Xylanase inhibitors bind to non-starch polysaccharides

Ellen Fierens, Kurt Gebruers^{*}, Christophe M. Courtin and Jan A. Delcour

Laboratory of Food Chemistry and Biochemistry, Department of microbial and molecular systems, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, Box 2463, 3001 Leuven, Belgium

^{*}Corresponding author: Tel.: +32(0)16321634, Fax: +32(0)16321997

e-mail of all authors : Ellen.fierens@biw.kuleuven.be

Kurt.gebruers@biw.kuleuven.be

Christophe.courtin@biw.kuleuven.be

Jan.delcour@biw.kuleuven.be

Abstract

This study is an in-depth investigation of the interaction between polysaccharides and the proteinaceous xylanase inhibitors, *Triticum aestivum* xylanase inhibitor (TAXI), xylanase inhibitor protein (XIP) and thaumatin-like xylanase inhibitor (TLXI). The binding affinities of all three known types of xylanase inhibitors from wheat are studied by measuring the residual xylanase inhibition activity after incubation of the inhibitors in the presence of different polysaccharides, such as β -glucans and (arabino)xylans. The binding affinities of all three xylanase inhibitors for (arabino)xylans increased with decreasing arabinose to xylose ratio (A/X-ratio). This phenomenon was observed both with water-extractable and water-unextractable (arabino)xylans. The inhibitors also interacted with different soluble and insoluble β -glucans. None of the inhibitors tested had the ability to hydrolyze the polysaccharides investigated. The present findings contribute to the unraveling of the function of xylanase inhibitors in nature and to the prediction of the effect of added xylanases in cereal based biotechnological processes, such as bread making and gluten-starch separation.

Keywords: wheat, xylanase inhibitors, β -glucan, xylan, binding, polysaccharide

Introduction

In the last decade, the occurrence of three types of xylanase inhibitors in cereals has been demonstrated, i.e. *Triticum aestivum* xylanase inhibitor (TAXI), xylanase inhibitor protein (XIP) and thaumatin-like xylanase inhibitor (TLXI) (1-3). The three types have distinct structures and differ in their specificities towards endo-beta-1,4-xylanases, further referred to as xylanases. They significantly impact the functionalities of a number of xylanases in cereal based biotechnological applications, such as bread making (4, 5) and gluten-starch separation (6).

The functions of these inhibitor proteins in the plant are not yet understood. Their lack of inhibition activity against endogenous xylanases disaffirms a regulatory role in plant development (3, 7, 8). However, based on their distinct specificities towards xylanases of microbial origin, the ability of TAXI to inhibit two xylanases of glycoside hydrolase family 11 of the cereal pathogen *Fusarium graminearum* (9), the fact that some TAXI and XIP genes are induced by pathogens and wounding (10, 11) and the homologies of TLXI and XIP with pathogenesis-related proteins of family 5 (PR-5) and 8 (PR-8), respectively, they are believed to be involved in plant defense. PR proteins, which have been classified as a major group of defense-related proteins, are proteins encoded by the host plant but induced in pathological or other stress situations. To date, PR proteins have been classified into 17 structurally and functionally distinct families (12-14), some of which consist of proteins with known biological functions.

The binding of XIP to polysaccharides has been reported. More exactly, XIP binds to Blue starch and wheat arabinoxylan but not to cellulose and chitin (15). Rouau and coworkers (16) reported that XIP binds to water-unextractable arabinoxylan (WUAX) and not to water-extractable arabinoxylan (WEAX). XIP loses its inhibition activity upon binding, in their view, resulting in a

41 strongly depressed inhibition activity in the vicinity of WUAX. In contrast, in the vicinity of
42 WEAX, the XIP activity is not affected. The xylanase is therefore proportionally more active on
43 WUAX (arabinoxylan solubilisation) than on WEAX (arabinoxylan depolymerisation). The net
44 effect is an increase in the soluble high molecular weight arabinoxylan levels, which are
45 beneficial for dough properties, and a larger decrease in WUAX levels, which are detrimental to
46 both dough and bread properties (17). The above opens perspectives for improving enzyme
47 performance in bread making, by steering the xylanase selectivity by a specific inhibitor.

48 In addition, insight in the binding of the xylanase inhibitors to polysaccharides can contribute to
49 our understanding of their functions in plants. After all, for the PR-5 protein family, binding to
50 polysaccharides has been reported to be involved in their antifungal mechanism. Members of this
51 family are also called the thaumatin-like proteins (TLPs) because they are all homologues to
52 thaumatin, a sweet tasting protein found in the arils of fruits of the African shrub
53 *Thaumatococcus daniellii* (18). Most TLPs are believed to be involved in plant defense because
54 their synthesis is induced upon challenge of the plant by pathogens or exposure to abiotic stress
55 factors (e.g. drought). In the last two decades, efforts have been directed towards unraveling the
56 exact physiological role of TLPs. For several TLPs, *in vitro* antifungal activity has been
57 discovered. Experiments with transgenic wheat and rice plants showed that TLPs enhance
58 resistance against various pathogens (19, 20). However, the underlying mechanism is still not
59 fully understood. Some TLPs function by dramatically increasing microbial membrane
60 permeability (21) and β -glucan binding. Also, several TLPs possess hydrolyzing activity (22-25).

61 In the light of the importance of the interaction of xylanase inhibitors with arabinoxylan and β -
62 glucan from a biotechnological as well as a plant physiological point of view, a thorough
63 investigation of the polysaccharide binding and hydrolyzing properties (if any) of the three

different xylanase inhibitors from wheat is important. After all, Rouau et al. (16) tested the binding of XIP for only a few polysaccharides. The fact that TLXI is homologous to the above cited TLPs further indicates the need for this investigation. In this context, the present report focuses on the affinity of all three types of xylanase inhibitors for both β -glucans and (arabino)xylans and whether the inhibitors can hydrolyze these polysaccharides.

Materials and Methods

Materials

Wheat (cultivar Soissons, AVEVE, Landen, Belgium) whole meal was prepared using a Cyclotec 1093 sample mill with a 1.0 mm sieve (Tecator, Hogänäs, Sweden). All electrophoresis media and molecular mass markers were from GE Healthcare (Uppsala, Sweden), unless specified otherwise. Glycoside hydrolase family 11 xylanase (pI 5.5) from *Trichoderma longibrachiatum* (also known as *T. reesei*), i.e. Xyn I (NCBI accession number CAA49294), Xylazyme AX tablets, azo-barley glucan, azo-wheat AX, CM-pachyman and oat β -glucan were from Megazyme (Bray, Ireland). Birchwood xylan, oat spelt arabinoxylan, xylose, zymosan (consisting of protein-carbohydrate complexes from the yeast cell wall of *Saccharomyces cerevisiae*), paramylon, cellulose, pullulan, laminarin, bovine serum albumin (BSA) and all other chemicals were from Sigma-Aldrich (Bornem, Belgium). Pullulan was from Calbiochem (Darmstadt, Germany). Water soluble birchwood xylan and oat spelt arabinoxylan were prepared as described by He et al. (26). Native wheat WE-AX and WU-AX were prepared as described by Moers et al. (27). The insoluble polysaccharides were washed by suspending the polysaccharide (0.5 g) in water (50.0 ml), shaking this suspension for 60 min and centrifugating (15000 g, 10

85 min, 20 °C). The supernatant was removed and this step was repeated twice with the precipitate.
86 The precipitate was freeze-dried before use in the assays.

87 ***Purification of xylanase inhibitors from wheat whole meal***

88 TAXI and XIP were purified from wheat whole meal as described earlier (28) as was TLXI (3).

89 ***Protein content determination***

90 Protein concentrations were determined by the Bradford Coomassie brilliant blue method with
91 BSA as standard (29). For pure TAXI, XIP and TLXI samples, protein concentrations were
92 determined by extinction measurements at 280 nm using specific absorbance values of 0.788,
93 2.442 and 1.457 AU for 1.0 mg/ml TAXI, XIP and TLXI, respectively (1.000 cm UV-cell path
94 length).

95 ***Xylanase inhibition assay (Xylazyme AX method)***

96 Inhibition activities were determined with the colorimetric Xylazyme AX method (28). The
97 xylanase solution was prepared in sodium acetate buffer (25 mM, pH 5.0) with BSA (0.5 mg/ml)
98 and contained 2.0 xylanase units per ml. Xylanase units were defined as described by Gebruers
99 *et al.* (28). Under the conditions of the assay, the xylanase concentration corresponding to 1.0
100 unit Xyn I was approximately 8.9 nM. All measurements were performed in triplicate.

101 ***Electrophoresis***

102 Regular SDS-PAGE

103 SDS-PAGE under non-reducing and reducing conditions was performed on 20 %
104 polyacrylamide gels with a PhastSystem unit, as described in the GE Healthcare separation
105 technique file 110. 2-mercapto-ethanol [5.0 % (v/v)] was added to the sample buffer as reducing

agent. All gels were stained with silver in accordance with the instructions of the manufacturer of the gels (development technique file no. 210).

Substrate SDS-PAGE

Substrate SDS-PAGE for the detection of xylanases was based on protocols by Biely et al. (30), Schwarz et al. (31) and Royer and Nakas (32), using a mini-Protean-3-system (Biorad, Nazareth, Belgium) with slab gels (140.0 mm x 160 mm x 1.5 mm) consisting of a stacking gel (4 % polyacrylamide) on top of a running gel (12 % polyacrylamide). The latter contained 0.28 % (w/v) azo-wheat arabinoxylan or azo-barley β -glucan as substrate. Separation was with a current of 20 mA per gel for 60 min (Power-Pac-200 power supply, Biorad). After separation, the gels were washed in 50 % (v/v) ethanol for 30 minutes and incubated in sodium acetate buffer (25 mM, pH 5.0) at 37 °C until hydrolysis zones became visible.

Analysis of the binding of xylanase inhibitors to polysaccharides

Glucans and insoluble (arabino)xylans

To avoid the unspecific binding of the xylanase inhibitor to the polysaccharides, casein and Tween 20 were used as blocking agents. The polysaccharides (5.0 mg) were suspended in casein/Tween solution [0.5 ml; 0.1 % (w/v) casein with 0.02 % (v/v) Tween in 25 mM sodium acetate buffer pH 5.0]. These suspensions were shaken at room temperature for 60 min. Subsequently, the inhibitor solution (200 μ l; 1.6 μ M in 25 mM sodium acetate buffer, pH 5.0) was added and the mixture was again incubated for 60 min at room temperature. The polysaccharides were removed by centrifugation (15000 g, 10 min, room temperature) and the inhibition activity against Xyn I of the resulting supernatant (100 μ l) was tested with the Xylazyme AX method (cfr. supra). Comparison with the inhibition activity of the reference sample, incubated in the absence of polysaccharides, allowed calculation of the fraction of

129 inhibitor inactivated by interaction with the polysaccharides. This fraction is defined as the
130 inhibitor bound to the polysaccharide. All measurements were performed in triplicate.

131 In addition, samples of the supernatant and the precipitate were analyzed on SDS-PAGE. To this
132 end, the precipitate was washed three times by the addition of sodium acetate buffer (1.0 ml; 25
133 mM, pH 5.0), shaking for 10 min and subsequent centrifugation (15000 g, 10 min, room
134 temperature). The washed precipitate was then incubated at 100 °C in a 1:1 dilution of SDS-
135 PAGE sample buffer (0.25 ml; 4.0 % SDS, 30 % glycerol, 0.04 % bromofenol blue in 125 mM
136 Tris, pH 6,8) in sodium acetate buffer (0.25 ml; 25 mM, pH 5.0) and analyzed on regular SDS-
137 PAGE.

138 Soluble (arabino)xylans – First, the inhibitors (10 µl; 0.5 µM TAXI, 0.2 µM XIP or 0.4 µM
139 TLXI in 25 mM sodium acetate buffer, pH 5.0) were preincubated with the soluble
140 (arabino)xylan (55 µl; 5 mg/ml in 25 mM in sodium acetate buffer, pH 5.0) for 60 min at room
141 temperature. The samples were then put at 40 °C for 10 min, after which the reaction was started
142 by the addition of the xylanase solution (15 µl; 0.25 µM of xylanase in 25 mM sodium acetate
143 buffer, pH 5.0 containing 1 mg/ml BSA). The samples were incubated for different periods of
144 time up to 20 min at 40 °C. The reaction was stopped by adding 120 µl of dinitrosalicylic acid
145 reagent (5.0 g dinitrosalicylic acid and 150.0 g potassium-sodium-tartrate in 500 ml 0.4 M
146 NaOH) and putting the samples on ice. After incubation at 100 °C (5 min) and cooling, the
147 samples were centrifuged for 5 min at 3000 g, 150 µl of each sample was transferred to a
148 microtiterplate and the absorbances were measured at 540 nm against a control (buffer instead of
149 enzyme and inhibitor). The inhibition activities were calculated from the difference in production
150 of reducing sugars between samples with and without inhibitor. The level of inhibitor bound to
151 the soluble substrate was determined by comparing this activity to the inhibition activity

obtained when the inhibitor and enzyme were preincubated and the soluble (arabino)xylan was added in a final step to start the reaction. All measurements were performed in triplicate.

Analysis of the hydrolysis of polysaccharides by xylanase inhibitors

The polysaccharides (5.0 mg) were suspended in the xylanase inhibitor solutions (1.0 ml; up to 2.6 μ M inhibitor in 25 mM sodium acetate buffer, pH 5.0 containing 0.05 % sodium azide). These suspensions were incubated at 40 °C up to 72 h. At different time intervals, samples (125 μ l) were withdrawn and immediately frozen in liquid nitrogen. At the end of the incubation time, all samples were rapidly defrosted and centrifuged (10000 g, 5 min, room temperature). The concentrations of reducing sugars in the supernatants were measured with tetrazolium blue. To this end, samples (50 μ l) were mixed with 4.0 ml tetrazolium blue solution [0.1 % (w/v) tetrazolium blue chloride in 50 mM NaOH containing 500 mM sodium potassium tartrate (33)]. After incubation at 100 °C for 3 min and cooling, the absorbances were measured at 660 nm against controls containing no inhibitor and which were incubated similarly. Standard curves with glucose and xylose were made for assessing the hydrolysis of glucans and (arabino)xylans, respectively (0-1.0 mg/ml).

Results

Binding of the xylanase inhibitors to (arabino)xylans

In a first experiment, the binding of TAXI, XIP or TLXI to different insoluble (arabino)xylans was visualised by comparing the SDS-PAGE profiles of the supernatants obtained after the incubation of xylanase inhibitors with insoluble (arabino)xylans with the SDS-PAGE profiles of the control samples. The fractions bound to the (arabino)xylans were equally analyzed. Figure 1 shows only the part of the SDS-PAGE gel where the inhibitor proteins are visible. These parts

174 correspond to 40 kDa, 30 kDa and 18 kDa, for TAXI, XIP and TLXI, respectively. For wheat
175 WUAX, oat spelt arabinoxylan and birchwood xylan, the inhibitors were clearly present in the
176 precipitate obtained after centrifugation and multiple washing steps. This was interpreted as
177 evidence for the binding of TAXI, XIP and TLXI to all three types of (arabino)xylans. Only for
178 TAXI and XIP, incubated with wheat WUAX, there was still inhibitor present in the supernatant.
179 For all other samples, no inhibitor was visible in this fraction, suggesting that all TAXI, XIP and
180 TLXI was bound.

181 To confirm these results the inhibition activities in the supernatants were compared to those in
182 the control samples. First, the interference of additional soluble arabinoxylan in the Xylazyme
183 assay was ruled out. Even when 750 µg additional WE-AX was added to the samples no
184 influence was seen on the xylanase activity measured with the Xylazyme tablet. The decrease in
185 xylanase activity measured when the supernatant fraction is added to the assay is thus solely
186 caused by the presence of the inhibitor and not by the solubilisation of part of the insoluble
187 arabinoxylan. The results of the inhibition activities in the supernatant show the same trend as
188 with SDS-PAGE (Figure 2), i.e. all types of inhibitors bound to all insoluble (arabino)xylans.
189 Only for wheat WUAX a significantly lower binding interaction was seen.

190 As Birchwood xylan has no arabinose substituent (A/X-ratio of 0.0), oat spelt arabinoxylan has
191 an A/X-ratio of approximately 0.1, and wheat WUAX has an A/X-ratio of approximately 0.5, the
192 results seem to indicate that the binding of the xylanase inhibitors to (arabino)xylans depends on
193 the A/X-ratio, with higher A/X-ratios resulting in reduced binding. However, for TLXI,
194 quantitative binding was also observed for wheat WUAX. The relative binding decreased with
195 increasing A/X-ratios. TAXI and XIP showed lower binding affinities for the three
196 (arabino)xylans than TLXI, but a similar trend was seen.

197 To assess the binding affinity of TAXI, XIP and TLXI for soluble (arabino)xylans, the inhibitors
198 were preincubated with either xylanase or (arabino)xylan for 60 min at room temperature. The
199 reaction was then started by adding the polysaccharide or xylanase, respectively. The increase in
200 the reducing sugar levels was measured for 20 min. Figure 3 shows a typical progress curve
201 obtained in this manner. The inhibition activities were calculated from the differences in the rate
202 of the release of reducing sugars. All three inhibitors were partially inactivated, probably by
203 being bound to the soluble (arabino)xylans (Figure 4). However, the trends were different from
204 those seen for their insoluble counterparts. Under the experimental conditions, the binding of
205 TLXI was still most prominent, but, in contrast to the insoluble (arabino)xylans, the binding of
206 TAXI was somewhat higher than that of XIP. The differences between the different
207 (arabino)xylans were less pronounced. The levels of the binding to birchwood xylan were still
208 the highest, but there was little if any difference between binding to oat spelt and wheat WEAX.

209 ***Binding of the xylanase inhibitors to glucans***

210 Table 1 presents the differences in structure and solubility of the glucans used in present study.
211 The SDS-PAGE profiles of the inhibitor fraction either bound or not bound to the insoluble
212 glucans were compared to those of the control inhibitor samples (Figure 5). In contrast to what
213 was observed with (arabino)xylans, in this case the inhibitors were present in both the
214 supernatant and the precipitate for almost all glucans tested. For some of the glucans, hardly any
215 binding was observed. Zymosan, in contrast, left no inhibitor in the supernatant. In general,
216 TLXI had the highest glucan binding affinity.

217 Much as done for the insoluble (arabino)xylans, a more accurate assessment was made by
218 comparing the inhibition activities in the supernatants after incubation of the xylanase inhibitors
219 with insoluble glucans with those of the control inhibitor samples (Figure 6). For the three types

of inhibitors, a high binding affinity for zymosan was observed. TLXI also bound to paramylon and, to a lesser extent, to oat β -glucan. In contrast to the high binding to zymosan, low binding percentages were seen for the binding of XIP to oat β -glucan and pustulan and for the binding of TAXI to paramylon and cellulose.

Comparison of the above with the results obtained for the soluble glucans (Figure 7) revealed that, in contrast to the insoluble β -(1,3)(1,6)-glucan (zymosan), no binding to the soluble β -(1,3)(1,6)-glucan (laminarin) was detected. The soluble β -(1,3)-glucan, i.e. CM-pachyman, bound with TAXI and TLXI and, to a smaller extent, with XIP. Note that the insoluble β -(1,3)-glucan (paramylon), did not bind to XIP. The binding for TAXI was lower than that for TLXI. Only XIP was able to bind to the α -(1,4)(1,6)-glucan (pullulan).

Hydrolysis of non-starch polysaccharides by the xylanase inhibitors

The ability of the inhibitors to hydrolyze (arabino)xylans and β -glucans was studied. The same polysaccharides used in the above binding experiments were tested with the same three xylanase inhibitors from wheat. None of the polysaccharide-inhibitor combinations resulted in detectable hydrolysis after 72 h of incubation. In the substrate gels with incorporated azo-wheat arabinoxylan or azo-barley- β -glucan, the xylanase inhibitors samples caused no hydrolysis spot became visible (results not shown).

Discussion

Interaction of the xylanase inhibitors with polysaccharides

The binding of XIP to wheat arabinoxylan has been described before. Sancho and co-workers (15) reported XIP to bind to WUAX. In SDS-PAGE, they observed that XIP disappeared from

the liquid phase after incubation with the insoluble non-starch polysaccharide. Rouau et al. (16) performed a more quantitative measurement of the binding of XIP to wheat arabinoxylan and made a distinction between WEAX and WUAX. They observed only a slight reduction of the inhibition activity in the presence of WEAX, while, in the presence of a crude cell wall material preparation [mainly composed of WUAX (53 %)] a strong reduction in inhibition activity was seen.

Our study not only confirmed these results but, in addition, demonstrated that the ability to bind (arabino)xylans is not restricted to XIP, but that TAXI and TLXI are also able to interact. For the three inhibitors, a similar trend was seen, i.e. the affinity increased with decreasing A/X-ratio. This trend was most clear with the insoluble (arabino)xylans. This dependency on A/X-ratio suggested that the interaction between the inhibitor and the xylan chain was more outspoken when no substituents are present. The highest level of binding was observed with TLXI. For XIP, the affinity for the insoluble polysaccharides was much higher than for their soluble counterparts. Though the ratios of inhibitor to polysaccharide used in the present study were in the same order of magnitude as the ones used by Rouau et al. (16), the differences between the binding of XIP to WE-AX and WU-AX were less pronounced in the present study. This is probably due to the use of crude wheat cell wall material by Rouau et al. (16) instead of purified WU-AX. The crude cell wall material contains WUAX with a different structure than the one used in the present study and it may contain other constituents which interfere with the interaction with XIP. It should be noted that the binding of TAXI to wheat arabinoxylan seems to be independent of the solubility of this polysaccharide, but that it was higher for the insoluble oat spelt arabinoxylan and the insoluble birchwood xylan than for their soluble counterparts. TAXI and XIP had comparable affinities for (arabino)xylans.

265 Sancho et al. (15) studied the interaction between XIP and starch (Blue Starch), cellulose and
266 chitin. While XIP was able to interact with starch, no interaction with XIP was observed for
267 chitin and cellulose. Our results were in line with the latter, as also here no interaction between
268 XIP and cellulose could be detected. However, after incubation with pullulan, a linear α -
269 (1,4)(1,6)-glucan, a decrease in inhibition activity was measured, suggesting an interaction
270 between XIP and this polysaccharide.

271 A number of reports describe the interaction of TLPs with β -(1,3)-glucans. Trudel et al. (22)
272 observed that three barley TLPs bind to β -(1,3)-glucans independent of the presence of β -(1,6)
273 linkages. TLP binding polysaccharides included paramylon, zymosan, pachyman and laminarin.
274 The authors did not detect binding to β -(1,3)(1,4)-, β -(1,4)- and β -(1,6)-glucans, like lichenan,
275 cellulose and pustulan, respectively. In line with the above, Osmond et al. (24) did not observe
276 any adsorption of barley PR-5 proteins to chitin, pustulan or cellulose. The observed adsorption
277 to pachyman and curdlan suggested a preference for polysaccharides that predominantly contain
278 linear β -(1,3)-glucans.

279 The results obtained here for TLXI, a wheat TLP, demonstrated a significant level of binding to
280 zymosan and no binding at all to laminarin, two glucans with β -(1,3)(1,6)-bonds. It may be that
281 the interaction between TLXI and zymosan was non-specific and that the binding to this
282 preparation was caused by impurities. After all, the zymosan samples used consisted of almost
283 the entire yeast cell wall of *Saccharomyces cerevisiae*. The results shown allow concluding that
284 TLXI binds to both soluble and insoluble β -(1,3)-glucans and to a lesser extent to insoluble β -
285 (1,3)(1,4)-glucans. For TAXI, low binding to soluble and insoluble β -(1,3)-glucans and insoluble
286 β -(1,4)-glucans was seen, while XIP bound to insoluble β -(1,6)- and β -(1,3)(1,4)-glucan and to
287 soluble β -(1,3)-glucan.

Possible structural features responsible for the interaction

Based on their homologies with PRs, hypotheses can be formulated on the binding sites responsible for the interaction with non-starch polysaccharides, for XIP and TLXI.

XIP shows structural homology to chitinases of class III, which belong to the group of PR-8 proteins. One of the major latex proteins, (*Hevea brasiliensis*) hevamine, displays lysozyme and chitinase activities (34). The structure of hevamine is similar to that of a bacterial chitinase from Glycoside hydrolase family 18 (35). Chitinases may protect the seed against the invasion by chitin-containing pathogenic fungi because the substrates for chitinase are found in some fungal cell walls, but not in plants. For XIP, however, no chitinase activity was observed (36). One of the two catalytic glutamic acid residues of chitinases is nevertheless conserved in the structure of XIP (Glu128_{XIP}) but its side chain is fully engaged in salt bridges with two neighboring arginine residues (Arg163_{XIP} and Arg187_{XIP}). The other catalytic residue, Asp125_{hevamine}, is replaced by Phe126_{XIP}. In addition, the Gly81_{hevamine}, located in the active site at subsite -1, is replaced by Tyr80_{XIP} (36). This tyrosine side chain fills the subsite area and makes a strong hydrogen bond with the side chain of the Glu190_{XIP} located at the opposite site of the cleft, preventing access of a polysaccharide to the catalytic glutamic acid. However, three aromatic residues (Trp255_{hevamine}, Tyr183_{hevamine} and Tyr6_{hevamine}) present in the active centre of hevamine are conserved in the XIP-structure and correspond to Trp256_{XIP}, Tyr189_{XIP} and Phe11_{XIP} (36). The aromatic side chains of Trp255_{hevamine} and Tyr6_{hevamine} stack against the hydrophobic face of chitin-backbone. The hydroxyl group of Tyr183_{hevamine} is within hydrogen-bonding distance of the -1 sugar residue of the substrate and contributes to the catalysis. Since these residues are conserved in the XIP-structure, it is very well possible that they are responsible for the interaction between XIP and polysaccharides. In addition to these three aromatic residues, there are several other residues

311 causing hydrogen-bonding contacts in the cleft of hevamine (atoms of the main chain of 5
312 residues and atoms of the side chain of 3 residues). In the corresponding cleft in the XIP
313 structure, the side chains of the corresponding residues show similar conformations (36).
314 For TLXI, a TLP, a similar exercise can be made. Indeed, for several TLPs the crystal structure
315 has been resolved. Osmond et al. (24) compared the amino acid sequences of two barley TLPs,
316 HvPR5b and HvPR5c. Although their sequences show 60 % homology, they largely differ in
317 their binding activity. While HvPR5c interacts tightly with β -glucans, the interaction for
318 HvPR5b is only weak. Both proteins consist of a flattened β -barrel adjacent to a more flexible
319 region rich in loops and coils which are stabilized by disulfide bonds. A large cleft is present
320 between these two regions. Calculations of the electrostatic potentials at the surfaces of the
321 proteins revealed a concentration of acidic amino acid residues in the cleft. This negatively
322 charged cleft is thought to be the binding site for polysaccharides. The lack of binding of
323 HvPR5b is likely to be explained by the absence of several amino acid residues along the cleft
324 that might form hydrogen bonds with the bound β -glucans. In contrast to these two proteins,
325 TLXI belongs to the group of smaller PR-5 proteins, which are mainly found in cereals (37).
326 These smaller TLPs miss the more flexible region, rich in loops and coils. Hence, the negatively
327 charged cleft is not as distinctive as for the larger TLPs. Reiss et al. (37) describe four of these
328 smaller TLPs from barley. TLXI shows the highest homology to TLP3. TLP3 clearly differs
329 from the other small barley TLPs (TLP1, 2 and 4), in that it lacks the acidic residues (Glu83_{TLP1},
330 Asp93_{TLP1}, Asp101_{TLP1}) present at identical positions in its structure. In TLP3, they are replaced
331 by Gln87_{TLP3}, Tyr95_{TLP3}, Lys103_{TLP3}, respectively. In contrast to what is the case for TLP3, one
332 of the acidic residues is conserved (Glu87_{TLXI}) in TLXI, but the two others are identical to those
333 of TLP3 (Tyr96_{TLXI} and Lys105_{TLXI}). According to Reiss and coworkers (37) these residues

might form the binding site for ligands unidentified at present, possibly β -glucans. In this view, the differences between the small barley TLPs might be an indication for distinct substrate binding specificities between these proteins.

For TAXI, it is more difficult to bring forward a hypothesis on the binding sites responsible for the interaction with non-starch polysaccharides, as it shows homology to aspartic proteases and not to polysaccharide binding proteins. However, non-polar and hydrogen-bond interactions are involved in the binding of proteins to polysaccharides. Over 64 % of the TAXI sequence consists of non-polar and over 20 % of uncharged polar residues (38). It is possible that a combination of some of these residues makes up a sugar binding region.

Relevance of the present findings

In the light of the results obtained here, a hypothesis on the relevance of binding of xylanase inhibitors to non-starch polysaccharides in biotechnological processes such as bread making can be brought forward. As Rouau et al. (16) and Sibbesen and Soerensen (39) already suggested, the difference in the binding affinity of the cereal xylanase inhibitors between soluble and insoluble xylans may be an interesting mechanism for steering the enzyme selectivity and improving the biocatalyst's performance in this process.

However, it should be noted that the conditions in the *in vitro* polysaccharide binding experiments are not the same as in bread making.

First of all, all *in vitro* binding experiments were performed on either soluble or insoluble xylan while, in wheat matrices, these two polysaccharide types coexist. Secondly, while in both systems a large excess of polysaccharide is present, the ratio of polysaccharide to inhibitor is approximately ten times higher in the *in vitro* experiments than that in wheat dough systems. Thirdly, the *in vitro* experiments were performed in excess of water, while in bread making the

357 level of free water is limited, which, on the one hand, may well reduce the mobility of the
358 inhibitors because of viscosity effects, but, on the other hand, because of concentration effects,
359 may well increase the probability of successful encounters and, hence, interactions between the
360 molecules involved. Finally, it is of note that in the *in vitro* experiments there was a
361 preincubation step allowing interaction between the inhibitor and the enzyme or the inhibitor and
362 the substrate, while this is not the case in the bread making process.

363 With regard to their function in the plant, a role in plant defence has been suggested for xylanase
364 inhibitors, though the exact mechanism is not yet understood. Since all three cereal xylanase
365 inhibitors are secreted outside the plant cell (8, 38, 40) and xylans and β -glucans occur in the
366 cereal cell wall, the binding of the inhibitors to these polysaccharides may result in a correct
367 localization of the inhibitors to defend the plant cell against invasion of pathogenic fungi. On the
368 other hand, β -glucans also occur in the cell wall of fungi. Since for TLPs, a relation exists
369 between the binding to these polysaccharides and their antifungal activity, the same
370 argumentation may be used for the xylanase inhibitors, which bind to β -glucans. Moreover,
371 recently, an extracellular peptidic bifunctional xylanase/aspartic protease inhibitor produced by
372 an extremophilic *Bacillus* sp. (ATBI) was shown to have antifungal properties (41). Fungal
373 growth inhibitions correlated well with the xylanase inhibition activities. This would mean that
374 the xylanase inhibitors may have a dual function in plant defence, i.e. the inhibition of the
375 xylanases produced by the intruders and the disturbance of the cell wall of the intruders.

376 ***Abbreviations***

377 WUAX: water-unextractable arabinoxylan; WEAX: water-extractable arabinoxylan; TAXI:
378 Triticum aestivum xylanase inhibitor; XIP: Xylanase inhibitor protein; TLXI: Thaumatin-like
379 xylanase inhibitor; PR: pathogenesis-related protein; TLP: Thaumatin-like protein
380

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Figure captions

Figure 1: SDS-PAGE patterns of TAXI, XIP and TLXI after incubation with insoluble (arabino)xylans. The first lane shows inhibitor control incubated in the absence of polysaccharide (C). The next lanes correspond to supernatant (SN) and precipitate (P) of wheat WUAX, oat spelt arabinoxylan and birchwood xylan after preincubation with inhibitor and centrifugation. The gels were run under reducing conditions and were silver-stained. On the right side of the gels the MM at which the proteins were visible is indicated.

Figure 2: Inactivation of TAXI, XIP and TLXI by interaction with insoluble (arabino)xylans: wheat WUAX (□), oat spelt arabinoxylan (■) and birchwood xylan (■). After one hour incubation of inhibitor with insoluble (arabino)xylans at 25 °C and pH 5.0, the percentage of bound inhibitor was estimated by comparing the inhibition activity in the supernatant of the polysaccharide-inhibitor mixture to the inhibition activity of a control sample.

Figure 3: Typical enzymatic hydrolyses of soluble (arabino)xylans by a *T. longibrachiatum* (Xyn I) xylanase for samples in which no inhibitor was added (■), in which xylanase and inhibitor were preincubated (◆) and in which inhibitor and substrate were preincubated (▲). The represented progress curves were plotted with XIP and wheat WEAX.

Figure 4: Inactivation of TAXI, XIP and TLXI by interaction with soluble (arabino)xylans: wheat WEAX (□), oat spelt arabinoxylan (■) and birchwood xylan (■). After one hour of preincubation of inhibitor with soluble (arabino)xylans at 25 °C and pH 5.0, the percentage of

bound inhibitor was estimated by comparing the inhibition activity in this mixture to the activity of inhibitor preincubated with xylanase instead of (arabino)xylan.

Figure 5: SDS-PAGE patterns of TAXI, XIP and TLXI after incubation with insoluble glucans. The first lane shows the control inhibitor incubated in absence of glucans (C). The next lanes correspond to supernatant (SN) and precipitate (P) of paramylon, oat β -glucan, zymosan, cellulose and pustulan, after incubation with inhibitor and centrifugation. The gels were run under reducing conditions and were silver-stained.

Figure 6: Inactivation of TAXI, XIP and TLXI by interaction with insoluble glucans: Paramylon ($\square$), oat β -glucan ($\blacksquare$), zymosan ($\blacksquare$), cellulose ($\blacksquare$) and pustulan ($\blacksquare$). After one hour of preincubation of inhibitor with insoluble glucans at 25 °C and pH 5.0, the percentage of bound inhibitor was estimated by comparing the inhibition activity in the supernatant of the polysaccharide-inhibitor mixture to the inhibition activity of a control sample.

Figure 7: Inactivation of TAXI, XIP and TLXI by interaction with soluble glucans: CM-pachyman ($\square$), laminarin ($\blacksquare$) and pullulan ($\blacksquare$). After one hour of preincubation of inhibitor with soluble glucans at 25 °C and pH 5.0, the percentage of bound inhibitor was estimated by comparing the inhibition activity in the supernatant of the polysaccharide-inhibitor mixture to the activity of inhibitor incubated similarly without polysaccharide.

542 **Tables**

543

544 Table 1: Structures, sources and solubilities of different glucans

Polysaccharides	Structures	Sources	Solubilities
Paramylon	β -(1,3)-glucan	<i>Euglena gracilis</i>	Insoluble
β -glucan	β -(1,3)(1,4)-glucan	Graminaceae (Oats)	Insoluble
Zymosan	Complex cell wall polysaccharide containing β -(1,3)- and β -(1,6)-glucan	<i>Saccharomyces cerevisiae</i>	Insoluble
Cellulose	β -(1,4)-glucan	Plants	Insoluble
Pustulan	β -(1,6)-glucan	<i>Umbilicaria papulosa</i>	Insoluble
CM-Pachyman	β -(1,3)-glucan	<i>Poria cocos</i>	Soluble
Laminarin	β -(1,3)-glucan, contains some β -(1,6)-linkages	<i>Laminaria digitata</i>	Soluble
Pullulan	α -(1,4)(1,6)-glucan	<i>Aureobasidium pullulans</i>	Soluble

Figures

Figure 1

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Figure 5

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Figure 7