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Xylanase Inhibitors Bind to Nonstarch 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

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 a decreasing arabinose/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 past 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 toward endo-β-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, on the basis of their distinct specificities toward 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 co-workers (*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 strongly depressed inhibition activity in the vicinity of WUAX. In contrast, in the vicinity of WEAX, the XIP activity is not affected. The xylanase is therefore proportionally more active on WUAX (arabinoxylan solubilization) than on WEAX (arabinoxylan depolymerization). The net effect is an increase in the soluble high-molecular-weight arabinoxylan levels, which are beneficial for dough properties, and a larger decrease in WUAX levels, which are detrimental to both dough and bread properties (*17*). The above opens perspectives for improving enzyme performance in bread making, by steering the xylanase selectivity by a specific inhibitor.

In addition, insight in the binding of the xylanase inhibitors to polysaccharides can contribute to our understanding of their functions in plants. After all, for the PR-5 protein family, binding to polysaccharides has been reported to be involved in their antifungal mechanism. Members of this family are also called the thaumatin-like proteins (TLPs) because they are all homologues to thaumatin, a sweet tasting protein found in the arils of fruits of the African shrub *Thaumatococcus daniellii* (*18*).

* To whom correspondence should be addressed. Telephone: +32(0)16321634. Fax: +32(0)16321997. E-mail: ellen.fierens@biw.kuleuven.be.

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Most TLPs are believed to be involved in plant defense because their synthesis is induced upon challenge of the plant by pathogens or exposure to abiotic stress factors (e.g., drought). In the last 2 decades, efforts have been directed toward unraveling the exact physiological role of TLPs. For several TLPs, *in vitro* antifungal activity has been discovered. Experiments with transgenic wheat and rice plants showed that TLPs enhance resistance against various pathogens (*19, 20*). However, the underlying mechanism is still not fully understood. Some TLPs function by dramatically increasing microbial membrane permeability (*21*) and β-glucan binding. Also, several TLPs possess hydrolyzing activity (*22–25*).

In the light of the importance of the interaction of xylanase inhibitors with arabinoxylan and β-glucan from a biotechnological as well as a plant physiological point of view, a thorough 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 paper 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 [National Center for Biotechnology Information (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). Pustulan was from Calbiochem (Darmstadt, Germany). Water-soluble birchwood xylan and oat spelt arabinoxylan were prepared as described by He et al. (*26*). Native wheat WEAX and WUAX were prepared as described by Moers et al. (*27*). The insoluble polysacharides were washed by suspending the polysaccharide (0.5 g) in water (50.0 mL), shaking this supension for 60 min, and centrifugating (15000*g*, 10 min, 20 °C). The supernatant was removed, and this step was repeated twice with the precipitate. The precipitate was freeze-dried before use in the assays.

Purification of Xylanase Inhibitors from Wheat Whole Meal. TAXI and XIP were purified from wheat whole meal as described earlier (*28*), as was TLXI (*3*).

Protein Content Determination. Protein concentrations were determined by the Bradford Coomassie Brilliant Blue method with BSA as the standard (*29*). For pure TAXI, XIP, and TLXI samples, protein concentrations were determined by extinction measurements at 280 nm using specific absorbance values of 0.788, 2.442, and 1.457 AU for 1.0 mg/mL TAXI, XIP, and TLXI, respectively (1.000 cm UV-cell path length).

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

Electrophoresis. *Regular Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE).* SDS−PAGE under nonreducing and reducing conditions was performed on 20% polyacrylamide gels with a PhastSystem unit, as described in the GE Healthcare separation technique file 110. 2-Mercapto-ethanol [5.0% (v/v)] was added to the sample buffer as a reducing agent. All gels were stained with silver in accordance with the instructions of the manufacturer of the gels (development technique file number 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 × 160 × 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 the 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 min 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 at pH 5.0]. These suspensions were shaken at room temperature for 60 min. Subsequently, the inhibitor solution (200 μL; 1.6 μM in 25 mM sodium acetate buffer at 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 μL) was tested with the xylazyme AX method (cfr. supra). A comparison with the inhibition activity of the reference sample, incubated in the absence of polysaccharides, allowed for a calculation of the fraction of inhibitor inactivated by the interaction with the polysaccharides. This fraction is defined as the inhibitor bound to the polysaccharide. All measurements were performed in triplicate.

In addition, samples of the supernatant and the precipitate were analyzed on SDS−PAGE. To this end, the precipitate was washed 3 times by the addition of sodium acetate buffer (1.0 mL; 25 mM, pH 5.0), shaking for 10 min and subsequent centrifugation (15000*g*, 10 min, room temperature). The washed precipitate was then incubated at 100 °C in a 1:1 dilution of SDS−PAGE sample buffer (0.25 mL; 4.0% SDS, 30% glycerol, 0.04% bromofenol blue in 125 mM Tris at pH 6.8) in sodium acetate buffer (0.25 mL; 25 mM, pH 5.0) and analyzed on regular SDS−PAGE.

Soluble (Arabino)xylans. First, the inhibitors (10 μL; 0.5 μM TAXI, 0.2 μM XIP, or 0.4 μM TLXI in 25 mM sodium acetate buffer at pH 5.0) were preincubated with the soluble (arabino)xylan (55 μL; 5 mg/mL in 25 mM in sodium acetate buffer at pH 5.0) for 60 min at room temperature. The samples were then placed at 40 °C for 10 min, after which the reaction was started by the addition of the xylanase solution (15 μL; 0.25 μM of xylanase in 25 mM sodium acetate buffer at pH 5.0 containing 1 mg/mL BSA). The samples were incubated for different periods of time up to 20 min at 40 °C. The reaction was stopped by adding 120 μL of dinitrosalicylic acid reagent (5.0 g of dinitrosalicylic acid and 150.0 g of potassium−sodium−tartrate in 500 mL of 0.4 M NaOH) and placing the samples on ice. After incubation at 100 °C (5 min) and cooling, the samples were centrifuged for 5 min at 3000*g*, 150 μL of each sample was transferred to a microtiter plate, and the absorbances were measured at 540 nm against a control (buffer instead of enzyme and inhibitor). The inhibition activities were calculated from the difference in production of reducing sugars between samples with and without inhibitor. The level of inhibitor bound to 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 μM inhibitor in 25 mM sodium acetate buffer at pH 5.0 containing 0.05% sodium azide). These suspensions were incubated at 40 °C up to 72 h. At different time

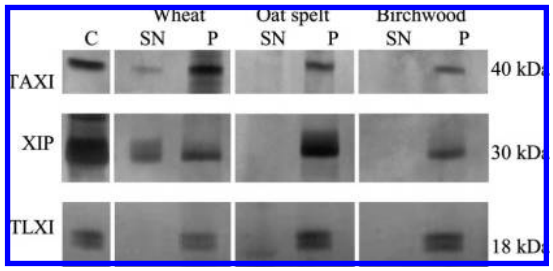

Figure 1. SDS−PAGE patterns of TAXI, XIP, and TLXI after incubation with insoluble (arabino)xylans. The first lane shows the inhibitor control incubated in the absence of polysaccharide (C). The next lanes correspond to the supernatant (SN) and precipitate (P) of wheat WUAX, oat spelt arabinoxylan, and 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 molecular mass (MM) at which the proteins were visible is indicated.

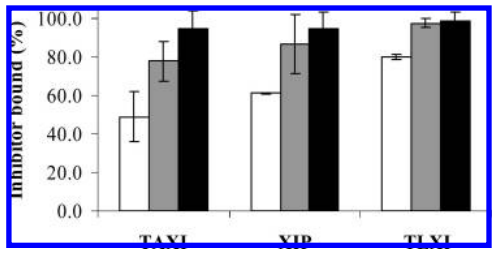

Figure 2. Inactivation of TAXI, XIP, and TLXI by the interaction with insoluble (arabino)xylans: wheat WUAX (white bar), oat spelt arabinoxylan (gray bar), and birchwood xylan (black bar). After 1 h of incubation of inhibitor with insoluble (arabino)xylans at 25 °C and pH 5.0, the percentage of bound inhibitor was estimated by comparing the inhibition activity in the supernatant of the polysaccharide−inhibitor mixture to the inhibition activity of a control sample.

intervals, samples (125 μL) 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 μL) 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 visualized 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 correspond to 40, 30, and 18 kDa for TAXI, XIP, and TLXI, respectively. For wheat WUAX, oat spelt arabinoxylan, and birchwood xylan, the inhibitors were clearly present in the precipitate obtained after centrifugation and multiple washing steps. This was interpreted as evidence for the binding of TAXI, XIP, and TLXI to all three types of (arabino)xylans. Only for TAXI and XIP, incubated with wheat WUAX, there was still inhibitor present

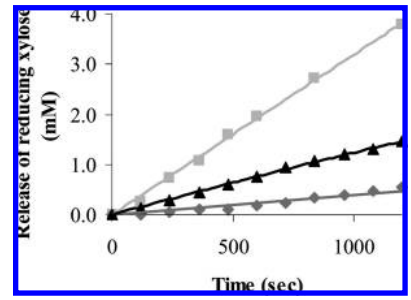

Figure 3. Typical enzymatic hydrolyses of soluble (arabino)xylans by a *T. longibrachiatum* (*Xyn* I) xylanase for samples in which no inhibitor was added (■), in which xylanase and inhibitor were preincubated (◆), and in which inhibitor and substrate were preincubated (▲). The represented progress curves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

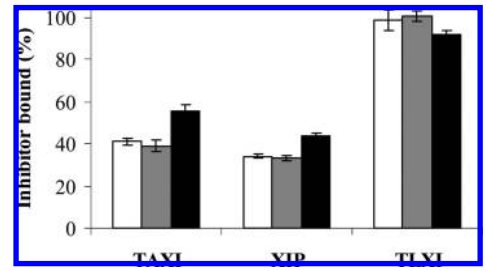

Figure 4. Inactivation of TAXI, XIP, and TLXI by the interaction with soluble (arabino)xylans: wheat WEAX (white bar), oat spelt arabinoxylan (gray bar), and birchwood xylan (black bar). After 1 h of preincubation of inhibitor with soluble (arabino)xylans at 25 °C and pH 5.0, the percentage of bound inhibitor was estimated by comparing the inhibition activity in this mixture to the activity of inhibitor preincubated with xylanase instead of (arabino)xylan.

in the supernatant. For all other samples, no inhibitor was visible in this fraction, suggesting that all TAXI, XIP, and TLXI were bound.

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

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

To assess the binding affinity of TAXI, XIP, and TLXI for soluble (arabino)xylans, the inhibitors were preincubated with either xylanase or (arabino)xylan for 60 min at room temper-

Table 1. Structures, Sources, and Solubilities of Different Glucans

polysaccharides	structures	sources	solubilities
paramylon	β-(1,3)-glucan	*Euglena gracilis*	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	*Saccharomyces cerevisiae*	insoluble
cellulose	β-(1,4)-glucan	plants	insoluble
pustulan	β-(1,6)-glucan	*Umbilicaria papulosa*	insoluble
CM-pachyman	β-(1,3)-glucan	*Poria coccos*	soluble
laminarin	β-(1,3)-glucan, contains some β-(1,6)-linkages	*Laminaria digitata*	soluble
pullulan	α-(1,4)(1,6)-glucan	*Aureobasidium pullulans*	soluble

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

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

Much as done for the insoluble (arabino)xylans, a more accurate assessment was made by comparing the inhibition activities in the supernatants after incubation of the xylanase inhibitors 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.

A 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 Nonstarch 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 to become 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 nonstarch 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

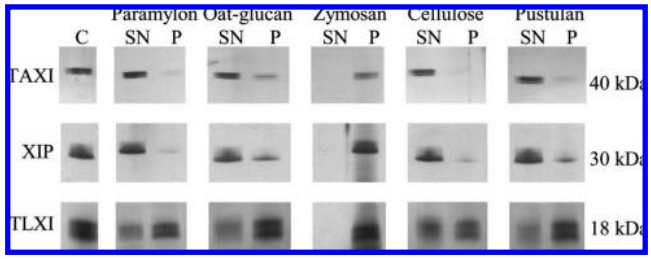

Figure 5. SDS−PAGE patterns of TAXI, XIP, and TLXI after incubation with insoluble glucans. The first lane shows the control inhibitor incubated in the absence of glucans (C). The next lanes correspond to the supernatant (SN) and precipitate (P) of paramylon, oat β-glucan, zymosan, cellulose, and pustulan,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

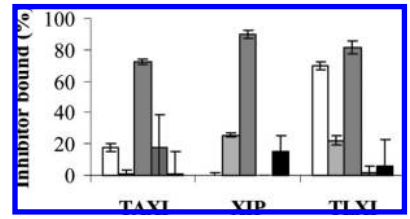

Figure 6. Inactivation of TAXI, XIP, and TLXI by the interaction with insoluble glucans: paramylon (white bar), oat β-glucan (light gray bar), zymosan (medium gray bar), cellulose (dark gray bar), and pustulan (black bar). After 1 h of preincubation of inhibitor with insoluble glucans at 25 °C and pH 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.

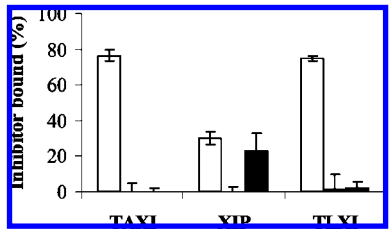

Figure 7. Inactivation of TAXI, XIP, and TLXI by the interaction with soluble glucans: CM-pachyman (white bar), laminarin (gray bar), and pullulan (black bar). After 1 h of preincubation of inhibitor with soluble glucans at 25 °C and pH 5.0, the percentage of bound inhibitor was estimated by comparing the inhibition activity in the supernatant of the polysaccharide−inhibitor mixture to the activity of inhibitor incubated similarly without polysaccharide.

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 a decreasing A/X ratio. This trend was most clear with the insoluble (arabino)xylans. This dependency on the 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. Although the ratios of inhibitor/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 WEAX and WUAX 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 WUAX. 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.

Sancho et al. (*15*) studied the interaction between XIP and starch (blue starch), cellulose, and chitin. While XIP was able to interact with starch, no interaction with XIP was observed for chitin and cellulose. Our results were in line with the latter, because no interaction between XIP and cellulose could be detected. However, after incubation with pullulan, a linear α-(1,4)(1,6)-glucan, a decrease in inhibition activity was measured, suggesting an interaction between XIP and this polysaccharide.

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

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

Possible Structural Features Responsible for the Interaction. On the basis of their homologies with PRs, hypotheses can be formulated on the binding sites responsible for the interaction with nonstarch 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 center 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 the chitin backbone. The hydroxyl group of $Tyr183_{hevamine}$ is within hydrogen-bonding distance of the −1 sugar residue of the substrate and contributes to catalysis. Because these residues are conserved in the XIP structure, it is very possible that they are responsible for the interaction between XIP and polysaccharides. In addition to these three aromatic residues, there are several other residues causing hydrogen-bonding contacts in the cleft of hevamine (atoms of the main chain of five residues and atoms of the side chain of three residues). In the corresponding cleft in the XIP structure, the side chains of the corresponding residues show similar conformations (*36*).

For TLXI, a TLP, a similar exercise can be made. Indeed, for several TLPs, the crystal structure has been resolved. Osmond et al. (*24*) compared the amino acid sequences of two barley TLPs, HvPR5b and HvPR5c. Although their sequences show 60% homology, they largely differ in their binding activity. While HvPR5c interacts tightly with β-glucans, the interaction for HvPR5b is only weak. Both proteins consist of a flattened β-barrel adjacent to a more flexible region rich in loops and coils, which are stabilized by disulfide bonds. A large cleft is

present between these two regions. Calculations of the electrostatic potentials at the surfaces of the proteins revealed a concentration of acidic amino acid residues in the cleft. This negatively charged cleft is thought to be the binding site for polysaccharides. The lack of binding of HvPR5b is likely to be explained by the absence of several amino acid residues along the cleft that might form hydrogen bonds with the bound β-glucans. In contrast to these two proteins, TLXI belongs to the group of smaller PR-5 proteins, which are mainly found in cereals (*37*). These smaller TLPs miss the more flexible region, rich in loops and coils. Hence, the negatively charged cleft is not as distinctive as the larger TLPs. Reiss et al. (*37*) describe four of these smaller TLPs from barley. TLXI shows the highest homology to TLP3. TLP3 clearly differs from the other small barley TLPs (TLP1, TLP2, and TLP4), in that it lacks the acidic residues ($Glu83_{TLP1}$, $Asp93_{TLP1}$, and $Asp101_{TLP1}$) present at identical positions in its structure. In TLP3, they are replaced by $Gln87_{TLP3}$, $Tyr95_{TLP3}$, and $Lys103_{TLP3}$, respectively. In contrast to what is the case for TLP3, one of the acidic residues is conserved ($Glu87_{TLXI}$) in TLXI, but the two others are identical to those of TLP3 ($Tyr96_{TLXI}$ and $Lys105_{TLXI}$). According to Reiss and co-workers (*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 nonstarch polysaccharides, because it shows homology to aspartic proteases and not polysaccharide binding proteins. However, nonpolar and hydrogen-bond interactions are involved in the binding of proteins to polysaccharides. Over 64% of the TAXI sequence consists of nonpolar residues, and over 20% of the TAXI sequence consists 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 light of the results obtained here, a hypothesis on the relevance of binding of xylanase inhibitors to nonstarch 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 performance of the biocatalyst 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. Second, while in both systems, a large excess of polysaccharide is present, the ratio of polysaccharide to inhibitor is approximately 10 times higher in the *in vitro* experiments than that in wheat dough systems. Third, the *in vitro* experiments were performed in excess of water, while in bread making, the level of free water is limited, which, on the one hand, may well reduce the mobility of the inhibitors because of viscosity effects but, on the other hand, because of concentration effects, may well increase the probability of successful encounters and, hence, interactions between the molecules involved. Finally, it is of note that in the *in vitro* experiments there was a preincubation step allowing for the interaction between the inhibitor and the enzyme or the inhibitor and the substrate, while this is not the case in the bread-making process.

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

ABBREVIATIONS USED

WUAX, water-unextractable arabinoxylan; WEAX, water-extractable arabinoxylan; TAXI, *Triticum aestivum* xylanase inhibitor; XIP, xylanase inhibitor protein; TLXI, thaumatin-like xylanase inhibitor; PR, pathogenesis-related protein; TLP, thaumatin-like protein.

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