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REVIEW

Proteinaceous Inhibitors of Microbial Xylanases

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Abstract—At the end of 1990s two structurally different proteinaceous inhibitors of xylanases were discovered in the grain of wheat (*Triticum aestivum*). They were named TAXI (*T. aestivum* xylanase inhibitor) and XIP (xylanase-inhibiting protein). Later it was shown that TAXI and XIP in wheat are present in several isoforms encoded by different genes. TAXI- and XIP-like inhibitors have also been found in other cereals—barley, rye, rice, maize, etc. All these proteins can specifically inhibit activity of fungal and bacterial xylanases belonging to families 10 and 11 of glycoside hydrolases, but they do not affect endogenous enzymes produced by plants. A common viewpoint is that the presence of proteinaceous inhibitors in cereals is a response of plants to pathogenic attack by microorganisms. A few years ago, an inhibitor of a third type was discovered in wheat. It was named TLXI (thaumatin-like xylanase inhibitor) because of its similarity to the thaumatin family of plant proteins. In this review, the occurrence of proteinaceous inhibitors of xylanases in different cereals, their specificity towards fungal and bacterial enzymes, as well as structural features responsible for enzyme sensitivity to various types of inhibitors are discussed.

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Xylanases (endo-1,4-β-xylanases, EC 3.2.1.8) are enzymes hydrolyzing β-1,4-xyloside bonds in xylans (hemicelluloses), which are among the main components of plant biomass [1, 2]. Xylanases are produced by various organisms: bacteria, fungi, plants, algae, insects, mollusks, worms, etc. [2, 3]. Microbial (bacterial and fungal) xylanases are the most studied ones; they participate in biological degradation of plant biomass and thus play an important role in the organic carbon cycle. Most microbial xylanases known by now belong to families 10 and 11 of glycoside hydrolases according to Henrissat [4]; his classification is commonly accepted for enzymes cleaving polysaccharides and other glycosides. Some bacterial xylanases and also xylanases from other organisms are classified in families 5, 8, and 43 of glycoside hydrolases (http://www.cazy.org/). Within one family, enzymes are similar in amino acid sequences and also have the same type of 3D folding of the polypeptide chain and similar structure of the active site. Xylanases of family 10 have 3D folding in $(\beta/\alpha)_8$-barrel and their molecular mass is usually ~30 kDa, whereas xylanases of family 11 are folded as β-jelly roll and their molecular mass is ~20 kDa, and high p*I* value is often typical of them [1-3].

In the last 20-30 years microbial xylanases have been industrially produced by various biotechnology companies because these enzymes have many applications. Xylanases are used for bio-bleaching of cellulose in the pulp and paper industry, added to feeds for agricultural animals and birds, used in baking and brewing and other areas of the foodstuff industry, where treatment of flour or grain of xylanase-rich cereals is required [1, 3-5]. The presence of xylanase inhibitors in wheat was first found at the end of the 1990s [6]. These inhibitors appeared to be proteins. Proteinaceous inhibitors of xylanases were then found also in other cereals—barley, rye, maize, etc. [7-9], and this initiated fundamental studies in this area.

It was shown that wheat (*Triticum aestivum*) contains two structurally different types of inhibitors, one of them named TAXI (*T. aestivum* xylanase inhibitor) [10] and another XIP (xylanase-inhibiting protein) [11]. It soon appeared that TAXI is not an individual protein but two proteins with different specificity of inhibitory action on xylanases; they were named TAXI-I and TAXI-II [12]. Later it was found that XIP in wheat is also represented by homologous proteins encoded by different genes, although XIP-I is mainly expressed [13, 14]. Recently, an inhibitor of a third type was found in wheat. It was named TLXI (thaumatin-like xylanase inhibitor) because of its similarity to the thaumatin family of plant proteins [15]. Proteinaceous inhibitors of xylanases found in other cere-

als are similar in structure and properties to XIP and TAXI from wheat. Thus they are often called XIP-like and TAXI-like proteins.

The data indicate that proteinaceous inhibitors from cereals can specifically effect microbial xylanases belonging to glycoside hydrolase families 10 and 11, but have no effect on endogenous xylanases produced by plants and also xylanases of other families [9, 16-19]. That is why it is a common viewpoint that the presence of proteinaceous inhibitors in cereals is a defensive response of plants to pathogenic attack by mold fungi and bacteria. Consistent with this viewpoint is the fact that proteinaceous inhibitors of other carbohydrases (α-amylases, pectinases, xyloglucanases, and so on) able to cleave polysaccharides of plant cell wall have been found in various plants [18, 20-23].

Microbial xylanases susceptible to the action of proteinaceous inhibitors differ in specificity and sensitivity to XIP, TAXI, and TLXI. These features depend on the glycoside hydrolase family to which any certain xylanase belongs and whether the xylanase is fungal or bacterial. Some microbial xylanases of families 10 and 11 are insensitive to proteinaceous inhibitors, but they are exceptions rather than the rule. From the elucidation of structure of proteinaceous inhibitors of cereals, the structure of the active sites of various xylanases, and the use of site-directed mutagenesis, the mechanisms of action of inhibitors of various types on xylanases of families 10 and 11 have recently been elucidated on the molecular level. Along with the fundamental knowledge, this is of practical importance because it opens a way to produce mutant enzymes insensitive to the action of proteinaceous inhibitors. Such mutant enzymes might be used in various biotechnological processes where enzymatic treatment of grain of wheat or other xylan-containing cereals is required.

In this review the occurrence of proteinaceous inhibitors of xylanases, their specificity towards fungal and bacterial enzymes belonging to glycoside hydrolase families 10 and 11, and structural features responsible for sensitivity of xylanases to various types of inhibitors are considered.

THE PRESENCE OF PROTEINACEOUS INHIBITORS OF XYLANASES IN CEREALS AND THEIR CLASSIFICATION AND MOLECULAR CHARACTERICTICS

Proteinaceous inhibitors of xylanases were discovered in wheat while studying the properties of beer wort composed of barley malt (60%) and wheat extract (40%) [6]. It was found that xylanase activity typical of malt decreased after addition of wheat extract. Inhibitors contained in the extract in turn lost their activity after high-temperature treatment of the wheat extract, this indicating their proteinaceous nature. Similar data were obtained studying the effect of xylanase (pentosanase) from *Aspergillus niger* on arabinoxylan isolated from wheat flour. It was shown that inhibitors are present in seed endosperm (the main part) as well as in bran [24].

TAXI-type inhibitors. The first discovered proteinaceous inhibitor of xylanases (TAXI) isolated from wheat and purified had molecular mass ~40 kDa and consisted of two molecular forms (A and B), the second being a product of limited proteolysis of form A [10]. After reduction by 2-mercaptoethanol, form B yielded two fragments with molecular masses ~10 and 30 kDa, whereas the molecular mass of form A remained unchanged. Later it was found that TAXI is not an individual inhibitor but consists of two different proteins, TAXI-I and TAXI-II. They have similar N-terminal amino acid sequences but different p*I* values (8.8 and 9.3, respectively) [12]. As shown by SDS-PAGE, after reduction by 2-mercaptoethanol each of these proteins partly breaks into fragments with molecular masses ~10 and 30 kDa as described above. The N-terminal amino acid sequences of TAXI-I and TAXI-II fragments with mass ~10 kDa were as follows: GAPVARAVEAVAPFGVXYDT and GAPVARAVIPVAPFELXYXTKSLGN. The N-terminal amino acid sequences of polypeptides with molecular masses 30 and 40 kDa were the same both for TAXI-I and TAXI-II (LPVLAPVTKDPATSLYTIPFXDXA and KGLPVLAPVTKDTATSLYTIPF, respectively). Based on these data, a molecular model of TAXI-I and TAXI-II was suggested (Fig. 1). Form A consists of one polypep-

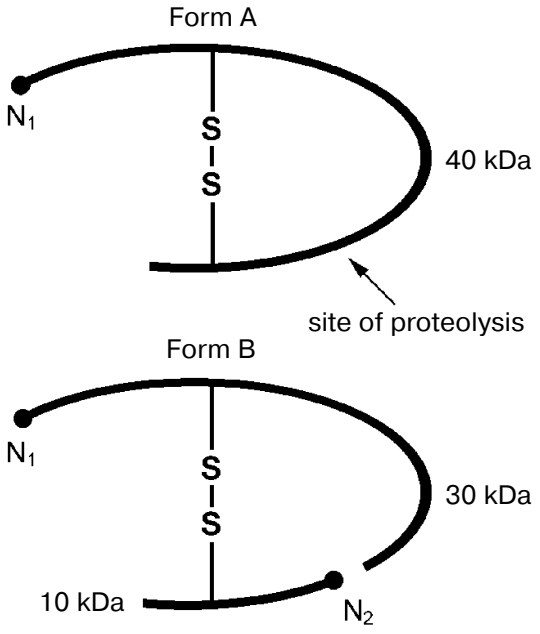

Fig. 1. Schematic structure of TAXI-I and TAXI-II molecules [10, 12, 17]. N_1 and N_2 are the N-termini of the polypeptide chains.

tide chain, and form B consists of two chains, and the latter does not dissociate under ambient conditions due to the disulfide bond between the two chains.

As a result of purification of TAXI-I and TAXI-II in the presence of protease inhibitors, it was shown that form B is not formed due to proteolytic activity during extraction and isolation of proteins but is initially present in wheat along with form A.

Using affinity chromatography on a column with xylanase from *Bacillus subtilis* immobilized on Sepharose, seven forms of inhibitors were isolated from wheat; six of them were isoforms (or multiple forms) of TAXI-I, and only one of TAXI-II [25]. All TAXI-I isoforms inhibited xylanases from *A. niger* and *B. subtilis* belonging to glycoside hydrolase family 11, but TAXI-II was active only against bacterial enzyme. Multiple forms and isoforms of TAXI-I and TAXI-II were also detected in other studies when 2D electrophoresis and tandem mass-spectrometry were used together [26, 27]. These forms are the products of closely related genes or the result of post-translational modifications. Isoforms of inhibitors encoded by different genes were named TAXI-IA, TAXI-IB, TAXI-IIA, and TAXI-IIB.

The complete nucleotide sequence of the gene encoding TAXI-I was published in 2003 (then it was named *TAXI-IA*, code AJ438880 in EMBL/GenBank/DDBJ) [28]. The nucleotide sequence did not contain introns, and it consisted of 68% G/C nucleotides. The gene was cloned in *Escherichia coli*, and the expressed recombinant protein completely retained specificity and inhibitory activity against xylanases of family 11. The protein encoded by the *Taxi-I* gene consists of 402 amino acid residues (a.a.), 21 of them being a signal peptide. The calculated molecular mass and p*I* of the mature protein, including 381 a.a., are 38.8 kDa and 8.24, respectively (Table 1).

One potential N-glycosylation site (Asn105) was found in the polypeptide. According to [29], six disulfide bridges (Cys39–Cys124, Cys50–Cys71, Cys55–Cys80, Cys66–Cys92, Cys282–Cys327, Cys167–Cys378) are present in TAXI-I. The first four disulfide bridges are inside the N-domain (subunit of form B with molecular mass 30 kDa), the fifth one inside the C-domain (subunit with mass 10 kDa), and the sixth one combines the two domains.

Similarity of TAXI-I to the known proteins was negligible except for a glycoprotein from carrot (code BAA03413 in GenBank, 45% similarity), which possibly has a protective function in the plant, and to a protein family from rice with unknown function (59-72% similarity) [12]. This suggests that TAXI-I is a representative of a new class of plant proteins whose function is to protect the plant from the action of glycoside hydrolases. Immediately after publishing of the data on the gene encoding TAXI-I, a proteinaceous inhibitor of specific xyloglucanases from tomatoes was described; its similarity to the amino acid sequence of TAXI-I is 44% [21].

Then the genes encoding TAXI-IB, TAXI-IIA, and TAXI-IIB were identified (Table 1) [30]. All of them have been successfully cloned in *Pichia pastoris*, and the recombinant proteins retained the inhibitory activity typical of the native proteins from wheat belonging to TAXI-I and TAXI-II subgroups.

TAXI-IA was also successfully expressed in the same yeast [31]. The degree of identity of amino acid sequences of proteins of the TAXI family in wheat varies in the range 86-94%. Compared with TAXI-IA and TAXI-IB, TAXI-IIA and TAXI-IIB have an additional 6-7 a.a. at the C-terminus–peptide 383FNFARST389 (complete alignment of amino acid sequences of the mentioned proteins has been published [30]). In all the four proteins a potential N-glycosylation site, 12 Cys residues forming six disulfide bridges, and also the site of proteolytic cleavage between the N-domain (30 kDa) and C-domain (10 kDa) are strictly conservative. The cleavage site is between

Table 1. Characteristics of TAXI inhibitors from various cereals

Inhibitor	GenBank code	Length of encoding sequence, bp	Mature protein, a.a.	Mass of protein, kDa*	p*I**	Reference
TAXI-IA	AJ438880	1206	381	38.8 (40)	8.24 (8.8)	[12, 28]
TAXI-IB	AJ697851	1203	382	39.5 (40)	8.41 (8.8)	[12, 30]
TAXI-IIA	AJ697849	1167**	389	40.2 (40)	8.41 (9.3)	[12, 30]
TAXI-IIB	AJ697850	1167**	389	40.2 (40)	8.41 (9.3)	[12, 30]
TAXI-III	AB114627	1206	382	39.4	8.55	[32]
TAXI-IV	AB114628	1227	389	40.2	8.54	[32]
HVXI	not given	1209	384	39.4 (40)	8.43 (9.3)	[7, 34]
SCXI-II/III	not given	1188	375	38.1 (40)	8.24 (>9.0)	[8, 34]

* Calculated parameters for the mature protein are given; experimental values are given in parentheses if they are given in the cited literature.
** Incomplete sequence.

Asn265 and Gly266 in TAXI-IA and between Asn267 and Gly268 in all the other proteins of the TAXI family [30].

Genes *Taxi-III* (AB114627) and *Taxi-IV* (AB114628) were identified in wheat [32], but Raedschelders et al. [30] consider that proteins TAXI-III and TAXI-IV encoded by these genes are isoforms of TAXI-I and TAXI-II because they have 99.6 and 99.8% identity with TAXI-IB and TAXI-IIB, respectively. This is supported by the fact that TAXI-III exhibited inhibitory properties similar to those typical of TAXI-I; properties of TAXI-IV were not studied [32].

Proteinaceous inhibitors similar in structure and properties to TAXI-I and TAXI-II were found in other cereals: barley (*Hordeum vulgare*) [7], rye (*Secale cereale*, four isoforms) [8, 33], and durum wheat (*Triticum durum*, two isoforms) [33]. Analogous to TAXI, these inhibitors were named HVXI, SCXI-I, SCXI-II, SCXI-III, SCXI-IV, TDXI-I, and TDXI-II, respectively. The following properties are typical of all these TAXI-like proteins: molecular mass ~40 kDa; isoelectric point in basic area (pI > 8.5); existence in two molecular forms (Fig. 1), the second being formed due to the limited proteolysis of the first; ability to inhibit xylanases of family 11 but not family 10 glycoside hydrolases. Proteins belonging to the TAXI-I subgroup generally inhibit xylanases with low as well as high pI values, whereas TAXI-II-like proteins inhibit only xylanases with high pI [17, 33].

Genes encoding proteinaceous inhibitor HVXI from barley and one of the inhibitors from rye (SCXI) were identified [34]. The fragments of translated amino acid sequence of protein from rye coincided with the experimentally determined N-terminal sequences of SCXI-II and SCXI-III [8] and also with their subunits with molecular masses 30 and 10 kDa, which is why Raedschelders et al. [34] failed to identify what inhibitor described earlier was encoded by the gene they sequenced, and they named it SCXI-II/III. They also identified fragments of genes encoding SCXI-I and SCXI-II/III/IV. The nucleotide sequence of the gene encoding the inhibitor from barley (HVXI) is 88.7 and 87.9% identical to *Taxi-I* and *Scxi-II/III*, respectively, whereas identity between *Scxi-II/III* and *Taxi-I* is 91.8% [34]. Some characteristics of genes and TAXI-like proteins encoded by them are presented in Table 1.

XIP-type inhibitors. Almost simultaneously with the isolation of TAXI [10], another proteinaceous inhibitor of xylanases named XIP-I was isolated from wheat [11]; it differed from TAXI both in structure and properties. XIP-I is a monomeric glycosylated protein with molecular mass 29 kDa and pI 8.7-8.9; its N-terminal sequence was found to be unique: AGGKTGQVTVFWGRN. XIP-I competitively inhibits fungal xylanases belonging to glycoside hydrolase families 10 and 11, but it does not affect bacterial xylanases of these families [11, 16]. The protein encoded by the *Xip-I* gene (AJ422119) consists of 274 a.a., 30 of them forming a signal peptide; the calculated molecular mass of the mature protein is 30.2 kDa; this is in good agreement with the experimental data [35]. Recombinant XIP-I expressed in *E. coli* initially exhibited inhibitory activity comparable with that of the native protein, but then rapidly lost it [35]. Loss of inhibitory activity was not caused by proteolysis of the protein or improper formation of disulfide bridges during polypeptide chain folding. Low stability of recombinant XIP-I was rationalized by the absence of glycosylation [35].

XIP-I is highly identical (63%) with chitinases belonging to glycoside hydrolase family 18 and with concanavalin B also belonging to this family; however, XIP-I as well as concanavalin B does not possess enzymatic activity typical of chitinases [35]. The crystal structure of XIP-I was determined in 2003 [36]; this explained why xylanase inhibitor does not exhibit chitinase activity. Similar to chitinases of family 18, the XIP-I molecule has 3D folding in the form of $(\beta/\alpha)_8$-barrel, one catalytic residue (Glu128) typical of chitinases being retained, but the side chain of this residue participates in ionic interactions with two neighboring Arg residues. The second catalytic residue (Asp) is substituted by Phe in XIP-I. Finally, mutation of Gly in substrate-binding subsite −1 to Tyr is responsible for the absence of chitinase activity in the xylanase inhibitor: the side chain of Tyr hinders formation of the reaction intermediate.

Later, when crystals of XIP-I complexes with xylanases from *A. nidulans* (glycoside hydrolase family 10) and *Penicillium funiculosum* (family 11) were obtained, it appeared that the inhibitor has two independent binding sites: one of them specifically interacts with xylanases of family 10 and the second with xylanases of family 11 [37].

Another property of XIP-I is its ability to inhibit not only xylanases but also some α-amylases belonging to glycoside hydrolase family 13 [38, 39]. It is noteworthy that the ability of barley α-amylase/subtilisin inhibitor (BASI) to partially inhibit xylanases of family 11 was also found. BASI is not structurally similar to XIP or TAXI. Such property – cross inhibition of xylanases and amylases by proteins from cereals – was possibly developed in the course of plant evolution as a protective function against external action of phytopathogens.

XIP-like xylanase inhibitors have been found in grain of other cereals: rye, barley [40, 41], durum wheat, maize [41], rice [42, 43], oat [44], sorghum [45], and Algerian pearl millet [46]. It should be noted that other xylanase inhibitors, namely TAXI- or TLXI-like proteins, were not found in the last two cereals. The presence of XIP-type inhibitors is also possible in leaves and roots of germinated maize [47]. All the mentioned proteins are homologous to chitinases of glycoside hydrolase family 18, with molecular masses in the range 29-32 kDa; for inhibitors from ordinary and durum wheat, rye, and barley, pI values are in the basic area (>8.5); for XIP-like proteins from maize and rice, pI is 7.0 and 5.6, respectively [11, 41, 42]. The degree of identity of the N-termi-

nal amino acid sequences for most inhibitors is in the range 75-92% [46]. Phylogenetic analysis of proteins belonging to glycoside hydrolase family 18 allowed their classification into four subgroups (subfamilies); XIP-like proteins and concanavalin B appeared in subgroup 1 together with chitinases assigned to hevamines (hevamine is enzyme from hevea (rubber tree)) [43].

The presence of genes encoding XIP-like proteins in various cereals was also shown by expression of short fragments of wheat cDNA *Xip-I* as a probe [9]. Fragments of genes homologous to *Xip-I* named *Xip-II* and *Xip-III* were found in wheat. *Xip-I* homologs were found in barley, rye, maize, and sorghum, whereas *Xip-II* and *Xip-III* homologs were found in rice and sorghum. The degree of identity between *Xip-I*-like nucleotide sequences encoding proteins from wheat, barley and rye is >90%, whereas for *Xip-II* from wheat and rice the degree of identity is 64% (for *Xip-II* from wheat and sorghum, 82%) [9].

Twelve closely homologous *Xip*-like genes named the *Xip-R* gene family were identified in hexaploid wheat [14]. It was shown that *Xip-I* transcription occurs to the maximal degree; transcription is induced by pathogens, and its level depends on the type of pathogen [13].

At least eight *Xip*-like genes have been found in rice (*Oryza sativa*); OsXIP protein encoded by gene AK073843 (GenBank code) is expressed to the maximal degree [48]. Expression of OsXIP was enhanced by the action of stress caused by ascorbic acid, sodium citrate, or sodium chloride. Data on germination of transgenic rice in which the corresponding gene was suppressed indicate that expression of OsXIP is not necessary for development and germination of rice seeds, but it occurs as a protective reaction of the plant to phytopathogens [49].

The complete nucleotide sequence of the *Xip-II* gene (AJ318884) from durum wheat was reported in 2009 [50]: Elliot et al. analyzed in detail the regulation and physiological role of this class of inhibitors, aligned complete amino acid sequences of known XIP-like proteins from various cereals, and elucidated the role of the main amino acid residues.

TLXI inhibitor from wheat. A proteinaceous xylanase inhibitor of a third type (TLXI) was discovered only in 2006. Initially the presence of the new type of inhibitor in wheat was shown using polyclonal antibodies from serum of rabbit immunized by TAXI and XIP (these inhibitors contained admixture of an unknown protein with molecular mass ~18 kDa) [44]. Then homogeneous TLXI was isolated from wheat using affinity chromatography on a column with xylanases from *B. subtilis* and *A. niger* (glycoside hydrolase family 11) immobilized on Sepharose [15]. The molecular mass of this protein (determined by SDS-PAGE) was ~18 kDa, and p$I \geq 9.3$. The protein encoded by the *Tlxi* (AJ786602) gene consisted of 177 a.a., 26 of them being a signal peptide and 151 a.a. the mature protein. The calculated molecular mass and p*I* were 15.6 kDa and 8.38, respectively, and 10 Cys residues potentially forming five disulfide bridges were detected in it. The amino acid sequence of TLXI demonstrated high identity (up to 60%) with the thaumatin family of plant proteins (Pfam00314). These proteins are considered to exhibit antifungal activity; it was also shown that one of them, zeamatin from maize, can inhibit α-amylases and trypsin [51]. TLXI has one N-glycosylation site (Asn95) in which glycans of variable composition were found [15]. Gene *Tlxi* was cloned in *P. pastoris*, the recombinant protein having molecular mass 21 kDa (determined by SDS-PAGE). Higher molecular mass of recombinant TLXI was caused by more intensive glycosylation of this protein. Similar to the TAXI family, TLXI inhibits xylanases belonging to glycoside hydrolase family 11 but has no effect on xylanases of family 10 [15]. Structure modeling of this inhibitor and the CD spectrum demonstrated that the protein is composed of antiparallel β-sheets, and it contains no α-helices. High thermal stability is typical of TLXI: its functional stability is retained for at least 2 h at 100°C [52].

There is now no reliable data on the presence of TLXI-type inhibitors in other cereals, although possible TLXI homologs were found in durum wheat by enzyme immunoassay [44].

Variation of composition of xylanase inhibitors in wheat. Variation of composition of proteinaceous xylanase inhibitors in different cultivars of wheat or in grains of a given breed depending on the month of harvesting and year of crop, different fractions of grain, etc. has been widely studied in the last five years [26, 27, 53-61]. Much attention has been given to development of methods for quantitative analysis of inhibitors of various types; for this, titration by purified xylanases (estimation of their residual activity), enzyme immunoassay, 2D electrophoresis, and mass-spectrometry have been used. These works are not considered here in details because this review is mainly directed to biochemical aspects of xylanase inhibition. However, it should be mentioned that TAXI, XIP, and TLXI content in various wheat breeds can differ up to 8, 5, and 3 times, respectively [53, 57, 60]. For example, TAXI content in flour of 20 different cultivars of wheat grown in France varied from 0.05 to 0.19 mg/g; for grain, this parameter varied from 0.07 to 0.20 mg/g. XIP-I content in flour and grain of the same cultivars of wheat varied in the range 0.12-0.6 and 0.21-0.56 mg/g, respectively [53].

SENSITIVITY OF XYLANASES FROM VARIOUS ORGANISMS TO PROTEINACEOUS INHIBITORS

After discovery of proteinaceous xylanase inhibitors of TAXI- and XIP-types, regularities of inhibition were studied for a relatively narrow range of microbial enzymes belonging to glycoside hydrolase families 10 and 11. It was found that **TAXI-like proteins** specifically inhibit fungal

and bacterial xylanases of family 11 but do not affect xylanases of family 10. Biological activity of TAXI-I manifested itself against all the tested enzymes of family 11, whereas TAXI-II did not inhibit some xylanases (or they were weakly inhibited) [9, 12, 17]. In particular, TAXI-II inhibited fungal xylanases from *T. viride* and *T. longibrachiatum* (Xyn2) having p*I* in the basic area (8.4 and 9.0, respectively) and also enzyme from the bacterium *B. subtilis* with p*I* 9.3, but did not inhibit xylanase from *A. niger* with acidic p*I* value (3.5) and weakly inhibited enzymes from *T. longibrachiatum* (Xyn1, p*I* 5.5) and *P. purpurogenum* (p*I* 5.9) [17]. Later, when sensitivity of other microbial xylanases to TAXI-I and TAXI-II was studied, the initially found regularities of inhibition were substantiated (Table 2). An exception to this was acidic XynC from *P. funiculosum* with p*I* 3.7, which was significantly inhibited by TAXI-II [62]. The inhibition constants (K_i) of xylanases belonging to family 11 varied in the range 2.2-165 nM (Table 2), in all cases inhibition was competitive; an exception was XynA from *B. subtilis*, when inhibition was noncompetitive with K_i = 2.2 nM [73]. XynB from *P. funiculosum* deserves special attention: in two works two essentially different K_i values, 2.9 and 165 nM, were obtained [63, 67]. This difference is possibly explained by the fact that in the first case [63] recombinant enzyme expressed in *P. pastoris* yeast was studied, whereas in the second case [67] the native fungal enzyme was used.

Table 2. Sensitivity of microbial xylanases to TAXI-type proteinaceous inhibitors from wheat

Source, enzyme	GenBank/ UniProtKB code	Family	p*I*	Fact of inhibition*	K_i, nM*	Reference
Fungi:						
P. funiculosum XynA	Q8WZJ4	7	3.6	yes/yes	46/46	[67]
A. aculeatus Xyl II	AAE69552	10	4.6	no/no	–	[9]
A. nidulans XlnC	Q00177	10	3.4	no/no	–	[9, 17]
A. niger XynA	CAA03655	10	6.0	no/no	–	[9, 17]
A. oryzae XynF1	O94163	10	n.d.	no/no	–	[9]
F. graminearum XylC**	XP_391663	10	8.8	no/no	–	[70]
F. graminearum XylD**	XP_391480	10	6.7	no/no	–	[70]
P. funiculosum XynD	Q5ZNB1	10	4.6	no/no	–	[67]
P. purpurogenum XynA	AAB35129	10	8.6	no/no	–	[9, 17]
T. emersonii XynA	AF439747	10	n.d.	no/no	–	[17, 73]
A. niger XylA	P55329	11	3.5	yes/no	20/–	[9, 72, 73]
B. cinerea XynBc1**	B3VSG7	11	n.d.	yes/no	6.0/–	[64]
F. graminearum XylA**	Q7ZA57	11	6.2	yes/n.d.	n.d./n.d.	[65]
F. graminearum XylB**	Q5NDZ1	11	9.2	yes/n.d.	n.d./n.d.	[65]
P. funiculosum XynB**	AJ489605	11	n.d.	yes/no	2.9/–	[63]
P. funiculosum XynB	AJ489605	11	5.0	yes/no	165/–	[67]
P. funiculosum XynC	Q9HFH0	11	3.7	yes/yes	17/16	[62]
P. purpurogenum XynB	Q96W72	11	5.9	yes/faintly	n.d./n.d.	[9, 17]
T. longibrachiatum Xyn1	P36218	11	5.5	yes/faintly	n.d./n.d.	[9, 17]
T. longibrachiatum Xyn2	P36217	11	9.0	yes/yes	n.d./n.d.	[9, 17]
T. viride Xyl	Q9UVF9	11	8.4	yes/yes	n.d./n.d.	[9, 17]
Bacteria:						
P. fluorescens XylA	CAA33469	10	n.d.	no/no	–	[43]
B. subtilis XynA	P18429	11	9.3	yes/yes	17/12	[9, 17]
					2.2/2.2	[73]

* Characteristics for TAXI-I and TAXI-II are given as numerator and denominator, respectively; n.d., not determined.
** Recombinant enzymes expressed in *E. coli* or *P. pastoris*.

Proteinaceous inhibitors from other cereals homologous to TAXI-I, namely HVXI from barley, four isoforms of SCXI from rye, and also TDXI-I from durum wheat exhibited the same biological activity as TAXI-I. They inhibited xylanases of family 11 from *T. viride*, *A. niger*, and *B. subtilis*, but did not inhibit xylanase from *A. aculeatus* belonging to family 10 [7, 8, 33]. Analogous to TAXI-II, TDXI-II from durum wheat significantly inhibited only basic xylanases with high p*I* values [33].

Initially it was found that **XIP-I from wheat** competitively inhibited fungal xylanases belonging to both glycoside hydrolase families 10 and 11 but did not affect bacterial xylanases belonging to these families [16]. An exception was xylanase II from *A. aculeatus* of family 10. Later, when a wider range of enzymes was studied (Table 3), other fungal xylanases of the mentioned families were found that appeared to be insensitive to XIP-I: xylanase from *Talaromyces emersonii* [9, 73] belonging to family 10 and also XylA and XylB from *Fusarium graminearum* [65, 66], XynA from *Neocallimastix patriciarum* [37, 69], XynB from *P. funiculosum* [67], and XynA and XynB from *P. griseofulvum* [68, 73] belonging to family 11. Along with this, two of three studied xylanases of family 10 isolated from the fungus *Chrysosporium lucknowense* appeared to be insensitive to the action of XIP-like proteins from rye extract possessing the same specificity as XIP-I from wheat [71]. Reasons (structure factors) for such exclusions are considered in the next chapter of this review. It should be noted that contrary to the native XynB from *P. funiculosum* [67], recombinant enzyme expressed in *P. pastoris* was inhibited by XIP-I [63]. We remind the reader that for the native and recombinant forms of this xylanase, the inhibition constants by TAXI-I differ significantly (Table 2).

An interesting phenomenon of sensitivity to XIP-I was observed in the case of XynD from *P. funiculosum*: the degree of inhibition depended on the sequence of mixing of components of the reaction mixture [67]. Inhibition was weak when the enzyme was added to the substrate and XIP-I mixture, but significant when XynD and XIP-I were premixed before adding the substrate. This seems to indicate different rates of formation of "enzyme–substrate" and "enzyme–inhibitor" complexes; in the latter case the equilibrium was established slowly.

Constants of competitive inhibition of xylanases by XIP-I varied from 2.1 to 610 nM, and in several cases $[I]/[E]_{50}$ parameter was determined showing the ratio of inhibitor and enzyme concentrations at which 50% decrease in enzyme activity was observed (Table 3). XIP-like proteins isolated from barley, rye, durum wheat, and maize exhibited almost the same biological activity as XIP-I from wheat, that is, they significantly inhibited Xyn1 and Xyn2 from *T. longibrachiatum* (family 11) and XlnC from *A. nidulans* (family 10), only slightly inhibited xylanases from *A. niger* and *T. viride* (family 11), and had no effect on xylanases from *A. aculeatus* (family 10) and *B. subtilis* (family 11) [41]. Qualitatively determined degree of inhibition of enzymes by the mentioned XIP-like proteins from various cereals correlates well with the inhibition constants of xylanases by XIP-I presented in Table 3. XIP-like proteins from rice had somewhat differing specificity. One of them isolated from rice by affinity chromatography and having molecular mass 32 kDa and p*I* 5.6 significantly inhibited xylanases from *A. niger* and *A. oryzae* (family 10) but did not inhibit xylanases from *A. niger*, *Trichoderma* sp., and *B. subtilis* (family 11) [42]. In contrast to XIP-I, the second inhibitor from rice, recombinant RIXI expressed in *P. pastoris* [43] and having p*I* ~ 9, did not effect xylanases of family 10 from *A. niger* and *A. nidulans* (Table 3, data given in parentheses). Differences from XIP-I were evidently not related with use of recombinant form of RIXI, because recombinant XIP-I was expressed under the same conditions, and it retained specificity typical of the native protein from wheat. XIP-like proteins isolated from sorghum and Algerian pearl millet [45, 46] were similar in properties to XIP-I, excluding the absence of inhibitory effect on xylanase from *A. niger* (family 11). Protein from millet by two orders of magnitude more efficiently inhibited Xyn1 from *T. longibrachiatum* than the protein from sorghum (parameter $[I]/[E]_{50}$ was 0.8 and 104, respectively) [46].

A special case is XynA from *P. funiculosum*, which belongs to glycoside hydrolase family 7 and is susceptible to inhibition by XIP as well as TAXI-I and TAXI-II (Tables 2 and 3) [67]. This enzyme is unique in family 7: it is the only xylanase, while other members of this family are cellulases – endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91). XynA from *P. funiculosum* is very similar to cellobiohydrolases of family 7, which is why this enzyme was classified as a xylanase/cellobiohydrolase. So far no other cases of inhibition of enzymes of family 7 by proteinaceous xylanase inhibitors have been found.

Thaumatin-like inhibitor TLXI from wheat exhibits specificity similar to the TAXI family of proteins, that is, it can inhibit fungal and bacterial xylanases belonging to family 11, but it does not affect xylanases of family 10 [15]. TLXI strongly inhibited Xyn1 from *T. longibrachiatum* ($[I]/[E]_{50}$ = 4.2) and to the significantly lower degree xylanases from *A. niger*, *T. viride*, *Thermobacillus xylanilyticus*, and *P. funiculosum* (XynC) for which $[I]/[E]_{50}$ varied in the range 135-290. Inhibitory effect was absent in the case of Xyn2 from *T. longibrachiatum* (p*I* 9.0) and xylanase from *B. subtilis* (p*I* 9.3). Thus, TLXI exhibited "mirror" specificity compared with TAXI-II (Table 2), that is, it preferentially inhibited acidic xylanases but did not affect basic (with high p*I* value) xylanases (an exception was an enzyme from *T. viride* with p*I* 8.4). Recombinant TLXI expressed in *P. pastoris* retained biological activity, but its inhibitory activity was lower than that of the native protein [15]. The degree of inhibition of xylanases by TLXI depends on temperature

Table 3. Sensitivity of microbial xylanases to XIP-I-type proteinaceous inhibitors from wheat and other cereals

Enzyme, source	GenBank/ UniProtKB code	Family	Fact of inhibition*	$[I]/[E]_{50}$*	K_i, nM*	Reference
Fungi:						
P. funiculosum XynA	Q8WZJ4	7	yes	6	106	[67]
A. aculeatus Xyl II	AAE69552	10	no (no)	–	–	[16, 43]
A. nidulans XlnC	Q00177	10	yes (no)	0.6	9	[16, 43]
A. niger XynA	CAA03655	10	yes (no)	0.7	n.d.	[9, 43]
A. oryzae XynF1	O94163	10	yes	1.5	17	[16]
C. lucknowense Xyl1	AAQ38147	10	yes***	n.d.	n.d.	[71]
C. lucknowense Xyl3	no	10	no***	–	–	[71]
C. lucknowense Xyl4	no	10	no***	–	–	[71]
F. graminearum XylC**	XP_391663	10	yes	n.d.	n.d.	[70]
F. graminearum XylD**	XP_391480	10	yes	n.d.	n.d.	[70]
P. funiculosum XynD	Q5ZNB1	10	yes****	n.d.	n.d.	[67]
T. emersonii XynA	AF439747	10	no	–	–	[9, 73]
A. niger XylA	P55329	11	yes (yes)	2.1-3.8	317	[16, 43]
B. cinerea XynBc1**	B3VSG7	11	yes	n.d.	2.1	[64]
F. graminearum XylA**	Q7ZA57	11	no	–	–	[65, 66]
F. graminearum XylB**	Q5NDZ1	11	no	–	–	[65, 66]
N. patriciarum XynA	P29127	11	no	–	–	[37, 69]
P. funiculosum XynB**	AJ489605	11	yes	n.d.	90	[63]
P. funiculosum XynB	AJ489605	11	no	–	–	[67]
P. funiculosum XynC	Q9HFH0	11	yes (yes)	1.0-1.6 (45)	3.4	[16, 43, 62]
P. griseofulvum XynA**	no	11	no	–	–	[68]
P. griseofulvum XynB**	no	11	no	–	–	[73]
T. longibrachiatum Xyn1	P36218	11	yes	n.d.	n.d.	[16]
T. longibrachiatum Xyn2	P36217	11	yes (yes)	1.1-5.3 (6.5)	20	[16, 43]
T. viride Xyl	Q9UVF9	11	yes	63	610	[16]
Bacteria:						
Bacillus sp. Xyl**	no	10	no	–	–	[16]
P. fluorescens XylA	CAA33469	10	no (no)	–	–	[16, 43]
B. agaradhaerens Xyl	CAB42305	11	no	–	–	[16, 73]
B. subtilis XynA	P18429	11	no (no)	–	–	[16, 43]
F. succinogens XynC-A	P35811	11	no	–	–	[16]
F. succinogens XynC-B	P35811	11	no	–	–	[16]

* Data for inhibitor from wheat are given, data for recombinant RIXI from rice and also parameter $[I]/[E]_{50}$ for RIXI showing the ratio of inhibitor and enzyme concentration at which 50% decrease in enzyme activity was observed are given in parentheses (if these data exist); n.d., not determined.

** Recombinant enzymes expressed in *E. coli* or *P. pastoris*.

*** Presence or absence of inhibition by XIP-like proteins from rye is indicated.

**** Inhibition depended on sequence of component mixing.

and pH, the maximal inhibition being observed at pH 5.0 and 40°C [52].

As mentioned above, XIP-I can inhibit not only xylanases but also α-amylases (in particular, AMY1 and AMY2 from barley) belonging to glycoside hydrolase family 13 [38, 39]. Inhibition of amylases proceeds via a complex mechanism, and due to this attempts to determine the corresponding constants failed. On hydrolysis of soluble starch, $[I]/[E]_{50}$ for AMY1 and AMY2 was 9.7 and 19.4, respectively [38]. Formation of the triple complex between substrate of amylases (starch), enzyme, and XIP-I was found, the interaction between starch and XIP-I being a necessary condition for inhibition. XIP-I binding to starch was shown by gel electrophoresis. Additional experiments demonstrated that XIP-I can form a complex with arabinoxylan from wheat but does not bind to chitin and cellulose [38]. Later it was found that other xylanase inhibitors (TAXI and TLXI) also can bind to soluble and insoluble arabinoxylans, and affinity of all inhibitors (including XIP-I) to polysaccharide increases with decreased ratio of arabinose and xylose residues in it. TLXI has the maximal affinity to arabinoxylans [74]. All types of inhibitors can also interact with several β-glucans (both soluble and insoluble), but they cannot hydrolyze polysaccharides. Since xylans and β-glucans are components of cell wall of cereals, and according to [74] secretion of xylanase inhibitors occurs out of the cell wall, such interaction between polysaccharides and inhibitors is necessary to provide their proper localization in the cell wall. The cell wall is the first to be subjected to the action of pathogenic microorganisms.

Fig. 2. Structure of TAXI-IA in complex with xylanase from *A. niger* (PDB code 1T6G) [29]. The inhibitor and enzyme molecules are shown in black and gray, respectively.

STRUCTURAL FACTORS GOVERNING SENSITIVITY OF XYLANASES TO PROTEINACEOUS INHIBITORS

Structural factors responsible for the inhibition of xylanases by TAXI- and XIP-like proteins have been determined by X-ray crystal structure analysis of "enzyme–inhibitor" complexes and by site-directed mutagenesis of the enzymes and inhibitors.

Crystal structures of **TAXI-IA** and complexes of the inhibitor with xylanases from *A. niger* and *B. subtilis* (family 11) were reported in 2004 [29, 75]. The inhibitor was found to be a bi-domain protein with both domains appearing as β-barrels, the protein being structurally homologous to the pepsin-like family of aspartic proteases. A cleft between the two domains in which the protease active site is located is retained in TAXI-IA structure. Analysis of the 3D structure of TAXI-IA in complex with xylanase from *A. niger* (PDB code 1T6G) demonstrated that the enzyme binding site in the inhibitor molecule is located on the molecule side opposite to the cleft (Fig. 2). The inhibitor completely blocks the active site of the xylanase (the contact surface area is 992 Å^2), the His374 residue from TAXI-IA being located between two catalytic residues of the enzyme (Glu79 and Glu170) and additionally interacting with Asp37, Arg115, and Tyr81 [29]. The Phe375, Thr376, and Leu292 residues of the inhibitor interfere with substrate-binding subsites −1 and −2 in the active site of the xylanase, interacting with its Glu129 and Arg115 residues. The inhibitor produces steric hindrances also for aglycone binding (subsites +1 and +2), the Gln187 residue of the inhibitor forming a hydrogen bond with Trp172 of the enzyme.

To clarify the role of His374 in TAXI-IA, this residue was replaced by Ala, Glu, or Lys by site-directed mutagenesis [76]. As a result of these mutations, the affinity of the inhibitor to xylanases from *A. niger*, *B. subtilis*, and *T. longibrachiatum* decreased; this manifested itself in increased (3-80-fold) rate constants of dissociation of the "inhibitor–enzyme" complex (the association rate constants decreased less – only up to 8-fold). These data indicate that His374 plays a key role in stabilization of the complex but not in its formation.

A mutant form of xylanase from *A. niger* (D37A) was expressed in *P. pastoris* [77]. As a result of the mutation, the enzyme lost its ability to be inhibited by TAXI-I as well as by XIP-I; this indicated direct interaction of Asp37 with both types of proteinaceous inhibitors. It should be noted that the D37A mutation also changed the

pH profile of xylanase activity; this is not surprising because this residue is located adjacent to a catalytic residue (Glu170) that acts as a general acid/base (Fig. 2).

For simplicity, 3D structures of family 11 xylanases are often presented as a bent right hand, the cleft of the active site being located between the "thumb" and the other "fingers" (Fig. 2). To clarify the role of amino acid residues adjacent to the cleft, 22 amino acid residues of xylanase from *B. subtilis* located on different "fingers" of the protein globule were subjected to site-directed mutagenesis [78]. A total of 62 mutations were obtained, and some of them resulted in significantly decreased inhibition of the enzyme by TAXI-I. Three mutations (D11F, D11Y, and D11K) of Asp11, a residue located on the "little finger", resulted in complete loss of inhibition, but the catalytic activity of the enzyme also decreased by 74-86%. Other mutant forms of the same bacterial xylanase were obtained by site-directed mutagenesis of amino acid residues in the active site of the enzyme as well as outside of it (there was also the task of retaining the catalytic activity of the xylanase) [79]. When Glu12 located on the "little finger" adjacent to Asp11 was replaced by bulky residues (Lys, Arg, or Trp), thus causing steric hindrances for binding of the inhibitor, the mutant forms of the enzyme practically lost sensitivity to TAXI-I but nevertheless retained catalytic activity as high as that of the wild type enzyme. The most successful was the G12W mutant, which exhibited the minimal sensitivity both to TAXI-I and TAXI-II. It should be noted that some mutations resulted in increased inhibition by TAXI-I and TAXI-II. As a result of various mutations, changes in sensitivity of recombinant forms of xylanase from *B. subtilis* to TAXI-I and TAXI-II correlated [79].

As mentioned above, xylanase from *A. niger* belonging to family 11 as well as some other xylanases with low p*I* value are not inhibited by TAXI-II, in contrast to TAXI-I (Table 2). After identification of genes encoding TAXI-IIA and TAXI-IIB [30], amino acid sequences of four proteins from wheat belonging to the TAXI family were aligned, and thus possible residues responsible for different specificity of TAXI-IA and TAXI-IB, on one hand, and TAXI-IIA and TAXI-IIB, on the other hand, to acidic xylanases were revealed. In particular, TAXI-IIA has Pro in position 294, whereas the other three proteins have Leu at the equivalent position. Leu292 in TAXI-IA interacts hydrophobically with substrate-binding subsite −2 in the active site of xylanase from *A. niger* [29]. TAXI-IIB has Gln at position 376, in contrast to other proteins of the TAXI family, the latter having a His residue in this position [30] (the role of His374 in TAXI-IA was already mentioned above, see also Fig. 2). After replacement of Pro294 for Leu in TAXI-IIA and the Q376H mutation in TAXI-IIB, both mutant proteins became able to inhibit acidic xylanase from *A. niger*; this supports the hypothesis about an important role of these residues in the TAXI family proteins.

After solving crystal structures of complexes of TAXI-IA and recombinant TAXI-IIA with xylanase from *B. subtilis* (PDB codes 2B42 and 3HD8) [72], the earlier found structural factors governing interaction between xylanases of family 11 and TAXI-like proteins were supported. Additionally, it was shown that as a result of complex formation with an inhibitor certain amino acid residues in the active site of the enzyme are subjected to conformational changes. An Asp or Asn residue adjacent to a catalytic residue of xylanases acting as a general acid/base appeared to be an important factor on which the degree of inhibition depended. This auxiliary residue (Asp37 in xylanase from *A. niger* or Asn35 in xylanase from *B. subtilis*), being a part of the catalytic triad, has a strong influence on the pH optimum of enzyme activity: the lower the pH optimum, the tighter is the interaction between the enzyme and inhibitor. The mechanism for interaction of inhibitors with acidic and basic xylanases belonging to family 11 is considered in detail in [72].

XIP-like proteins, as mentioned above, inhibit fungal xylanases belonging to glycoside hydrolase families 10 and 11 but do not affect bacterial xylanases of these two families; however, as the range of studied enzymes became wider, exceptions to this rule accumulated. It should also be noted that number of bacterial xylanases tested for susceptibility to inhibition by XIP-I is rather limited (Table 3); this prevents concluding that absolutely all bacterial enzymes are insensitive to this type of proteinaceous inhibitors.

The XIP-I molecule appears as a $(\beta/\alpha)_8$-barrel, that is, its structure is typical of proteins belonging to glycoside hydrolase family 18 [36]. There are two disulfide bridges binding Cys25 and Cys66 and also Cys164 and Cys195 residues in XIP-I. When 3D structures of XIP-I complexes with xylanases from *A. nidulans* (glycoside hydrolase family 10) and *P. funiculosum* (family 11) were solved, it appeared that the inhibitor has two independent binding sites: one of them specifically interacts with xylanases of family 10 and another with xylanases of family 11 [37]. Structures of these complexes are presented in Figs. 3a and 3b, while Fig. 3c represents the structure of a hypothetical triple complex of XIP-I with both xylanases of different families obtained by superposition of the first two structures (in fact, formation of such triple complex has not been detected experimentally). As shown in Fig. 3c, sites of XIP-I interaction with enzymes of families 10 and 11 are on the opposite sides of the inhibitor molecule.

On interaction with xylanase from *A. nidulans* (family 10), α-helix 7 of XIP-I (a.a. 232-245) interacts with peptide loops forming a "cleft" of the active site of the enzyme (a.a. 46-47 in β_2-α_2 loop, 87-90 in β_3-α_3 loop, 131-146 in β_4-α_4 loop, and 269-281 in β_8-α_8 loop), thus shielding the central part of the cleft , that is, substrate-binding subsites from −3 to +2. This results in the burial of 1130 Å^2 of accessible surface area [37]. The Lys246, Tyr238, Lys234, Asn235, and His232 residues from XIP-I

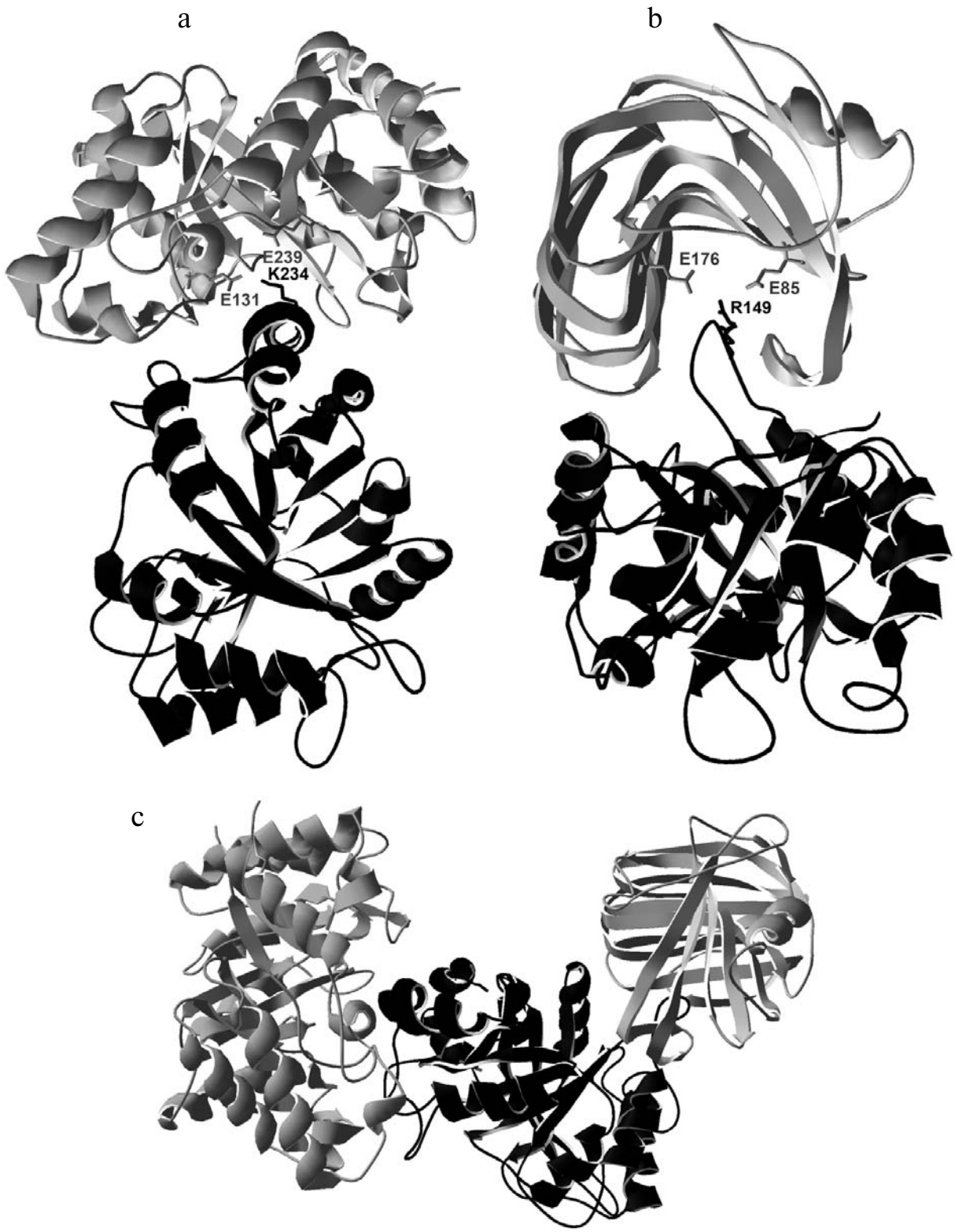

Fig. 3. Structures of XIP-I complexes with xylanases belonging to glycoside hydrolase families 10 and 11 (PDB codes 1TA3 and 1TE1) [37]: a) XIP-I in complex with XlnC from *A. nidulans* (family 10); b) XIP-I in complex with XynC from *P. funiculosum* (family 11); c) a hypothetical triple complex of XIP-I with both xylanases (XlnC from *A. nidulans* on the left) obtained by superposition of 1TA3 and 1TE1 via the XIP-I molecule. Molecules of inhibitor and enzyme are shown in black and gray, respectively. Numeration of amino acid residues in XlnC (a) is given according to [37]; it differs by 3 a.a. from the numeration of residues in the 1TA3 PDB file.

interact with the above-mentioned subsites, respectively. The Lys234 residue forms a hydrogen bond with catalytic residue Glu131 of xylanase functioning as general acid/base and also a hydrogen bond via a water molecule with the catalytic nucleophile Glu239 (Fig. 3a). Clusters of aromatic residues Tyr237, Tyr238, His232, Trp230, and Tyr273 in XIP-I and residues Trp269, His209, Trp277, Tyr174, and Arg278 in the active site of the xylanase play an important role in the "inhibitor–enzyme" interaction [37].

Alignment of amino acid sequences of fungal and bacterial xylanases belonging to family 10 and analysis of their 3D structures revealed the reason for the absence of inhibition of bacterial enzymes by XIP-I [37]. As mentioned above, in xylanase from *A. nidulans* β_2-α_2, β_3-α_3, β_4-α_4, and β_8-α_8 loops combining the corresponding β-sheets and α-helices interact with XIP-I. The alignment demonstrated that in bacterial xylanase from *P. fluorescens* insensitive to the action of the inhibitor there are two additional residues (Ser and Tyr) in the β_3-α_3 loop compared with the enzyme from *A. nidulans*; β_4-α_4 and β_8-α_8 loops also appeared to be longer by 10 and 5 a.a., respectively [37]. Elongation of β_4-α_4 and β_8-α_8 loops in the bacterial enzyme results in steric hindrances for penetration of α_7-helix of XIP-I into the active site of the xylanase; as a result, the latter is insensitive to the action of the inhibitor. A similar situation is typical for other bacterial xylanases of family 10 as well as for fungal xylanases from *A. aculeatus* (Xyl II) and *C. lucknowense* (Xyl3 and Xyl4) that are not susceptible for inhibition (Table 3) and which have an insertion in the β_8-α_8 loop (also 5 a.a.) compared with xylanase from *A. nidulans* and Xyl1 from *C. lucknowense* (Fig. 4) [37, 71]. The latter two enzymes are significantly inhibited by XIP-like proteins.

As for interaction of XIP-I with xylanases belonging to family 11, study of 3D structure of the inhibitor complex with XynC from *P. funiculosum* showed that ∏-like loop $\alpha_4\beta_5$ of XIP-I (a.a. 148-154) enters the cleft between the "thumb" and other "fingers" of the enzyme molecule, blocking access of substrate to the active site (Fig. 3b). This results in the burial of 870 Å^2 of accessible surface area [37]. Amino acid residues of the ∏-like loop of XIP-I interact with substrate-binding subsites from −3 to −1, and Arg149 of the inhibitor forms hydrogen bonds with a catalytic residue Glu176 and Tyr87 of the enzyme.

Xylanases of family 11 have a conservative residue Gly129 at the "thumb end" (numeration of residues as in XynC from *P. funiculosum*). The fact that the xylanase from *B. subtilis* is not inhibited by XIP-I is rationalized by the insertion of Asp after Gly129 and replacement of the Thr next after it with Arg [37]; this causes steric hindrances for binding of the inhibitor. This hypothesis is supported by the absence of inhibition of fungal xylanase from *N. patriciarum* (family 11), which also has an insertion after the residue equivalent to Gly129 in XynC.

To reveal the key residues responsible for interaction with XIP-I, site-directed mutagenesis of family 11 xylanases (mainly by replacing amino acid residues in the "thumb" area of protein globule) was performed. As a result of the replacement N117A, the xylanase from *A. niger* became insensitive to XIP-I, but its catalytic activity was completely retained [80]. XylA and XylB from *F. graminearum*, which initially were not inhibited by XIP-I, were subjected to site-directed and combinatory mutagenesis [66]. Only as a result of the replacement V151T (on the "thumb" of XylA) the latter gained the capacity to be inhibited by XIP-I; this was rationalized by the impossibility for Val151 in the wild type enzyme to form a hydrogen bond with Asn147 of the inhibitor. Combinatory mutagenesis of amino acid residues on various "fingers" of XylB showed that the absence of inhibition is provided by residues on the "thumb", namely, deletion of Asp148 and also mutations C141N and C149T made xylanase sensitive to XIP-I; however, the thus arising inhibition was weaker than in the case of the V151T mutant of XylA [66]. Combinatory mutagenesis of seven residues on the "thumb" of Xyn2 from *T. longibrachiatum* gave 62,208 mutant forms of the enzyme [81]. Asn124 and Thr131 appeared to be the most important residues governing sensitivity of xylanase to XIP-I.

As a result of mutations T131R, N124A, and N124D the inhibition of the enzyme by XIP-I was abolished. On

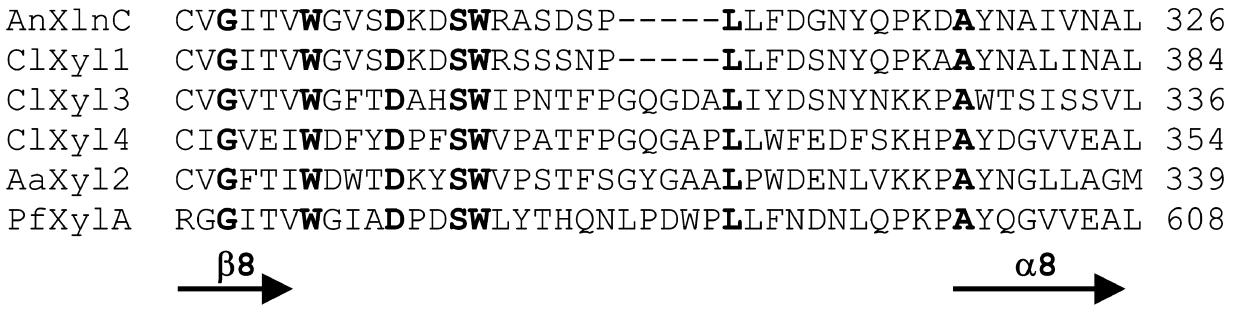

Fig. 4. Alignment of fragments of amino acid sequences of xylanases belonging to family 10. AnXlnC is xylanase C from *A. nidulans* (Q00177); C1Xyl1, C1Xyl3, and C1Xyl4 are xylanases I, III, and IV from *C. lucknowense* [71]; AaXyl2 is xylanase II from *A. aculeatus* (AAE69552); PfXylA is xylanase A from *Pseudomonas fluorescens cellulosa* (CAA33469).

replacements V123A and V123D the enzyme retained sensitivity to XIP-I, but inhibition by TAXI-I was significantly weaker. As a result of multiple replacements V123N-N124D-Q125A-I129D-T131R-A132S-Y135D, inhibition by XIP-I completely disappeared, but inhibition by TAXI-I was enhanced. Mutant D119V-D121∅-R121T-T123S-T126N of XynA from *S. subtilis* became susceptible to inhibition by XIP-I, in contrast to the wild type enzyme [81].

The causes of insensitivity of XynA and XynB from *P. griseofulvum* to XIP-I were studied in [82, 83]. Deletion of the Asp130 residue on the "thumb" of XynA did not change the properties of the enzyme, whereas the S129G replacement and also double mutation S129G/S44N (the second of the residues was in the active site of xylanase) caused the enzyme to be inhibited by XIP-I with K_i = 82 and 90 nM, respectively. Inhibition was enhanced as a result of double mutation S129G/ΔD130 (K_i = 3.9 nM) [82]. Structure modeling of XynB from *P. griseofulvum* and its comparison with XynC from *P. funiculosum* suggests that the additional Pro at position 130 and also Lys131 and Lys132 (XynC has less bulky Ser residues at the equivalent positions) are responsible for the insensitivity of XynB to XIP-I. Neither deletion of Pro130 nor the double mutation K131S/K132S changed the insensitivity of XynB to the action of the inhibitor, whereas the triple mutation ΔP130/K131S/K132S made the enzyme sensitive to XIP-I, K_i = 16.5 nM [83]. These phenomena were rationalized by disappearance of steric hindrances in the "thumb" area of the xylanases on their binding to the inhibitor [82, 83].

As for the interaction of **TLXI** (proteinaceous inhibitor of the third type from wheat) with xylanases belonging to family 11, modeling of inhibitor structure and site-directed mutagenesis of His22 showed that this residue plays an important role in binding to the enzymes [84]. The mutant form of the inhibitor expressed in *P. pastoris* could not inhibit family 11 xylanases. As of now (March 2010) other structural data on features of inhibition by TLXI have not been published.

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