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ARTICLE

Algerian Pearl Millet (*Pennisetum glaucum* L.) Contains XIP but Not TAXI and TLXI Type Xylanase Inhibitors

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An XIP (xylanase inhibiting protein) type xylanase inhibitor was purified from Algerian pearl millet (*Pennisetum glaucum* L.) grains and characterized for the first time. Cation exchange and affinity chromatography with immobilized *Trichoderma longibrachiatum* glycoside hydrolase (GH) family 11 xylanase resulted in electrophoretically pure protein with a molecular mass of 27−29 kDa and a p*I* value of 6.7. The experimentally determined N-terminal amino acid sequence of the purified XIP protein is 87.5%, identical to that of sorghum (*Sorghum bicolor* L.) XIP and 79.2% identical to that of wheat (*Triticum aestivum* L.) XIP-I. The biochemical properties of pearl millet XIP are comparable to those described earlier for sorghum XIP, except for the higher specific activity toward a *T. longibrachiatum* GH family 11 xylanase. On the basis of immunoblot neither TAXI nor TLXI type xylanase inhibitors were detected in pearl millet grains.

KEYWORDS: Pearl millet; xylanase inhibitor; XIP

INTRODUCTION

FAO estimated the global production of millets in 2007 at 31.9 million tonnes, ranking them as the sixth most cultivated cereals after maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and sorghum (*Sorghum bicolor* L. Moench). Millets can be found in parts of Asia, Africa, China, and Russia (*1*, *2*). They are well suited for cultivation on less fertile soils and under poor growing conditions, such as intense heat and drought (*3*, *4*). These small-seeded cereal crops are harvested for food [leavened or unleavened baked goods, porridges, boiled, or steamed foods (couscous, pasta), snacks], beverage (alcoholic and nonalcoholic), and feed purposes (*2*). They include species in several genera, mostly in the subfamily Panicoideae of the grass family Poaceae (*5*). The Panicoideae subfamily also includes cereals such as maize (*Z. mays* L.) and sorghum (*5*). Widely cultivated millet species in terms of worldwide production are, in decreasing order, pearl millet (*Pennisetum glaucum* L.), foxtail millet (*Setaria italica* L.), proso millet (*Panicum miliaceum* L.), and finger millet (*Eleusine coracana* L.) (*2*, *6*). Pearl millet is very often the most productive cereal crop on the driest and most infertile soils in India and Africa. Finger millet belongs to the Chloridoideae subfamily.

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In the past decade, the presence of significant levels of proteinaceous xylanase inhibitors in a number of cereals has been demonstrated (see below) (*7*−*14*). These proteins prevent the internal hydrolysis of the xylan backbone of cereal cell wall arabinoxylan (AX) by xylanases. To date, three types of cereal proteinaceous xylanase inhibitors have been successfully purified and characterized. They typically have alkaline p*I* values. The *T. aestivum* xylanase inhibitor (TAXI) type proteins occur in common (*8*, *15*, *16*) and durum wheat (*Triticum durum* Desf.), rye (*Secale cereale* L.), and barley (*10*, *12*), have a molecular mass (MM) of approximately 40 kDa, and inhibit xylanases belonging to glycoside hydrolase (GH) family 11. The xylanase inhibiting proteins (XIPs), studied in wheat (*17*, *18*), rye, barley, rice, maize (*11*, *13*), and sorghum (*9*), have MMs of approximately 30 kDa and inhibit microbial GH family 10 and 11 xylanases (*19*). The recently identified thaumatin-like xylanase inhibitor (TLXI) type proteins are approximately 18 kDa, inhibit GH family 11 xylanases, and have, so far, been revealed only in common wheat (*7*). Cereal xylanases, belonging to GH family 10, are not affected by any of the three types of inhibitors.

Studies have in the meantime generated insight into the potential physiological role and technological relevance of xylanase inhibitors. Indirect evidence for the involvement of xylanase inhibitors in plant defense has been provided (*20*, *21*). They would prevent pathogenic micro-organisms from penetrating the outer tissues of the host by preventing hydrolysis of AX. From a

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technological point of view, they strongly compromise the action of some microbial xylanases used in cereal-based applications to improve processing and/or final product quality (*22*−*25*). Xylanases are routinely used in breadmaking (*22*), where they support gluten functionality by affecting the structure of AX (*26*, *27*). In millet or pearl millet wheat composite bread systems (*2*, *28*), which evidently contain lower gluten levels than regular wheat breads, xylanases may have a strong impact on dough and bread characteristics.

As AX is also the major cell wall component in pearl millet (*28*−*30*), the presence of xylanase inhibitors in this cereal can be expected. Until now, however, they have not been identified in pearl millet. Therefore, the aim of this study is to assess the occurrence of xylanase inhibitors in this crop and to purify and biochemically characterize them. The results obtained can be of immediate use in directing xylanase technologies in composite breadmaking systems.

MATERIALS AND METHODS

Materials. Pearl millet was from Ain Salah, Algeria (harvest year 2005). The grains were ground into whole meal using a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden). All electrophoresis and chromatographic media and MM and p*I* markers were from GE Healthcare (Uppsala, Sweden), unless specified otherwise. The GH family 11 xylanases from *Aspergillus niger* (Quest International, Naarden, The Netherlands) (NCBI accession no. CAA01470) and *Bacillus subtilis* (Danisco, Brabrand, Demark) (AAA22897) were those purified by Gebruers et al. (*15*, *17*). The GH family 10 xylanases of *Aspergillus oryzae* (BAA75475) and *Penicillium purpurogenum* (AAF71268) were kindly made available by Prof. Maija Tenkanen (University of Helsinki, Finland) and Prof. Jaime Eyzaguirre (Departamento de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile), respectively. *A. niger* GH 10 xylanase was purified from an *A. niger* CBS 110.42 culture filtrate (*24*). An *Aspergillus aculeatus* GH family 10 xylanase [similar to xylanase II described by Kofod et al. (*31*), AAC12003] was supplied by F. Arnaut (NV Puratos, Groot-Bijgaarden, Belgium). GH family 11 xylanases from *Trichoderma viride* (CAB60757) and *Trichoderma longibrachiatum* [p*I* 5.5 (CAA46294) and p*I* 9.0 (CAA49293)], and Xylazyme-AX substrate tablets were purchased from Megazyme (Bray, Ireland). Activated nitrocellulose and polyvinylidene fluoride (PVDF) membranes were from Schleicher & Schuell (Dassel, Germany). Goat anti-rabbit antibodies conjugated to horseradish peroxidase, substrate (3,3′,5,5′-tetramethylbenzidine) for the horseradish peroxidase, bovine serum albumin (BSA), and all other reagents and chemicals were from Sigma-Aldrich (Bornem, Belgium) and of analytical grade.

Extract Preparation. Whole meal was extracted for 16 h at 7 °C in 5 volumes of sodium acetate buffer (25 mM, pH 5.0), centrifuged (10000*g*, 7 °C, 30 min), and filtered. The pH of the extract was adjusted to 5.0 with 1.0 M HCl.

Protein Quantification. Protein concentrations were determined according to the Bradford Coomassie brilliant blue method with BSA as standard (*32*). For purified XIP samples, protein concentrations were determined by measuring extinctions at 280 nm using a specific absorbance value of 2.348 AU for 1.0 mg/mL XIP (1.000 cm UV-cell path length), calculated from the complete sorghum XIP amino acid sequence (*9*) using the ProtParam tool (*33*). This sequence was used because of its high similarity with the pearl millet XIP sequence at the N-terminus (see below).

Immunoblot. The rabbit polyclonal antibodies (PAbs) against native TAXI, XIP, and TLXI used in this study were those prepared by Beaugrand et al. (*34*). Crude whole meal extract samples of pearl millet (50.0 μg of protein) and pure TAXI (0.50 μg), XIP (0.50 μg), and TLXI (0.50 μg) from wheat (controls) were separated with SDS-PAGE in 12% polyacrylamide gels under reducing conditions [5% (v/v) 2-mercaptoethanol] using a Bio-Rad (Hercules, CA) Mini Protean 2 device. The proteins were electroblotted at room temperature for 25 min at 16 V onto activated nitrocellulose membranes (Protran Schleicher and Schuell, Dassel, Germany) using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Nazareth, Belgium) and were subsequently probed with anti-TAXI, anti-XIP, and anti-TLXI PAbs as described by Beaugrand et al. (*34*). MultiMark colored MM standards from 6 to 250 kDa (Invitrogen, Carlsbad, CA) were used. These standards were used only to estimate the efficiency of electrotransfer onto the nitrocellulose membrane. Use of these markers tends to overestimate the MM (see manufacturer's guidelines) of 40 (TAXI) and 30 kDa (TAXI and XIP) polypeptides.

Xylanase Inhibition Assay. Inhibition activities were determined with a colorimetric Xylazyme-AX method (*8*). All xylanase solutions were prepared in sodium acetate buffer (25 mM, pH 5.0) with BSA (0.5 mg/mL) and contained 2.0 xylanase units per 1.0 mL. Xylanase units were as defined by Gebruers et al. (*15*, *17*) and, under the conditions of the assay, the xylanase concentrations corresponding to 1.0 unit were approximately 5.1 nM for the *A. niger*, 8.9 nM for the *T. longibrachiatum* (p*I* 5.5), 2.1 nM for the *T. longibrachiatum* (p*I* 9.0), 11.6 nM for the *T. viride*, and 5.8 nM for the *B. subtilis* GH family 11 xylanases. The corresponding concentrations for the GH family 10 xylanases were 36.6 nM for the *A. niger*, 2.9 nM for the *A. oryzae*, 17.7 nM for the *P. purpurogenum*, and 53.7 nM for the *A. aculeatus* enzymes. All measurements were performed in triplicate. Xylanase inhibition activity is often expressed in xylanase inhibitor units (XIU), corresponding to the amount of inhibitor needed to inhibit a given xylanase for 50% in the Xylazyme-AX method. The $[I]/[E]_{50}$, that is, the molar inhibitor (I) over enzyme (E) ratio resulting in 50% inhibition, is a measure for the efficiency of an inhibitor.

To determine the temperature and pH conditions for maximal inhibition activity, the Xylazyme-AX assay was performed at different temperatures at pH 5.0 and at different pH conditions at 40 °C, respectively. For the latter, universal buffers of pH 3.0−8.0 instead of the above-cited sodium acetate buffer were used. The buffers were prepared from a stock solution of 30 mM citric acid, 30 mM KH_2PO_4, 30 mM H_3BO_3, and 20 mM diethyl barbituric acid by adding HCl or NaOH (2.0 M) solutions.

Affinity Matrix Preparation. Before immobilization, *T. longibrachiatum* GH 11 xylanase (p*I* 5.5) was dialyzed overnight against deionized water. The affinity matrix was prepared with *N*-hydroxysuccinimide activated Sepharose 4 Fast Flow resin (5.0 mL) as described by Gebruers et al. (*9*).

Purification of Xylanase Inhibitor. The proteins with xylanase inhibition activity were purified from the above-described crude extract (approximately 5 L). A protein fraction enriched in pearl millet XIP was retained by cation exchange chromatography (CEC) on a SP Sepharose Big Beads column [180 × 130 mm, equilibrated with sodium acetate buffer (25.0 mM, pH 4.5)]. Elution of the bound protein fraction was performed in one step with NaCl solution (1.0 M, 1.0 L), resulting in the CEC protein fraction. This fraction was divided into 50 mL portions and kept in the freezer until further processing.

After thawing, the portions were diluted 1:2 in sodium acetate buffer (25.0 mM, pH 5.0) and loaded separately on the *T. longibrachiatum* xylanase affinity column [10 × 65 mm, equilibrated with sodium acetate buffer (25.0 mM, pH 5.0, containing 0.2 M NaCl), flow rate of 0.33 mL/min]. XIP proteins were eluted from the column with Tris solution (250 mM, pH 11.0, 5.0 mL, flow rate of 1.0 mL/min) and immediately neutralized with acetic acid solution (1.0 M). The protein fraction eluted during affinity chromatography (AC) is further referred to as the AC protein fraction or purified pearl millet XIP.

Electrophoresis. SDS-PAGE (*35*) under reducing conditions was performed on 20% polyacrylamide gels with a PhastSystem unit and low MM markers (14.4−97.0 kDa) (GE Healthcare, separation technique file 110). 2-Mercaptoethanol (5.0% v/v) was used as reducing agent. The p*I* was determined with the same instrument using polyacrylamide gels containing ampholytes (pH 3.0−9.0) and appropriate standards (broad p*I* kit: p*I* 3.5−9.3) (GE Healthcare, separation technique file 100). The gels were silver stained as described in GE Healthcare development technique file 210.

Protein Sequencing. To determine the N-terminal amino acid sequence, inhibitor protein (approximately 50 μg) was separated under reducing conditions [5% (v/v) 2-mercaptoethanol] with SDS-PAGE in a 12% polyacrylamide gel using the Hoeffer Mighty Small unit (GE Healthcare) (electric potential difference of 160 V, 1 h, room temperature),

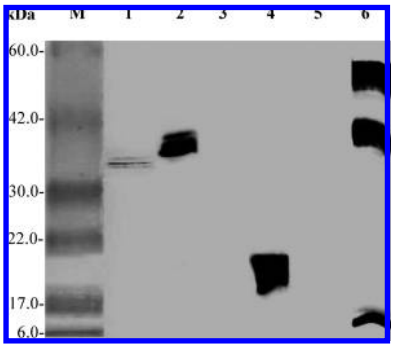

Figure 1. Immunoblot of pearl millet whole meal crude extract proteins (lanes 1, 3, and 5) and pure XIP (lane 2), TLXI (lane 4), and TAXI (lane 6) proteins from wheat as controls. Probing was performed with PAbs from rabbits immunized with the purified wheat inhibitors. Lanes: M, MultiMark colored MM standards (5 μL); 1, pearl millet crude extract proteins (~50 μg) probed with anti-XIP PAbs; 2, wheat XIP protein (~0.5 μg) probed with anti-XIP PAbs; 3, pearl millet crude extract proteins (~50 μg) probed with anti-TLXI PAbs; 4, wheat TLXI protein (~0.5 μg) probed with anti-TLXI PAbs; 5: pearl millet crude extract proteins (~50 μg) probed with anti-TAXI PAbs; 6, wheat TAXI protein (~0.5 μg) probed with anti-TAXI PAbs. The MultiMark colored MM standards were used only to estimate the efficiency of electrotransfer onto the nitrocellulose membrane. Use of these markers tends to overestimate MM (see manufacturer's guidelines) of the 40 (TAXI) and 30 kDa (TAXI and XIP) polypeptides.

electroblotted onto a PVDF membrane with the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) (electric potential difference of 10 V, 1 h, room temperature), and subjected to Edman degradation after protein visualization by Coomassie staining. Sequence analysis was performed on a Procise cLC 491 Sequencer (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Xylanase Inhibitor Detection in Pearl Millet Grains. Figure 1 shows the immunoblot of pearl millet grain extract proteins and pure wheat TAXI, XIP, and TLXI (controls) probed with rabbit PAbs raised against the purified wheat xylanase inhibitors (*34*). Probing of the pearl millet proteins of crude extract (50 μg) with anti-XIP PAbs resulted in a faint triplet (**Figure 1**, lane 1) at slightly lower MM than that of wheat XIP (**Figure 1**, lane 2). However, no bands appeared when the millet proteins were probed with anti-TLXI and -TAXI PAbs (**Figure 1**, lanes 3 and 5, respectively). The intense bands on immunoblot with wheat TLXI and TAXI demonstrate that the antibodies are highly reactive (**Figure 1**, lanes 4 and 6, respectively). Earlier work by Beaugrand et al. (*34*) showed that the antibodies very well recognize TLXI and TAXI proteins from other cereals. These results suggest that pearl millet, in analogy with sorghum (*9*), contains only XIP type xylanase inhibitors. The lack of TAXI proteins in pearl millet is supported by the absence of inhibition activity toward *B. subtilis* GH family 11 xylanase in grain crude extracts. After all, the *B. subtilis* xylanase is strongly inhibited by all TAXI type proteins isolated from different cereal species (*8*, *10*). In contrast, the activity of *P. purpurogenum* GH family 10 xylanase was strongly affected by pearl millet grain proteins, which is typical for XIP type inhibitors (*19*). The pearl millet whole meal contained approximately 1580 XIU/g toward the *P. purpurogenum* enzyme. Similar to sorghum, very low, if any, inhibition activity could be measured toward the *A. aculeatus* GH family 10 and *A. niger* GH family 11 xylanases under the assay conditions (*9*).

The above-discussed results demonstrate that pearl millet grains contain only XIP type xylanase inhibitors in levels detectable with immunoblot and the colorimetric Xylazyme-AX inhibitor assay.

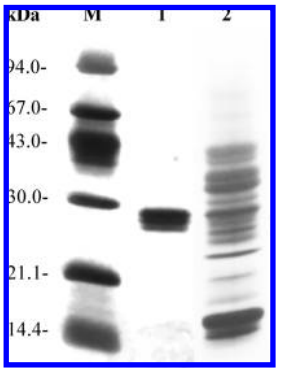

Figure 2. SDS-PAGE profiles of affinity-purified pearl millet XIP (AC fraction, lane 1) and pearl millet crude extract proteins (lane 2). The sizes of the MM markers (lane M) are indicated on the left.

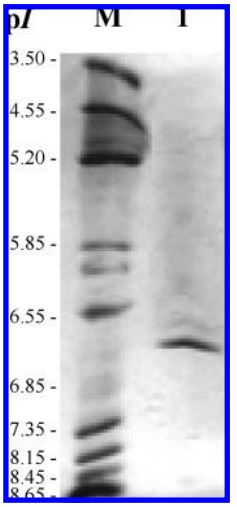

Figure 3. Isoelectric focusing gel of purified pearl millet XIP (lane 1). The p*I* values of the markers (lane M) are indicated on the left. The band visible between p*I* 6.55 and 5.85 in lane M is an artifact and corresponds to the site of sample application.

Xylanase Inhibitor Purification. The pearl millet XIP proteins were purified from whole meal using CEC and *T. longibrachiatum* GH family 11 xylanase (p*I* 5.5) AC. The latter enzyme was chosen because the inhibition activity toward *A. niger* GH family 11 xylanase in pearl millet whole meal extracts, if present, was very low, whereas considerable inhibition activity toward the *T. longibrachiatum* xylanase (p*I* 5.5) could be measured (results not shown). A similar protocol was used earlier for the purification of sorghum XIP (*9*), whereas XIP proteins were isolated from wheat, barley, and rye using *A. niger* GH family 11 xylanase AC (*11*, *17*).

Analysis of the purified pearl millet xylanase inhibitor by SDS-PAGE under reducing conditions (**Figure 2**) resulted in a triplet at 27−29 kDa, which is somewhat lower than the MM of XIP proteins from other cereals (29−32 kDa) (*11*, *13*, *17*). This is in line with the above-described observations on immunoblot. The occurrence of multiple bands with SDS-PAGE is typical for XIP proteins as they often carry glycan moieties (*36*, *37*). Pearl millet XIP gives only one band with isoelectric focusing that corresponds to an estimated p*I* value of approximately 6.7 (**Figure 3**), which is rather low for XIP proteins but in the same order of magnitude as p*I* values of XIP proteins from maize and sorghum, which belong to the same grass subfamily (see above).

Table 1. Overview of the Purification of XIP from Pearl Millet Whole Meal Monitored by Assessment of Total Protein and Inhibition Activity toward *P. purpurogenum* GH Family 10 Xylanase; Specific Inhibition Activity and Recovery of Inhibition Activity Are Also Presented

	total protein (mg/kg)	total inhibition activity ($\times 10^3$ XIU[a]/kg)	specific inhibition activity (XIU/mg)	recovery (%)
crude extract	3150	750	240	100
CEC[b] fraction	550	340	620	45
AC[c] fraction	12	59	4900	8

[a] One XIU (xylanase inhibitor unit) corresponds to the amount of inhibitor needed for 50% inhibition of a xylanase according to the Xylazyme-AX inhibition assay described under Materials and Methods. [b] CEC, cation exchange chromatography. [c] AC, affinity chromatography.

Table 2. Experimentally Determined Pearl Millet (*Pennisetum glaucum* L.) XIP N-Terminal Amino Acid Sequence Aligned with Sequences of (Putative) XIPs from Other Cereals

	NCBI Accession no.	Amino acid sequence	Identity[a] (%)	Similarity[a] (%)
Pennisetum glaucum*	n.a.[i]	AGNKTGQVTVFXGRNKDEXTLREA	--	--
Panicum virgatum	FL886376	AGNKTGQVTVFWGRNKDEGTLREA	91.7	91.7
P. virgatum	FL883377	AGNKTGQVTVFWRRNKDEGTLREA	87.5	87.5
Sorghum bicolor*	CN138888	AGNKTGQVTVFWGRNKAEGTLREA	87.5	87.5
Zea mays[b]	DQ245902	AGNKTGQVTVFWGRNKAEGTLREA	87.5	87.5
Triticum aestivum	CA486546	AGNKTGQVTVFWGRNKAEGTLREA	87.5	87.5
Saccharum officinarum	CA095754	AGNKTGQVTVFWGRNKAEGTLREA	87.5	87.5
Oryza sativa[b]	CT836240	AVGKTGQVTVFWGRNKDEGTLREA	83.3	83.3
***T. aestivum* (XIP-I)**[c,*]	AJ422119	AGGKTGQVTVFWGRNKAEGSLREA	79.2	83.3
Z. mays[d,*]	n.a.	AGRKTGQVTVFXXR	78.6	85.7
Hordeum vulgare[d,*]	BY845439	AGGKTGQVTVFWGRNKAEGSL	76.1	81.0
***T. aestivum* (XIP-III)**[e,*]	AB204556	AGKTGQVTVFWGRNKAEGSLREA	73.9	78.3
***T. aestivum* (XIP-R1)**[f,*]	AB302972	ATGKTGQVAVFWGRNKNEGSLREA	70.8	79.2
***T. aestivum* (XIP-R2)**[f]	AB302973	ATGKTGQVAVFWGRNKNEGSLREA	70.8	79.2
Triticum durum[d]	n.a.	AGKTGQVTVFWGRNKAEGSL	70.0	75.0
***T. durum* (XIP-II)**[e]	AJ318884	KQTGQLTVFWGRNAGEGTLREA	68.2	77.3
***O. sativa* (RIXI)**[g,*]	AB027415	AAGKTGQMTVFWGRNKNEGTLKET	66.7	79.2
O. sativa[h,*]	AK064356	DDPGLAVYWGRHKEEGSLREA	42.9	66.7

* Proteins proven to have inhibition activity. [a] Pairwise sequence identity and similarity percentages of the overlapping regions were determined using the "align-needle algorithm" at the EBI Web site (http://www.ebi.ac.uk). [b] Reference (*9*). [c] Reference (*37*). [d] Reference (*11*). [e] Reference (*12*). [f] Reference (*41*). [g] Reference (*42*). [h] Reference (*13*). [i] n.a. = not available.

Table 1 shows a brief overview of the purification of XIP from pearl millet grains monitored by assessment of total extractable protein and inhibition activity toward *P. purpurogenum* GH family 10 xylanase and also presents the specific inhibition activity and recovery of inhibition activity. Only about 8% of the initial inhibition activity was recovered. A considerable amount of protein precipitated after freezing and thawing of the CEC fraction. Recoveries obtained earlier were 3% for sorghum (*9*), 9% for barley, and 26, 21, and 60% for rye, durum wheat, and maize XIP type proteins, respectively (*11*). From 1.0 kg of pearl millet whole meal approximately 12 mg of XIP was isolated (**Table 1**), which is of same order of magnitude as the yields described for XIP from wheat (15 mg/kg) (*17*), rye (18 mg/kg) (*11*), and sorghum (7 mg/kg) (*9*) whole meal. Much lower XIP yields were obtained for durum wheat, barley, and maize, that is, 2.5, 0.6, and 0.6 mg/kg, respectively (*11*). The specific activity of pearl millet XIP (AC fraction) toward the *P. purpurogenum* GH family 10 xylanase was approximately 4900 XIU/mg (**Table 1**).

Sequence Determination. The N-terminal amino acid sequence of the purified pearl millet protein (AGNKTGQVTVFX$_1$GRNKDEX$_2$TLREA, where X is an unidentified amino acid), confirmed its identity as a XIP type xylanase inhibitor. All three XIP bands visible on blot (similar to that on SDS-PAGE, **Figure 2**, lane 3) gave the same results. However, the amino acid residue N3 was detected only for the lower band. As the NetNGlyc 1.0 tool (*38*) indicates that N3 is a potential glycosylation site, this might explain why it was not detected for the two pearl millet XIP bands with slightly higher MM. BLASTp and tBLASTn database searches (*39*) did not yield pearl millet sequences with significant similarities.

The pearl millet XIP N-terminal amino acid sequence was compared with sequences reported for XIP proteins from different cereals and other sequences identified by tBLASTn database searches. Sequence identity and similarity percentages were calculated using the "align-needle algorithm" at the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.be). The pearl millet sequence is 91.7 and 87.5% identical with translated EST sequences of switchgrass (*Panicum virgatum* L.) (FL886376 and FL8833777, respectively) (**Table 2**). In addition, 87.5% identity was found with sequences from sorghum (CN138888), maize (DQ245902), wheat (CA486546) and sugar cane (*Saccharum officinarum* L.) (CA095754) (**Table 2**). Less than 80% identity was observed with the N-terminal amino acid sequences of wheat XIP-I (AJ422119), XIP-III (AB204556), XIP-R1 (AB302972), and XIP-R2 (AB302973), barley XIP (BY845439), and durum wheat XIP-II (AJ318884) and of RIXI from rice (AB027415) (**Table 2**).

Table 3. $[I]/[E]_{50}$ Values for the Inhibition of Different GH Family 10 and 11 Xylanases by Pearl Millet XIP Compared with Values Reported for Sorghum XIP and Wheat XIP-I

		$[I]/[E]_{50}$		
	NBCI accession no.	pearl millet XIP	sorghum XIP[a]	wheat XIP-I[b]
GH 10 xylanases				
Aspergillus niger	CAA03655	1.3	0.9	nd[c]
Penicillium purpurogenum	AAF71268	0.9	1.3	nd
Aspergillus oryzae	BAA75475	1.1	2.2	1.5
GH 11 xylanases				
A. niger	CAA01470	ni[d]	ni	3.8
Trichoderma ongibrachiatum (p*I* 5.5)	CAA46294	0.8	104.0	nd
T. longibrachiatum (p*I* 9.0)	CAA49293	3.4	6.1	5.3
Trichoderma viride	CAB60757	49.1	37.0	63.0
Bacillus subtilis	AAA22897	ni	ni	ni
Penicillium funiculosum	CAC15487	nd	nd	1.0

[a] Data described by Gebruers et al. (*9*). [b] Data described by Flatman et al. (*19*). [c] nd, not determined. [d] ni, not inhibited.

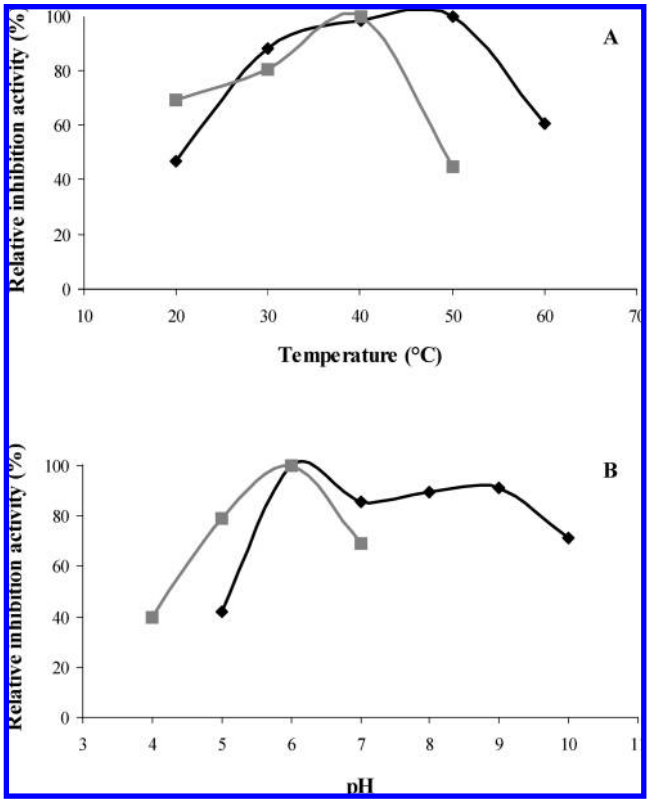

Figure 4. Relative xylanase inhibition activity (%) of pearl millet XIP toward *P. purpurogenum* GH family 10 xylanase (black diamonds) and *T. longibrachiatum* GH family 11 xylanase (p*I* 5.5) (gray squares) at various temperature (**A**) and pH (**B**) conditions as determined with the Xylazyme-AX method.

The first (X_1) and second (X_2) unidentified amino acids in the experimenally determined pearl millet XIP N-terminal sequence (see above) are probably W and G, respectively. These two amino acids occur on these positions in all of the other sequences listed in **Table 2** and are thus conserved. In addition, a close look at the N-terminal sequence analysis data revealed a very weak G signal for X_2. However, in the case of X_1, no such signal could be seen for W.

Biochemical Characterization. As already mentioned, pearl millet XIP inhibits both GH family 10 and 11 xylanases, as do wheat XIP-I (*19*) and sorghum XIP (*9*).

The $[I]/[E]_{50}$ values measured with the GH family 10 xylanases from *A. niger*, *P. purpurogenum*, and *A. oryzae* are approximately 1.0 (**Table 3**). Such low values indicate tight-binding inhibition (*40*) and are of the order of magnitude observed by Flatman et al. (*19*) (wheat XIP-I) and Gebruers et al. (*9*) (sorghum XIP). The former reported $[I]/[E]_{50}$ values of 0.6 and 1.5 for the GH family 10 xylanases of *Aspergillus nidulans* and *A. oryzae*, respectively, whereas the latter reported values of 0.9, 1.3, and 2.2 for the enzymes of *A. niger*, *P. purpurogenum*, and *A. oryzae*, respectively (**Table 3**). For pearl millet XIP and the GH family 11 xylanases analyzed here, comparable $[I]/[E]_{50}$ values were observed as for sorghum XIP (*9*), except for the *T. longibrachiatum* xylanase (p*I* 5.5), which resulted in a much lower value with pearl millet XIP (**Table 3**). Similar to what was the case for sorghum XIP, no significant inhibition could be detected toward *A. niger* GH family 11 xylanase under the experimental conditions used, whereas Flatman et al. (*19*) reported a $[I]/[E]_{50}$ value of 3.8 for wheat XIP-I and the *A. niger* enzyme.

Pearl millet XIP has >80% of its maximal activity toward *P. purpurogenum* GH family 10 xylanase at temperatures between 30 and 50 °C (**Figure 4A**) and at pH values from 6 to 9 (**Figure 4B**). These results correspond with those for sorghum XIP (*9*). The *T. longibrachiatum* GH family 11 xylanase (p*I* 5.5) is maximally inhibited at 40 °C (**Figure 4A**) and pH 6.0 (**Figure 4B**). For wheat XIP-I and *A. niger* GH family 11 xylanase, the inhibition activity increases from pH 4.5 to 6.5 (*19*).

In conclusion, pearl millet grains contain XIP proteins with biochemical properties comparable to those of wheat XIP-I and sorghum XIP. No TAXI and TLXI proteins could be detected in the grains. Insight into the presence and properties of xylanase inhibitors in pearl millet may help to understand the functionality of xylanases in the production of pearl millet-based products.

ABBREVIATIONS USED

AC, affinity chromatography; BSA, bovine serum albumin; CEC, cation exchange chromatography; EST, expressed sequence tag; GH, glycoside hydrolase; $[I]/[E]_{50}$, molar inhibitor over enzyme ratio resulting in 50% inhibition; MM, molecular mass; PAbs, polyclonal antibodies; TAXI, *Triticum aestivum* xylanase inhibitor; TLXI, thaumatin-like xylanase inhibitor; XIP, xylanase inhibiting protein; XIU, xylanase inhibitor unit.

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