KVASNÝ PRŮMYSL

Kvasny prumysl (2019) 65: 127–133
https://doi.org/10.18832/kp2019.65.127

Microbial xylanases and their inhibition by specific proteins in cereals

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Abstract

Arabinoxylans (AXs) belong to the components of plant cells which are mainly degraded by microbial xylanases during colonization of grain by phytopathogens. For the defence, cereals contain proteinaceous xylanase inhibitors (XIs), namely xylanase inhibitor protein (XIP), *Triticum aestivum* xylanase inhibitor (TAXI) and thaumatin-like xylanase inhibitor (TLXI). Their presence in cereals in high levels can be a serious problem in different industrial applications. XIs regulate AX hydrolysis and participate in plant defence mechanisms. XIs have various specificity against microbial xylanases from the glycoside hydrolase (GH) families of GH10 and GH11. Therefore, this review brings new information about the function of XIs as defence responses to pathogen infection of plants and as a problem in plant material processing in different industrial applications.

Key words: xylanase inhibitor, cereals, plant defence, xylanases.

1 Introduction

Arabinoxylans (AXs) and glucans are non-starch polysaccharides present in cereal grains and these cell wall components were also confirmed in barley (Egi et al., 2004). AXs are mainly presented in the cell wall of aleurone tissues, half of AXs in barley grains is located in the husk and a significant content of AXs was found in the endosperm walls (60–70%) (Saulnier et al., 2007). They are hydrolysed by xylanases, namely endo-(1-4)-β-xylanases (EC 3.2.1.8), β-D-xylosidases (EC 3.2.1.37), and α-L-arabinofuranosidases (Goesaert et al., 2001). The majority of xylanases degrading AXs are produced by microbial contaminates of barley grains. For the regulation of their activity, cereals produce their own xylanases in the form of the xylanase precursor in the aleurone cells (Caspers et al., 2001; Simpson et al., 2003; Selvaraj et al., 2010) and xylanase inhibitors (XIs) controlling the AX hydrolysis and the utilization of starchy resources (Caspers et al., 2001; Evers and Millar, 2002). These processes play the key role in the natural defence to disease (Payan et al., 2004; Dornez et al., 2010; Zhan et al., 2017a; Sun et al., 2018).

More than 90% of total xylanase activity in cereals has a microbial origin (Dornez et al., 2008). Microbial xylanases are included in the glycoside hydrolase (GH) family, specifically GH10 and GH11 families (Simpson et al., 2003). Generally, the GH10 xylanases have higher molecular weights (>30 kDa) than xylanases of the GH11 family (~20 kDa). Microbial xylanases are produced by bacteria and fungi (*Fusarium graminearum, Botrytis cinerea, Magnaporthe grisea and Aspergillus fumigatus*) and ensure the release of nutrients from the outer kernel layers of barley grains. Some of these organisms such *Aspergillus* sp. and *Trichoderma* sp. have been used for industrial production of xylanases resulting of the improval of end-product quality (Wu et al., 1997; Brutus et al., 2005; Paper et al., 2007). Generally, microbial xylanase activity is significantly affected by proteinaceous XIs. These proteins have various specificities and were detected in seed endosperm and bran (Rouau and Surget, 1998). XIs are specified for xylanases produced by microorganisms and the inhibition of endogenous plant xylanases was not observed (Hou et al., 2013).

Research Institute of Brewing and Malting, Plc.
Published online: 15 August 2019

Levels of XIs can be a serious problem in different industrial applications in which the addition of exogenous xylanases affects the final quality of products (Dornez et al., 2011; Smeets et al., 2014). The high content of XIs in certain cereals reduces the xylanase efficiency in bread making, and a higher enzyme dosage should be used (Dornez et al., 2011). The supplementation of xylanases in broiler feeds is commonly used to improve feed digestion. Krogh Madsen et al. (2018) observed that broilers fed with wheat containing the highest xylanase inhibitory activity resulted in the lowest growth rates. Inhibitory activity of these proteins was not affected during the digestion process, and feed pelleting at 85 °C had no effect on their activity (Smeets et al., 2014). Similarly, xylanase inhibition makes a problem in the processing of cereals including malting and brewing (Dornez et al., 2009), gluten-strach separation (Frederix et al., 2004) and refrigerated dough (Simsek and Ohm, 2009).

Therefore, the aim of this study was to describe the function of XIs against xylanases of the GH10 and GH11 families and defence responses to pathogen infection of plants.

2 Xylanase inhibitors

XIs are involved in the development and germination of the cereal grains (Gebruers et al., 2004; Croes et al., 2009a). The well-known inhibitors of microbial xylanases are xylanase inhibitor protein (XIP), *Triticum aestivum* xylanase inhibitor (TAXI) and thaumatin-like xylanase inhibitor (TLXI) (Debyser et al., 1997; McLauchlan et al., 1999; Fierens et al., 2007). Lin et al. (2013) described the fourth type of xylanase inhibitor, XILP (xylanase inhibitor-like protein), homologous to XIs from wheat and rice, but the molecular weight of XILP is lower than that of wheat and rice inhibitors. XILP inhibits fungal xylanases and exhibits antifungal activities, inhibits proliferation of various cancer cell lines and reduces HIV-1 reverse transcriptase.

The content of XIP, TAXI and TLXI in cereals varies. The content of XIP varied from 0.12 to 0.6 mg/g plant material, TAXI content ranges from 0.05 to 0.3 mg/g plant material (Bonnin et al., 2005; Dornez et al., 2006; Croes et al., 2009a) and the content of TLXI varied from 0.51 to 1.50 (Croes et al., 2009a). TAXI and XIP proteins are mainly located in the outer layers of grains (Mendis et al., 2013). TLXI was almost exclusively found in seeds of cereals while the presence of XIP and TAXI was also observed in roots and shoots of the growing seedlings. Moreover, the highest XI levels were observed at later stages of maturation. This finding suggests that these proteins can play a role in the growth and development of plants. A significant loss of inhibitor signals was observed in fourth day of germination (Croes et al., 2009b). Also, XIs exhibit different effectiveness against bacterial and fungal xylanases and also differ from each other by the mechanism of enzyme inhibition (Gebruers et al., 2001; Flatman et al., 2002; Dornez et al., 2010; Gusakov, 2010).

2.1 *Xylanase inhibitor protein (XIP)*

XIP is an extracellular protein isolated for the first time from wheat (*Triticum aestivum* var. Soisson) flour (McLauchlan et al., 1999) and belongs to the best-characterized XIs. XIPs are usually glycosylated monomeric basic proteins with the average molecular weight of 30 kDa and with pI values in the range from 7.0 to 9.0 (McLauchlan et al., 1999; Elliot et al., 2002; Juge et al., 2004). This plant-derived protein has been shown to inhibit fungal endo-β-(1,4)-xylanases belonging to the GH10 and GH11 families (Flatman et al., 2002). Also, it has been reported that XIP does not inhibit both bacterial and endogenous plant xylanases (Juge et al., 2004). XIP from wheat has biochemical properties similar to XIP-type inhibitors from other cereals.

XIP exists in different isoforms; Gebruers et al. (2002) identified the presence of five XIP-I isoforms. The presence of various XIP variants can be affected by a modification during the storage of cereals, the purification of these proteins or can be the results of post-translational modifications (glycosylation), or is the product of multiple *xip* genes (McLauchlan et al., 1999; Flatman et al., 2002). Homologous fragments to *xip-I* gene were found in barley, rye and corn while homologous fragments to *xip-II* and *xip-III* genes were only shown in rice and sorghum (Goesaert et al., 2004). Four XIP-type inhibitors have been reported in rice, namely rice XIP, RIXI, OsXIP and OsHI-XIP (Zhan et al., 2017a). Twenty homologous *xip* genes called *xip-R* gene family were identified in the roots of hexaploid wheat (Takahashi-Ando et al., 2007). XIP-Is were identified in four plant species, including sorghum, rice, maize and *Brachypodium* (Sonah et al., 2016). These authors also found that the *xip* genes generally have few or no introns, and about 75% of total *xip-I* genes identified in cereals are without introns.

XIP-I is encoded by a *xip-I* gene and is related to chitinases of the family GH18 (Sonah et al., 2016). XIP-I shows no chitinase activity. Juge et al. (2004) suggested that insufficient chitinase activity can be caused by the XIP-I binding cleft. *Xip-I* gene encodes 274 amino acids. The signal peptide forms 30 of these amino acids (Elliot et al., 2002). XIP-I molecule consists of $(\beta/\alpha)_8$-barrel fold (Terwisscha van Scheltinga et al., 1996). This inhibitor contains two conventional regions and two non-proline cis-peptide bonds, namely Ser-36-Phe and Trp-256-Asp (Terwisscha van Scheltinga et al., 1996; Drouillard et al.,

1997). Using the 3D structure of XIP-I in complexes with *Aspergillus nidulans* xylanase (GH10) and *Penicillium funiculosum* xylanase (GH11), XIP-I have been shown to have two independent binding sites. One of them specifically interacts with xylanases of the GH10 family and another with xylanases of the GH11 family. The insertion of an XIP-I Π-shaped loop into the enzyme active site causes the inhibition of GH11 xylanases whereas the residues in the helix α7 of XIP-I mediate the reversible inhibition of GH10 xylanase (Payan et al., 2004).

The specificity of XIPs was determined against various bacterial and fungal xylanases from the GH10 and GH11 families (Flatman et al., 2002; Furniss et al., 2002; Chang et al., 2016). Structural analyses of XIP-I suggest that this protein is able to interact with GH10 and GH11 xylanases of bacterial and fungal origins (Payan et al., 2004). Indeed, all tested fungal xylanases were inhibited by XIP-I, except for xylanase from *Aspergillus aculeatus* belonging to the GH10 family. XIP-I inhibition was competitive for all tested xylanases, indicating the protein binding near or directly at the active site (Flatman et al., 2002; Furniss et al., 2002). XIP-I did not inhibit bacterial xylanases (Flatman et al., 2002). XIP-I recognizes the difference between bacterial and fungal xylanases belonging to the same GH family. This specificity is a unique property of XIP (Gebruers et al., 2001). XIP-I is also able to inhibit certain α-amylases from the GH13 family and this dual inhibitory function of XIP-I can be potentially important for biotechnological applications (Sancho et al., 2003; Juge et al., 2004). Also, XIP-II shows inhibition against the fungal GH11 xylanases (Elliot et al., 2009).

In addition, the preparation of recombinant XIP was also investigated (Elliot et al., 2002; Takahashi-Ando et al., 2007; Liu et al., 2017). XIP-I protein was expressed in *Escherichia coli* and was then able to inhibit endo-1,4-β-D-xylanase activity. The recombinant protein rapidly lost its biological activity. This could be caused by insufficient or incomplete glycosylation (Elliot et al., 2002). Also, recombinant XIP-R protein isolated from hexaploid wheat was expressed in *E. coli* without the loss of biological activity (Takahashi-Ando et al., 2007). XIP identified in rice (RIXI, rice xylanase inhibitor) was expressed in *E. coli* (reERIXI) and this protein inhibited fungal GH11 xylanases (Huo et al., 2018). Surprisingly, recombinant RIXI inhibited both fungal and bacterial xylanases (Hou et al., 2013; Huo et al., 2018). Liu et al. (2017) observed that recombinant XIP inhibited xylanase from the rumen bacterium *Paenibacillus* sp. This xylanase has five highly conserved amino acids which can be the key residues in the interaction of XIP with xylanase (Payan et al., 2004; Liu et al., 2017). The inhibition of bacterial xylanases by XIPs was only observed within recombinantly prepared XIP, no native XIPs could inhibit bacterial xylanases.

Two XIP proteins were also expressed by *Pichia pastoris*. Although overglycosylation of XIP recombinant proteins was observed, inhibitory activity was not affected and both recombinant XIPs inhibited xylanases of the GH10 and GH11 families (Liu et al., 2017). *P. pastoris* and *E. coli* were also used by Hou et al. (2013) for recombinant RIXI protein production. The level of xylanase inhibitory activities and molecular weight of recombinant RIXIs were similar but *E. coli* is more suitable for recombinant production due to the production rate and costs.

The presence of XIPs in plants could be caused by their evolution as part of plant pathogen protection, predominantly against fungal pathogens (Zhan et al., 2017a). The expression of XIPs was also associated with stress-induced factors, namely herbivore infestation, mechanical wounding or the presence of methyl jasmonate, and caused the accumulation of XIPs in roots and partially in shoots. It has been assumed that XIP production is involved in plant defence responses via methyl jasmonate signal pathway (Zhan et al., 2017a; Zhan et al., 2017b). Payan et al. (2004), Takahashi-Ando et al. (2007) and Sun et al. (2018) similarly reported that XIPs are involved in plant defence against a pathogen and are probably overexpressed in plant as the response to pathogen attack. The production of XIP-I can prevent cell death and could limit *F. graminearum* infection via the inhibition of xylanase activity (Tundo et al., 2015). *Osxip* expression in transgenic plants was mainly observed in the shoot and root tissues after the exposure of two-week-old seedlings of rice to *Nilaparvata lugens* (Sun et al., 2018). Some researchers suggest that transgenic plants with over-expression of XIP may be more resistant to pathogens than wild-type plants (Hou et al., 2014; Tundo et al., 2015; Zhan et al., 2017a).

2.2 Triticum aestivum *xylanase inhibitor (TAXI)*

TAXI protein inhibitors were discovered and described in 1997 (Debyser et al., 1997). TAXI is a 40 kDa polypeptide (Gebruers et al., 2001). TAXI consists from two different proteins, namely TAXI-I and TAXI-II with 86% sequence identity. These types of TAXI are differ in N-terminal amino acid end and their inhibitory specificity (Gebruers et al., 2001). TAXI I preferably inhibits xylanases with high and low values of pI and TAXI-II inhibits only xylanases with high pI values (Goesaert et al., 2003). TAXI-I and TAXI-II have pI values of 8.8 and 9.3, respectively (Debyser et al., 1999).

All isoforms of TAXI-I inhibit xylanases from *Aspergillus niger* and *Bacillus subtilis* belong to the family of GH11 but TAXI-II only inhibits bacterial xylanases from the

same GH family. These forms are the products of similar genes or the results of post-translational modification. Isoforms of inhibitors encoded by different genes were called TAXI-IA, TAXI-IB, TAXI-IIA and TAXI-IIB. Nucleotide sequence of TAXI-I does not contain introns, and G/C nucleotides form 68% of total nucleotides (Fierens et al., 2003). Sansen et al. (2004) found that TAXI-I is composed of 6 disulfide bridges. The similarity of TAXI-II with other known proteins is negligible to the carbohydrate-derived glycoproteins (Gebruers et al., 2001).

The specific *taxi*-III and *taxi*-IV genes were identified in wheat (Igawa et al., 2004), but Raedschelders et al. (2004) considered that the TAXI-III and TAXI-IV proteins encoded by these genes are TAXI-I and TAXI-II isoforms because they have 99.6 and 99.8% similarity to TAXI-IB and TAXI-IIB, respectively. Protein inhibitors similar to the structure and properties of TAXI-I and TAXI-II have also been found in other cereals, namely rye, durum wheat and barley. The properties are very similar and typical for TAXI-like proteins, with molecular weight of 40 kDa and alkaline pI value (> 8.5) (Goesaert et al., 2001; Goesaert et al., 2002; Goesaert et al. 2003).

3D structure of TAXI is similar to pepsine-like aspartate peptidases (Sansen et al., 2004; Dornez et al., 2010) but has no proteolytic activity. TAXI protein consists of two β-barrel domains with several helical segments divided by an extended cleft (Dornez et al., 2010). TAXI inhibitor binds at the active site of xylanase, resulting in competitive inhibition of the enzyme (Tundo et al., 2015). TAXI-I was able to inhibit recombinant xylanase from *Penicillium funiculosum* (GH11) although it did not inhibit recombinant xylanase from *P. occitanis* (GH11). The inability of the inhibition of GH11 xylanase can be explained by the lack of twenty amino acids in the thumb of recombinant xylanase resulting in steric clashes (Driss et al., 2013).

Recombinant TAXI inhibitor was successfully produced by *E. coli* and *P. pastoris* (Fierens et al., 2004). Similar biochemical properties were measured for rTAXI-I expressed in both *E. coli* and *P. pastoris* and natural TAXI-I, although the pI value of rTAXI-I was higher (9.3) than the pH value of natural TAXI-I (8.8). Fierens et al. (2004) confirmed that rTAXI and natural TAXI can inhibit bacterial and fungal GH11 xylanases while GH10 xylanases were not inhibited. rTAXI expressed in *P. pastoris* was glycosylated but this had no effect on xylanase inhibitory activity.

The presence of TAXI inhibitors is significantly affected under stress conditions, specifically by pathogen infection (Gebruers et al., 2002; Moscetti et al., 2013). As mentioned above, it seems that XIs play a key role in plant defence (Elliot et al., 2009; Bellincampi et al., 2014). Moscetti et al. (2013) proved that wheat transgenic plants with over-expression of TAXI-III limited *F. graminearum* infection. Igawa et al. (2004) observed over-expression of TAXI-IB/III and TAXI-IIB/IV in wheat leaves after the infection by the fungus *Blumeria graminis*. Tundo et al. (2015) confirmed the dual function of XIs, namely TAXI-III and XIP. These proteins competitively inhibited *F. graminearum* xylanase activity (GH11) and also prevented host cell death activities caused by xylanases. Based on these data, TAXI is a potential representative of a new class of plant proteins whose function is to protect plants from the xylanase effects (Sun et al., 2018).

2.3 TLXI

TLXI and TLXI-like proteins were identified in wheat, rye and durum wheat (Fierens et al., 2007; Dornez et al., 2010). The inhibitor is a small variously glycosylated protein with molecular weight of 18–21 kDa and alkaline pI (≥9.3). The inhibitor is named as tautine-like proteins due to its highly homologous amino acid sequence (more than 60%) with these proteins (Fierens et al., 2007; Rombouts et al., 2009). Although XIP and TAXI belong to competitive XIs, TLXI is a non-competitive inhibitor of the GH11 family xylanases. The inhibitory effect of TLXI on xylanases of the GH10 family was not described (Fierens et al., 2007).

The 3D structure of TLXI contains two domains. The first domain is a β-sandwich built up of β strands and the second domain consists of β-hairpin turn followed by an extended loop with His-22. This residue, key for inhibitory activity, interacts with the negatively charged residue located at the thumbs or on the fingers of the GH11 xylanases (Dornez et al., 2010). Vandermarliere et al. (2009) suggested that the whole loop region might be involved in inhibitory activity of TLXI. The gene sequence of *tlxi* consists of 177 amino acids and no introns. The signal sequence encodes 26 amino acids followed by 151-amino acid mature protein. TLXI have one glycosylation site at Asn-95 in which glycans of different compositions were detected. TLXI contains ten cysteine residues involving in five intramolecular disulfide bridges. Due to this conformation, TLXI xylanase inhibitor is more stable at extreme pH and temperature (Fierens et al., 2007).

Inhibition of the TLXI inhibitor has been described in a number of different xylanases. TLXI strongly inhibits xylanase isolated from *Trichoderma longibrachiatum*, similar to xylanases produced by the filamentous fungi *A. niger, Trichoderma viride* and *P. funiculosum* or bacteria *Thermobacillus xylanilyticus* and *B. subtilis*. TLXI preferentially inhibits acidic xylanases (Fierens et al., 2007). The inhibition rate of TLXI xylanases depends on temperature and pH, maximum inhibitory activity was observed at pH 5.0 and 40 °C (Fierens et al., 2009).

The recombinant TLXI expressed in *P. pastoris* resulting in a 21 kDa protein retains biological activity but its inhibitory activity was lower than that of the native protein (Fierens et al., 2007). Although this protein has been discovered in 2007 (Fierens et al., 2007), little information exists about its specificity as well as its involvement in plant defence to stress conditions.

3 Conclusion

Insufficient quality of cereal grains has a negative impact on consumer health. It seems that XIs can be a suitable tool for cereal grain defence to pathogenesis and accumulation of mycotoxins. XIP and TAXI type of XIs belong to potential candidates to prevent infection due to the inhibition of bacterial and fungal xylanases from the GH10 and GH11 families. The response of plants to stress factors or the tools of recombinant techniques of DNA can be used for increasing of XI production. This can lead to improved plant defence to pathogens. Plant-derived xylanases can be an appropriate alternative to currently used microbial xylanases in cereal processing because no inhibitory effect of XIs on their activities was observed.

4 Acknowledgment

This work was supported by the grants of APPV-16-0088 and APVV-14-0393.

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