYGIENPG-TKSTAVLQLVSNL 208
MtXyl4  RESVFYKVLGEDYIKLAFETAAK-----VDPHAKLYYNDYNLESPS-AKTEGAKRIVKML 211
AaXyl2  RTNIFYTTIGEAYIPIAFAAAAA-----ADPDAKLFYNDYNLEYGG-AKAASARAIVQLV 193
MtXyl3  RDNVFLRTFGTDYFALSFNLAKK-----ADPDTKLYYNDYNLEYNQ-AKTDRAVELVKMV 194
          .::   :*  :. ::*  *           .:*: ***.::     *       *.

TrXyn3  ISQGVPIDGIGSQSHLSGGG--GSGTLGALQQLATVPVTELAITELDIQG---------- 271
PcXylA  LAAGIPIDGIGSQTHLGAGA--GSAVAGALNALASAGTKEIAITELDIAG---------- 243
AnXlnC  LAEGVPIDGIGSQAHYSSSHWSSTEAAGALSSLANTGVSEVAITELDIAG---------- 243
PcXylE  RKRGIRIDGVGLESHFIVGETPSLADQLATKQAYIKANLDVAVTELDVRFSTVPYYTAAA 268
MtXyl4  KDAGIRIDGVGLQAHLVAESHPTLDEHIDAIKGFTELGVEVALTELDIRL-SIPANA-TN 269
AaXyl2  KNAGAKIDGVGLQAHFSVGTVPSTSSLVSVLQSFTALGVEVAYTEADVRI-LLPTTA-TT 251
MtXyl3  QAAGAPIDGVGFQGHLIVGSTPTRSQLATALQRFTALGLEVAYTELDIRHSSLPASS-SA 253
           *  ***:* : *                        ::* ** *:

                                                    ↓
TrXyn3  --APTTDYTQVVQACLSV-SKCVGITVWGISDKDSWRASTNP-----LLFDANFNPKPAY 323
PcXylA  --ASSTDYVNVVNACLNQ-AKCVGITVWGVADPDSWRSSSSP-----LLFDGNYNPKAAY 295
AnXlnC  --AASSDYLNLLNACLNE-QKCVGITVWGVSDKDSWRASDSP-----LLFDGNYQPKDAY 295
PcXylE  QKQQAEDYYVSVASCMNAGPRCIGVVVWDFDDAYSWVPSAFAGQGGACLFNNTLEAKPAY 328
MtXyl4  LAQQREAYKNVVGACVQV-RGCIGVEIWDFYDPFSWVPATFPGQGAPLLWFEDFSKHPAY 328
AaXyl2  LAQQSSDFQALVQSCVQT-TGCVGFTIWDWTDKYSWVPSTFSGYGAALPWDENLVKKPAY 310
MtXyl3  LATQGNDFANVVGSCLDT-AGCVGVTVWGFTDAHSWIPNTFPGQGDALIYDSNYNKKPAW 312
               :   : :*:.    *:*. :*.  *  **             :      : *:

TrXyn3  NSIVGILQ        331
PcXylA  NAIANAL         302
AnXlnC  NAIVNALS        303
PcXylE  YAVADALEGKPCSVC 343
MtXyl4  DGVVEALT...VWKA 356
AaXyl2  NGLLAGMG...LKKK 417
MtXyl3  TSISSVLA...WQCL 396
         .:   :
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Fig. 1. CLUSTAL OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/) alignment of amino acid sequences of GH10 family xylanases. TrXyn3, *T. reesei* xylanase III (GenBank accession XP_006962419); PcXylA, *P. canescens* xylanase A (AAV65488); AnXlnC, *A. nidulans* xylanase C (ABF50851); *P. canescens* xylanase E (ACP27611); MtXyl3 and MtXyl4, *M. thermophila* (*C. lucknowense*) xylanases 3 and 4 (AEN99941 and AEO58598); AaXyl2, *A. aculeatus* xylanase II (AAE62343). Amino acid insertions in xylanases resistant to cereal proteinaceous inhibitors are shown in bold. Numeration of residues is given for mature proteins without signal peptides.

3.2. Expression and purification of recombinant PcXylA and TrXyn3

Genes encoding the recombinant PcXylA and TrXyn3 of wild type and their engineered forms with the GQGGA insertion (PcXylA-ins and TrXyn3-ins) were cloned and expressed in *P. verruculosum* B1-537 and *P. canescens* RN3-11-7 host strains, respectively. It should be noted that initially the *P. verruculosum* B1-537 was used as a recipient strain for heterologous production of both xylanases, but since the expression of TrXyn3 was not very high in this host (data not shown), then we used the *P. canescens* RN3-11-7 as a recipient strain for production of TrXyn3 recombinant forms, the latter being more successful. The secreted into culture broths proteins were separated using anion-exchange and hydrophobic-interaction chromatography. SDS-PAGE data for purified recombinant xylanases are shown in Fig. 3. All the enzymes under study

Table 1
Sequences of oligonucleotides.

No.	Primer name	Structure of oligonucleotides from 5′- to 3′- end
1	TrXyn3-INS-fwd	**ggt-cag-ggt-ggt-gcc**-ctt-ctg-ttt-gac-gca-aac-ttc
2	TrXyn3-INS-rev	**ggc-acc-acc-ctg-acc-**agg-gtt-ggt-gct-ggc-acg-cca-cga
3	TrXyn3-LIS5	gca-cag-gca-gca-gga-gct-ctc-ccc-acc-gaa-acc-atc-cac-ct
4	TrXyn3-LIS3	ag-agc-aag-ccg-agc-agg-tta-ttg-taa-gat-gcc-aac-aat-gct
5	PcXylA-INS-fwd	**ggt-cag-ggt-ggt-gca**-ctg-ctg-ttt-gac-ggc-aac-tac-aac
6	PcXylA-INS-rev	**tgc-acc-acc-ctg-acc**-agg-gga-aga-gct-gga-gcg-cca-tga-atc
7	PcXylA-LIS5	caa-aca-gaa-gca-acc-gac-aca-atg-gtt-caa-ctc-aag-act-gct
8	PcXylA-LIS3	ga-gga-gaa-gcc-cgg-tta-aag-cgc-att-ggc-gat-ag

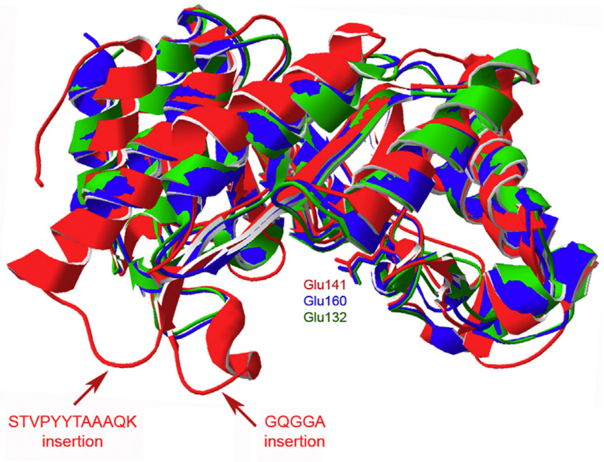

Fig. 2. Superimposed crystal structures of *P. simplicissimum* XylA (1BG4.PDB, shown in green, the enzyme is identical to PcXylA), TrXyn3 (4XV0.PDB, shown in blue) and PcXylE (4F8X.PDB, shown in red). The insertions in PcXylE amino acid sequence are shown with arrows. Glu catalytic residues, interacting with Lys234 of XIP-I according to Payan et al. (2004), are marked.

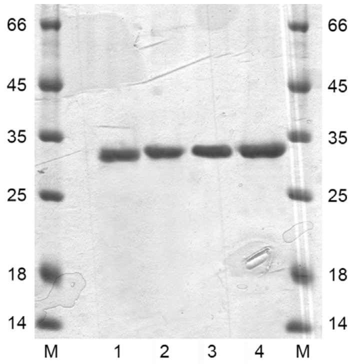

Fig. 3. SDS-PAGE of purified recombinant xylanases. *1*, PcXylA; *2*, PcXylA-ins; *3*, TrXyn3; *4*, TrXyn3-ins. Left and right lanes represent molecular markers shown in kDa.

were homogeneous according to the data of electrophoresis.

3.3. Enzyme identification

The enzyme identification was carried out by MALDI-TOF MS peptide fingerprinting. The results of MS data analysis using the MASCOT server are shown in Table 2. The recombinant PcXylA and TrXyn3 of wild type and their engineered forms were identified with a high score. In comparison with the wild-type enzymes, the PcXylA-ins and TrXyn3-ins were identified by MASCOT with a lower score and a lower degree of protein sequence coverage because of the insertions. However, in the latter two cases additional peptides carrying the GQGGA insertion were then identified, that is, additional peaks with m/z of 1996.0 and 3174.7 were found in the mass spectra of tryptic digests of PcXylA-ins and TrXyn3-ins (a theoretical m/z of the corresponding peptides predicted by the PeptideMass on-line tool is 1995.9 and 3174.6, respectively), and their analysis using the MS/MS fragmentation confirmed the presence of the GQGGA insertion. With those additional peptides, the sequence coverage for PcXylA-ins and TrXyn3-ins significantly increased (Table 2).

Table 2
Identification of recombinant xylanases by MALDI-TOF MS peptide mass fingerprinting using the MASCOT search (http://www.matrixscience.com/) in the NCBIprot database.

Enzyme	GenBank accession number	MASCOT score	Mass values matched	Protein sequence coverage (%)
PcXylA	**AAV65488**	154	19	72
PcXylA-ins	**AAV65488**	114	16 (17)[a]	55 (61)[a]
TrXyn3	**XP_006962419**	147	17	53
TrXyn3-ins	**XP_006962419**	118	16 (17)[a]	46 (55)[a]

[a] Data taking into account additional peptides with the GQGGA insertion, found using the PeptideMass tool (https://web.expasy.org/peptide_mass/), are given in parentheses.

3.4. Enzyme pH-optima, specific activities and thermostability

The GQGGA insertion did not affect the pH-optima of PcXylA and TrXyn3 that were observed at pH 5.0–6.0 and pH 5.5–6.5, respectively (data not shown). However, the insertion led to a notable decrease in specific activity of engineered xylanases against glucuronoxylan from birch wood, while the activity toward arabinoxylan from wheat was less affected in comparison with that for the wild-type enzymes (Table 3). The engineered forms of both xylanases also displayed lower thermostability; the latter parameter was characterized by the enzyme melting temperature (T_m) determined by differential scanning microcalorimetry and by the enzyme half-life times ($t_{1/2}$) during incubation at 55 and 60 °C (Table 3).

3.5. Effect of xylanases on the viscosity of rye extracts

In order to evaluate the influence of the 5 aa insertion in PcXylA and TrXyn3 on susceptibility of the enzymes to the cereal proteinaceous inhibitors, we used a previously developed method (Gusakov and Ustinov, 2009) based on measuring the effect of a xylanase on viscosity of the native extract of rye flour and that subjected to boiling in order to inactivate its proteins. During preparation of the native extract, the proteinaceous inhibitors and viscous xylans from the rye flour are dissolved in water. As a result of the heat treatment, the inhibitors are subjected to thermal denaturation, thus losing their power. According to this method, only xylanases resistant to the proteinaceous inhibitors may effectively reduce the viscosity of the native rye extract because of the enzyme action on xylans, while all xylanases by definition reduce the viscosity of the boiled extract containing the inactivated inhibitors.

In our previous studies (Gusakov and Ustinov, 2009; Denisenko et al., 2015), we used an Ostwald viscometer to measure the changes in

Table 3
Specific activity and thermostability of recombinant xylanases.

Enzyme	Activity toward glucuronoxylan (U/mg)	Activity toward arabinoxylan (U/mg)	T_m (°C)	$t_{1/2}$, 55 °C (min)	$t_{1/2}$, 60 °C (min)
PcXylA	29.2 ± 0.9	28.6 ± 1.0	56.1	14	2.2
PcXylA-ins	22.8 ± 1.0	27.1 ± 0.6	54.1	5	1.0
TrXyn3	60.1 ± 2.1	58.8 ± 1.5	61.4	62	28
TrXyn3-ins	43.9 ± 1.6	54.4 ± 1.9	59.8	17	4.1

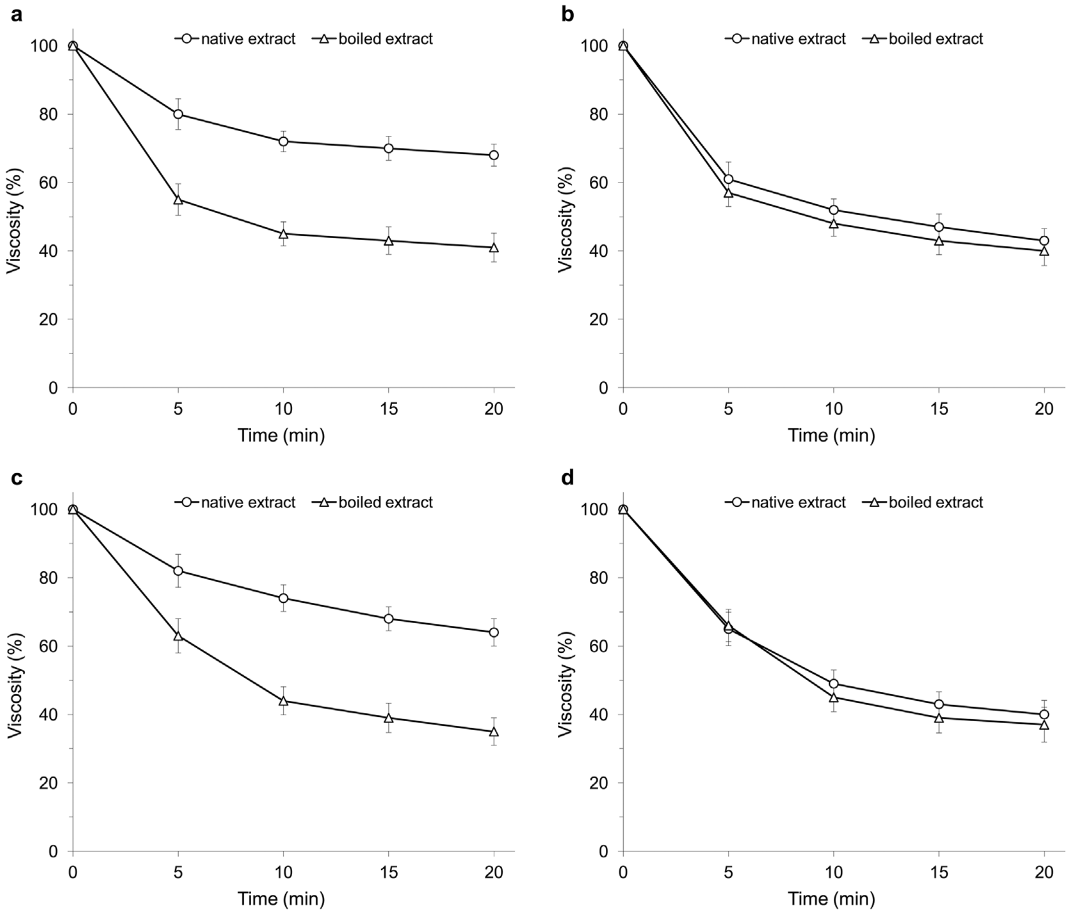

Fig. 4. Changes in viscosity of the native and boiled rye extracts (as % of the initial value) under the action of PcXylA (**a**), PcXylA-ins (**b**), TrXyn3 (**c**) and TrXyn3-ins (**d**) at pH 5.0, 40 °C and xylanase activity loading of 5 U/mL.

viscosity of rye extracts. In this work we modified the method by using an A&D Vibro Viscometer SV-10 for this purpose. Fig. 4 shows changes in viscosity of the native and boiled rye extracts under the action of all recombinant xylanases under study. If the wild-type PcXylA and TrXyn3 could reduce the viscosity of the native rye extract only to a limited extent (not exceeding 30% during 20-min measurements of the enzymatic reaction) while acting much more effectively on xylans in the boiled extract, both recombinant xylanases carrying the 5 aa insertion reduced the viscosity of the native and boiled extracts equally well (by 57–63% after 20 min of the reaction). Thus, the elongation of the peptide loop adjacent to the enzyme active site led to alleviation of the inhibition of xylanases under study by XIP-type proteins from rye.

4. Discussion

Although the inhibition of GH10 and GH11 family xylanases by TAXI-type and XIP-type proteinaceous inhibitors from cereals has been intensively studied during the first decade of the 21st century, the number of publications based on protein engineering approach is quite limited. Practically all of those papers have been focused on site-directed mutagenesis of GH11 xylanases in order to find out factors affecting their inhibition by XIP-type (Tahir et al., 2002, 2004; Beliën et al., 2007a, 2007b; André-Leroux et al., 2008; Tison et al., 2009) and TAXI-type proteins (Tahir et al., 2004; Fierens et al., 2005; Sørensen and Sibbensen, 2006; Bourgois et al., 2007; Rasmussen et al., 2010). However, knowledge obtained on how structural features of the GH11 xylanases influence their sensitivity to XIP-type inhibitors cannot be applied to the GH10 enzymes, since the XIP has two independent sites for interaction with GH10 and GH11 xylanases located on the opposite sites of the inhibitor molecule (Payan et al., 2004). According to the hypothesis of Payan et al. (2004), two amino acid insertions in the primary structure of bacterial GH10 xylanases, also present in a fungal *A. aculeatus* Xyl2, are responsible for their resistance to XIP-type proteins. A few other exceptions of the fungal xylanases, resistant to the cereal inhibitors, confirm this hypothesis, since in all of them similar two insertions (shown in bold) take place (Fig. 1). Our previous theoretical analysis (Gusakov and Ustinov, 2009; Denisenko et al., 2015) anticipated that the second, 5 aa insertion, is more likely responsible for the lack of the inhibition of these xylanases by XIP than the first, longer insertion. However, no experimental evidence to support this assumption has been demonstrated so far.

In this paper, by introducing the GQGGA insertion, that is intrinsically present in the XIP-resistant PcXylE, into the equivalent positions of two recombinant fungal GH10 xylanases (PcXylA and TrXyn3), we showed that this insertion alone is sufficient for gaining the resistance of both xylanases against proteinaceous XIP-type inhibitors from rye. As a result of the 5 aa insertion, the elongation of a respective peptide loop forming the enzyme active site cleft took place (Fig. 2), and it caused a steric hindrance for binding the XIP-type inhibitor to PcXylA-ins and TrXyn3-ins. At the same time, this insertion led to 22–27% decrease in specific activity of both xylanases under study against glucuronoxylan as well as to some decrease in their thermostability (Table 3). So, a tradeoff between this type of activity of xylanases and their susceptibility to cereal inhibitors was observed. At the same time, activities of the engineered xylanases against arabinoxylan were very close to those for the wild-type enzymes (the difference did not exceed 5–7%). This is particularly important for cereal-based xylanase applications since arabinoxylans are the component of cereals while glucuronoxylans are typical for hardwoods.

Still the role of the first 10–12 aa insertion in xylanases resistant to XIP-type inhibitors (Fig. 1), also present in many bacterial xylanases (Payan et al., 2004), is unclear. We observed a moderate decrease in thermostability for PcXylA-ins and TrXyn3-ins as a result of the 5 aa insertion. The respective elongated loop (Fig. 2), not structured into α-helix or β-sheet, seems to introduce some disordering of the protein structure resulting in its destabilization. Perhaps, a longer peptide loop containing the first (10–12 aa) insertion and located close to the loop with the 5 aa insertion (Fig. 2) helps to stabilize the enzyme. However, additional studies are needed to find out how the structural elements of GH10 xylanases influence the enzyme properties.

Declarations of interest

None.

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