JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Isolation and Characterization of Two Endoxylanases from *Fusarium graminearum*

Xinrong Dong, Steven W. Meinhardt,* and Paul B. Schwarz

Department of Plant Sciences, Dept. 7670, North Dakota State University, Fargo, North Dakota 58108-6050, United States

ABSTRACT: This paper reports the first isolation from cultures of two endoxylanases secreted by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch]. When *F. graminearum* is grown on wheat bran hydrated with a modified synthetic medium, high xylanase activity can be extracted. The two endoxylanases were identified by LC-MS/MS as the products of genes FGSG_6445 (Genbank gene id 2788192) (xylanase 1) and FGSG_3624 (GenBank accession no. AJ863566) (xylanase 2) with 61 and 51% sequence coverage, respectively. Both enzymes showed a pH optimum at pH 6, with xylanase 1 exhibiting a wider active pH range (5.5−9) than xlylanase 2 (5.5−7.5). Their temperature dependences were similar, >60% between 35 and 60 °C, with optimal temperatures of 45 °C for xylanase 1 and 50 °C for xylanase 2. Kinetic studies found that both enzymes had a lower K_m for linear beachwood xylan than arabinoxylan. For xylanase 2, the V_{max} increased with arabinoxylan, but decreased for xylanase 1.

KEYWORDS: *xylanase characterization, Michaelis−Menten kinetics, pH dependence*

INTRODUCTION

Fusarium head blight (FHB) causes severe damage to wheat and barley crops each year in many regions of the world.[1,2] Because of the economic impact, concerted efforts have been focused on the development of FHB-resistant cultivars since the mid-1990s. Despite this work, only a few wheat or barley cultivars with improved resistance to FHB or DON accumulation have been released. This can be attributed to the small number of resistance sources/genes that are, in turn, difficult to incorporate into commercially acceptable cultivars, the complex genetics of FHB resistance,[3] and a limited understanding of FHB pathogenesis.[4]

In the United States, *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch] has been identified as the primary FHB pathogen.[5] It exhibits a hemibiotrophic relationship with the host, starting out as biotrophic, but later shifting to necrotrophic.[4] The necrotrophic attack mechanism may involve the secretion of mycotoxins and cell wall degrading enzymes.[6] Cell wall degrading enzymes have long been thought to be important for the infection process[7] and may represent pathogenicity or virulence factors.[8] The pathogen may enter the host through openings such as glume stomata[9] or directly through epidermal cell walls using short infection hyphae.[10] As the cross-linked polysaccharides of graminaceous cell walls are principally glucuronoarabinoxylans,[11] xylolytic enzymes should be of importance in this process.

Empirical evidence for the role of xylanases (EC 3.2.1.8) in infection has been suggested by a number of studies. Klechkovskaya et al.[12] inoculated wheat cultivars with differing susceptibilities for *F. graminearum* and measured the levels of hydrolase enzymes. They found in susceptible cultivars that the levels of protease, cellulase, and especially xylanase activity were considerably higher at pathogen contact sites following 15 days of infection. Schwarz et al.[13] found that mature grain from barley plants that were inoculated with *F. graminearum* and *Fusarium poae* in the greenhouse contained highly elevated amounts of proteinase, endoxylanase, and an endo-β-glucanase activity. Kang and Buchenauer[14] observed the infection of wheat spikelets using immunogold labels prepared against cellulose, xylan, and pectin. Host cell walls in the ovary, lemma, and rachis in direct contact with the pathogen surface had reduced gold labeling density when compared to cell walls in noncolonized tissues. Results of this and similar studies by the same group have suggested *F. graminearum* and *Fusarium culmorum* produce cellulases and xylanases during infection and that both are involved in the infection and colonization of wheat spikes tissues.[10,15]

In studies in which cell wall degrading enzymes have been inactivated through DNA technologies, the pathogenicity or virulence has often not been reduced, although this may be due to the redundancy of enzymes for these processes.[16−18] In *F. graminearum*, 30 different xylanase metabolism-related genes, including multiple xylanases, are transcribed during growth on hop cell walls.[19] Proteomic studies have demonstrated the secretion of numerous and redundant cell wall degrading enzymes from *F. graminearum* when grown on plant cell walls or in planta.[20,21] Jenczmionka and Schafer[22] and Jenczmionka et al.[23] conducted studies to elucidate the importance of cell wall degrading enzymes during infection of wheat spikes by *F. graminearum*. Urban et al. investigated the regulation of enzymes via the Gpmk1 mitogen-activated protein (MAP) kinase pathway.[24] They analyzed disruption mutants of Gpmk1 that had completely lost their ability to infect wheat spikes for their ability to produce cell wall degrading enzymes in vitro. Although the production of polygalacturonases or amylases was

Received: August 24, 2011
Revised: February 6, 2012
Accepted: February 7, 2012
Published: February 7, 2012

dx.doi.org/10.1021/jf203407p | J. Agric. Food Chem. 2012, 60, 2538−2545

not affected, Gpmk1 was found to regulate the early induction of extracellular endoglucanase, xylanase, and protease activities.

Additional evidence for the role of xylanases in infection has come from the study of endogenous inhibitors present in cereals. The proteinaceous endoxylanase inhibitors described in the literature belong to the *Triticum aestivum* xylanase inhibitor (TAXI), the xylanase inhibiting protein (XIP), or the thaumatin-like xylanase inhibitor (TLXI) families.[8,25,26] Igawa et al.[27] showed *F. graminearum* induced expression of TAXI III and TAXI IV in wheat leaves. They found that recombinant TAXI III protein was active against xylanases of *F. graminearum*, suggesting that this inhibitor may have a role in plant defense. Dornez et al.[28] later showed a 2−3-fold up-regulation of several TAXI forms and an XIP in wheat plants that were treated with *F. graminearum* ΔTri5. Finally, Gebruers et al.[29] have reported genotypic differences in TAXI and XIP levels in grain from different wheat types.

To date, four *F. graminearum* xylanase genes have been cloned and expressed and the biochemical properties of the proteins studied.[30,31] The xylanase enzymes xylanase A and xylanase B had calculated molecular masses of 24 and 23 kDa, with theoretical p*I* values of 6.2 and 9.2, respectively. Both enzymes were inhibited by TAXI I, but not XIP I. Xylanases C and D had molecular masses of 34.2 and 39.7 kDa and p*I* values of 8.8 and 6.7, respectively. Given the probable importance of xylanases in the infection process, we have investigated the two xylanases that are produced when *F. graminearum* is grown on wheat bran.

MATERIALS AND METHODS

Fungal Strain. *F. graminearum* strain 172 originally obtained from barley by single-spore isolation was provided by Dr. Stephen Neate (Department of Plant Pathology, North Dakota State University). The stock culture was maintained on potato dextrose agar (PDA) at 4 °C.

Media and Growth Conditions. *F. graminearum* was cultured on PDA (Difco) for 4−6 days at room temperature (25 ± 2 °C) with an alternating 12 h light/dark cycle. A loop of mycelia and spores from the PDA plate was used to inoculate a 250 mL flask containing 50 mL of synthetic medium (7.4 mM KH_2PO_4, 2 mM $MgSO_4·7H_2O$, 1.8 mM $CaCl_2$, 0.035 mM $FeSO_4·7H_2O$, 0.35 mM $ZnSO_4·7H_2O$, pH 5.0, and 0.3% peptone (w/v), pH 4.5) supplemented with 20 g/L of glucose. This culture was grown at room temperature for 3 days with 120 rpm agitation and was used to inoculate various media for xylanase production.

Preliminary experiments were conducted to identify the optimal medium for xylanase production. Initially, the media tested for xylanase production included potato agar (PA), synthetic medium agar, and modified synthetic medium agar. Each of these contained 1.5% agar and was supplemented with either 1% beechwood xylan (Sigma, St. Louis, MO) or 2% hard red spring wheat bran obtained from the North Dakota State University milling laboratory. To prepare the potato agar, (Russet) white potatoes (200 g) were peeled, sliced, and boiled in 1 L of water until soft. The liquid was strained through four layers of cheesecloth and the pH adjusted to 5.0 with HCl. Fifteen grams of agar was added to the liquid and made up to 1 L with distilled water. Synthetic medium agar was prepared using the synthetic medium (see above). The modified synthetic medium was made by adding 0.2 mL of the trace elements solution (260 mM citric acid, 10 mM $CuSO_4·5H_2O$, 2 mM $MnSO_4·7H_2O$, 8 mM H_3BO_4, and 2 mM $Na_2MoO_4·2H_2O$) to 1 L of synthetic medium.

Inoculated plates, 70 × 15 mM, were grown at 25 °C for 7 days with alternating 12 h light/dark cycles. After 7 days, the agar in a single plate, 12 mL, was sliced and extracted by placing the pieces in 15 mL of 100 mM sodium acetate, pH 4.5, and shaking at 120 rpm for 3 h. The solids were removed by centrifugation for 30 min at 10400*g* and 4 °C. This extract was then tested for xylanase activity.

On the basis of the results from the preliminary tests described above, we tested a solid medium consisting of wheat bran (100 g) mixed with 100 mL of either synthetic medium, modified synthetic medium, or water and autoclaved. Four grams of each sterile bran preparation was placed in separate Petri dishes and inoculated with 0.2 mL of the liquid culture. The plates were grown for 5−6 days at 25 °C with an alternating 12 h light/dark cycle. Each plate was extracted as described above with 70 mL of 100 mM sodium acetate, pH 4.5. The growth medium used for xylanase production throughout the rest of the study consisted of the solid medium of wheat bran mixed with modified synthetic medium.

Protein Determination and Xylanase Activity Assays. Three different protein assay methods were used to determine the protein concentration. The Lowry method[32,33] was used during studies of xylanase production on different culture media. During the purification procedure, the Bio-Rad Quick Start Bradford Dye reagent (Bio-Rad Laboratories, Hercules, CA) was used. The purified proteins were measured with the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used as the standard for all protein assays.

For most applications, xylanase activity was assayed using azo-wheat arabinoxylan according to the manufacturer's instructions (Megazyme, Bray, Ireland). Briefly, 40 μL of the sample was dissolved in 260 μL of 100 mM sodium acetate buffer, pH 5, preincubated at 40 °C. To this solution, 300 μL of prewarmed substrate was added, and the sample was incubated at 40 °C for 10 min. The reactions were terminated by vigorously stirring in 2.5 mL of 95% ethanol. After 10 min, the tubes were again mixed and centrifuged at 1500*g* for 10 min. The absorbance of the supernatant was measured at 590 nm against the reagent blank prepared with assay buffer instead of enzyme solution. *Trichoderma* sp. xylanase (255 units/mg) (Megazyme, Bray, Ireland) was used as the standard to measure the xylanase activity.

In kinetic and substrate specificity studies, the amount of reducing sugars released by the enzymes was measured by the dinitrosalicylate (DNS) assay.[34] The purified enzymes were diluted 5−10-fold with 100 mM potassium phosphate buffer, pH 6.0, and 50 μL of the diluted enzyme was added to 0.45 mL of the prewarmed substrate, 50 °C, and 1−10 mg/mL of either beechwood xylan (Sigma, St. Louis, MO) or arabinoxylan (Megazyme, Wicklow, Ireland). The samples were incubated at 50 °C for 5 min, then 0.75 mL of the DNS reagent was added and mixed, and the samples were placed in boiling water for 5 min. The samples were rapidly cooled in cold water and then allowed to attemperate to room temperature, after which the absorbance was measured at 540 nm. D-(+)Xylose (Sigma) was used as the standard. One enzyme unit (U) was defined as liberating 1 μmol of reducing sugars per minute.

Xylanase Isolation. *F. graminearum* was cultured on wheat bran hydrated with modified synthetic medium with 4 g per Petri dish. The crude extract was obtained by slicing the bran−fungal mat and extracting the pieces with 100 mL of 100 mM sodium acetate, pH 4.5, containing 38 mM 6-aminohexanoic acid and 4 mM benzamidine (protease inhibitors) with shaking (120 rpm) for 3 h. The supernatant was filtered through a 0.45 μm nylon filter (Pall Co., East Hills, NY) and used as the source of the xylanase. Preliminary experiments had determined that endogenous proteases were problematic and that the protease inhibitors listed above were effective in their inhibition. Protease activity was determined by using Quanti-Cleave Protease Assay Kit (Pierce Chemical Co., Rockford, IL). The extract was stored at −20 °C for up to 1 month.

The crude extract, 100 mL, was diluted 6-fold with 10 mM sodium acetate, pH 5.0, and loaded onto a 75 mL (1.6 × 37 cm) SP-Sepharose Fast Flow column (GE Healthcare, Piscataway, NJ) at a flow rate of 2 mL/min. After loading, the column was washed with 80 mL of 20 mM sodium acetate, pH 5.0, at a flow of 2 mL/min. The flow was changed to 1 mL/min and the column eluted with a linear gradient from 20 mM sodium acetate to 20 mM sodium acetate/50 mM NaCl, pH 5.0, over 20 min. The salt concentration remained at 50 mM NaCl for 20 min and was followed by a linear gradient from 50 to 200 mM NaCl/20 mM sodium acetate, pH 5.0, over 70 min. Two xylanases, hereafter referred to as xylanases 1 and 2, were separated, and fractions containing activity for each xylanase combined. Samples of each xylanase from up to three SP-Sepharose columns were combined and concentrated to 0.5 mL using a Centriprep YM3 (Millipore Co., Billerica, MA).

Each concentrated xylanase sample was injected into a 1.0 × 30 cm Superdex-75 column (10 × 300 mm) (GE Healthcare) equilibrated with 100 mM sodium acetate/150 mM NaCl, pH 4.5. Active fractions of each enzyme were combined and diluted 10-fold with 20 mM sodium acetate and separated by HPLC ion-exchange chromatography on a Shodex IEC SP-825 column (8 × 75 mm) (Showa Denko K.K., Kawasaki, Japan). The buffers used were 20 mM sodium acetate, pH 5.0, and 20 mM sodium acetate with 500 mM NaCl, pH 5.0. Each enzyme was eluted at a flow of 1 mL/min with a linear gradient from 0 to 25 mM NaCl over 5 min followed by a linear gradient from 25 to 275 mM NaCl over 50 min.

Both xylanase enzymes were further purified by hydrophobic interaction chromatography using a 1 mL phenyl-Separose HP column (GE Healthcare). The sample was dissolved with an equal volume of 20 mM sodium acetate/3 M ammonium sulfate, pH 5.0, and then injected into the column. The column was washed at a flow rate of 0.5 mL/min with 1.5 mL of 1.5 M ammonium sulfate, 20 mM sodium acetate, pH 5.0. Xylanase 1 (Xyl1) was eluted with a linear gradient from 1.5 M to 750 mM ammonium sulfate over 6 min followed by a linear gradient from 750 to 300 mM ammonium sulfate over 30 min. Xylanase 2 (Xyl2) was eluted with a linear gradient from 450 to 0 mM ammonium sulfate over 30 min. The active fractions for each xylanase were combined and mixed with an equal volume of 50% glycerol/100 mM sodium acetate, pH 5.0, and stored at −80 °C.

Biochemical Characterization of Purified Xylanases. The relative molecular weight was determined by denaturing SDS−polyacrylamide gel electrophoresis (SDS-PAGE) (12%:3%) using the Bio-Rad Mini-Protean II (Bio-Rad Laboratories, Hercules, CA)[35] and stained with colloidal Coomassie blue. Bio-Rad Kaleidoscope Prestained Standards (Bio-Rad Laboratories) were used for determination of the relative molecular weights.

Effect of pH and Temperature. The pH dependence was measured with the following buffers, all at 100 mM: sodium acetate (pH 4.03, 4.51, 5.01, 5.51); MES (pH 5.52, 6.01, 6.51); MOPS (pH 6.50, 7.02, 7.48); Tricine (pH 7.50, 7.99, 8.48); Bicine (8.50, 9.01); and CHES (pH 9.00, 9.49). Cold enzyme samples, 10 μL for Xyl1 and 5 μL for Xyl2, were brought to 0.3 mL with prewarmed buffer, and 0.3 mL of prewarmed azo-wheat arabinoxylan substrate added. The samples were incubated for 10 min at 40 °C and the reactions stopped by adding 1.5 mL of 95% ethanol. The samples were incubated at 4 °C for 10 min to allow for precipitation and then centrifuged at room temperature for 10 min at 1500*g*. The supernatants were removed, and the absorbance was read against the reagent blank at 590 nm. The pH dependence was measured twice with duplicate samples.

The pH stability was measured at pH 5.5 and 8.5. The pH values of each of the buffers were set such that the desired pH of 6 would be obtained after the addition of the enzymes. The purified enzymes were incubated at room temperature (20 ± 2 °C) for 0, 1, 2, and 5 h. At pH 5.5, 5 μL of the enzyme was added to 20 μL of 100 mM MES, pH 5.75, mixed, and incubated. The pH was adjusted to 6.0 for the xylanase activity assay by adding 275 μL of 100 mM MES, pH 6.0. At pH 8.5, 5 μL of the enzyme was added to 20 μL of 100 mM Bicine, pH 8.53, mixed, and incubated. The pH was adjusted to pH 6 by adding 275 μL of 100 mM MES, pH 5.97, and the xylanase activity was assayed. Assay conditions were described as above.

The temperature dependence of the activities was measured at pH 6 with 100 mM MES. The temperature dependence of the pK_a of MES was taken into account when the buffers were prepared such that they would be at pH 6 at the selected temperature. A series of MES buffers were made at pH 5.95, 6.00, 6.05, 6.11, 6.17, 6.22, 6.27, 6.31, 6.36, 6.41, and 6.45 (measured at 20 °C) for the assay temperatures of 20−70 °C at 5° increments, respectively. Cold enzyme samples, 10 μL for Xyl1 and 5 μL for Xyl2, were brought to 0.3 mL with the appropriate prewarmed buffer, and 0.3 mL of prewarmed azo-wheat arabinoxylan substrate was added. The samples were incubated for 10 min at the indicated temperature and the reactions stopped by adding 1.5 mL of 95% ethanol. The samples were incubated in ice water for 10 min to allow for precipitation and then centrifuged for 10 min at 1500*g*. The supernatants were removed, and the absorbance was read against the reagent blank at 590 nm. A nonenzyme control was prepared at each temperature and its absorption subtracted from the sample readings.

The temperature stability was determined by incubating the purified enzymes in 100 mM MES, pH 6.0, at 35, 55, and 65 °C for 5, 10, 15, and 30 min. When the buffers were prepared, the temperature dependence of the buffer was taken into account. Ten microliters of Xyl1 and 5 μL of Xyl2 were brought to 0.3 mL with buffer warmed to the appropriate temperature, mixed, and incubated for the indicated time. The assay was performed as described previously. The experiment was performed two to three times with duplicate samples.

Michaelis−Menten Parameters. Experiments were performed twice with duplicate samples. Beechwood xylan (Sigma) and high-viscosity wheat arabinoxylan (Megazyme, Wicklow, Ireland) were used as substrates. Potassium phosphate buffer (100 mM, pH 6.0) was used as the assay buffer. The beechwood xylan substrate was made by suspending 1.0 g of beechwood xylan in 80 mL of assay buffer (100 mM potassium phosphate, pH 6.0) at 60 °C and stirred until boiling. The preparation was cooled to room temperature with continuous stirring overnight. After cooling, the total volume was brought to 100 mL with the same assay buffer. For arabinoxylan, a 1% stock solution was prepared using the assay buffer. Assay solutions of 1, 2, and 5 mg/mL were made by diluting the stock solutions. The reducing sugars released were assayed by the DNS method. For kinetic studies, Xyl1 was diluted 40-fold and Xyl2 was diluted 75-fold in 100 mM potassium phosphate buffer, pH 6.0. K_m and V_{max} were determined from a Linewaver−Burk plot.

Mass Spectrometry. Mass spectrometry was performed at the University of Minnesota Center for Mass Spectrometry and

Table 1. Quantification[a] of Crude Xylanase Activity Extracted from *F. graminearum* Cultured on Various Media

	medium/inducer					
	potato/xylan	potato/bran	synthetic/xylan	synthetic/bran	modified/xylan	modified/bran
xylanase (mU/mL)	0.12 ± 0.01	0.50 ± 0.02	0.53 ± 0.01	1.11 ± 0.18	0.50 ± 0.02	1.10 ± 0.10
total protein (mg/mL)	0.59 ± 0.02	1.16 ± 0.11	1.00 ± 0.03	1.57 ± 0.21	0.90 ± 0.02	1.49 ± 0.30
specific activity (mU/mg)	0.20 ± 0.01	0.43 ± 0.06	0.53 ± 0.03	0.71 ± 0.11	0.56 ± 0.03	0.74 ± 0.10

[a]Mean and standard deviation of three replications.

Proteomics (St. Paul, MN). The purity of the isolated xylanases was checked by SDS-PAGE and stained with colloidal Coomassie blue. The bands were excised from the gel, trypsin digested, and injected into a Qstar ESI-MS/MS mass spectrometer. Fragments were identified by Scaffold (Proteome Software, Portland, OR) using both Sequest and X!Tandem database searching.

RESULTS AND DISCUSSION

Development of Culture Condition for Xylanase Production. Several culture conditions were explored to determine the optimal medium for xylanase production (Table 1). Three media were used, each supplemented with either 1% beechwood xylan (w/v) or 2% wheat bran (w/v). The lowest total xylanase production and specific activity were observed in the medium containing potato extract supplemented with xylan. When the potato extract medium was supplemented with wheat bran, the total production increased 4-fold, and both the specific activity and total protein doubled. Synthetic medium supplemented with xylan produced as much activity as bran-supplemented potato extract medium. When bran was added to the synthetic medium, the specific activity increased 1.5-fold relative to the xylan-supplemented synthetic medium. The modified synthetic medium gave results similar to the synthetic medium when supplemented with either xylan or wheat bran. In general, bran-supplemented media produced on average twice the total activity and increased the specific activity by 1.5-fold over xylan-supplemented media.

From these results, it was clear that the bran provided the best induction of xylanase production. We therefore investigated growing *F. graminearum* directly on bran hydrated with various media (water, synthetic medium, and modified synthetic medium) and extracting the activity from the bran by soaking it in buffer (Table 2). This approach produced a 400−600-fold increase in total activity, with the modified synthetic medium producing the greatest amount. Bran hydrated with the modified synthetic medium was chosen for further xylanase production.

Table 2. Quantification[a] of Crude Xylanase Activity Extracted from *F. graminearum* Cultured on Wheat Bran-Based Media

	additional component		
	water	synthetic medium	modified medium
xylanase (mU/mL)	471.9 ± 12.7	420.6 ± 21.9	678.3 ± 17.7
total protein (mg/mL)	422.2 ± 16.2	198.7 ± 14.9	409.7 ± 15.6
specific activity (mU/mg)	1.11 ± 0.08	2.11 ± 0.05	1.66 ± 0.19

[a]Mean and standard deviation of three replications.

Purification of the Xylanases. The above extracts contained a substantial level of protease activity. Using a protease inhibitor panel (Sigma), it was determined that the majority of the protease activity was inhibited by adding 38 mM 6-aminohexanoic acid (chymotrypsin and lysine carboxy peptidase protease inhibitor) and 4 mM benzamidine (trypsin-like and serine protease inhibitor). These inhibitors were added during extraction and to the active fractions after low-pressure ion-exchange and gel filtration chromatography.

The two major xylanases present in the extracts were concentrated and separated by the first cation exchange column (Figure 1). The xylanases eluted from the column at approximately 80−104 mM for Xyl1 and from 112−152 mM NaCl for Xyl2. Separating the xylanases at this point resulted in the best purification (Table 3), and all subsequent steps were performed separately for each enzyme.

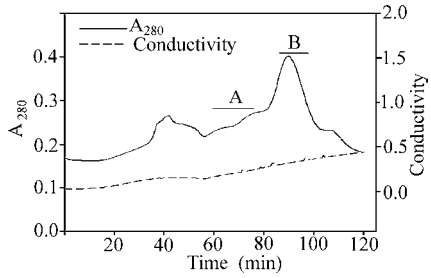

Figure 1. Low-pressure cation exchange chromatography of the crude extract. One hundred milliliters of diluted crude extract was loaded onto a SP-Sepharose Fast Flow column and eluted with a NaCl gradient. The horizontal lines indicate the time at which xylanase 1 (A) and xylanase 2 (B) eluted.

The active fractions for each xylanase were combined, concentrated to 0.5 mL, injected onto a Superdex-75 column, and eluted at a flow of 0.5 mL/min. Xylanase 1 eluted from 24 to 27 min, whereas Xyl2 eluted between 30 and 33 min (Figure 2A,D). This gel filtration step was effective in removing the bulk of the contaminating protein (Table 3). Combined active fractions from gel filtration were diluted 10-fold with 20 mM sodium acetate, pH 5, and loaded onto the HPLC cation exchange column at a flow of 1 mL/min. Xyl1 eluted between 75 and 90 mM, and Xyl2 between 172 and 187 mM NaCl (Figure 2B,E). SDS-PAGE of the samples following cation exchange HPLC indicated the presence of a small amount of contamination (data not shown), and phenyl-Sepharose HR hydrophobic interaction chromatography was employed to remove the last contaminants. Xyl1 eluted at high ammonium sulfate concentrations, from 650 to 560 mM, whereas Xyl2 eluted between 385 and 345 mM (Figure 2C,F). The purified proteins each exhibited a single band in SDS-PAGE (Figure 3). The isoelectric point of both xylanases was determined to be greater than pH 8.5 (data not shown).

The estimated molecular weights were 41000 for Xyl1 and 17000 for Xyl2. Predicted molecular weights for these proteins are 37200 and 22600, respectively. ESI-MS/MS of the

dx.doi.org/10.1021/jf203407p | J. Agric. Food Chem. 2012, 60, 2538−2545

Table 3. Purification of Xylanases 1 and 2 from *F. graminearum*

purification step	vol (mL)	total activity (U/mL)	total protein (mg)	specific activity (U/mg)	purity (fold)	recovery (%)
xylanase 1						
diluted extract	3600	137	221.8	0.62	1.00	100
SP-Sepharose	0.5	104.6	1.09	9.6	15.47	7.6
Superdex 75	0.9	8.8	0.46	19.3	31	6.4
SP-HPLC	2	3.6	0.1	35.1	56.5	2.4
phenyl-Sepharose HPLC	1.5	5.8	0.07	77.8	125	4.2
xylanase 2						
diluted extract	3600	137	221.8	0.62	1.00	100
SP-Sepharose	0.52	203.2	1.09	18.6	30	14.8
Superdex 75	1.2	13.8	0.15	90.2	49	10
SP-HPLC	2.5	3.7	0.03	109.7	176	2.7
phenyl-Sepharose HPLC	1.5	3.5	0.02	166.6	268	2.6

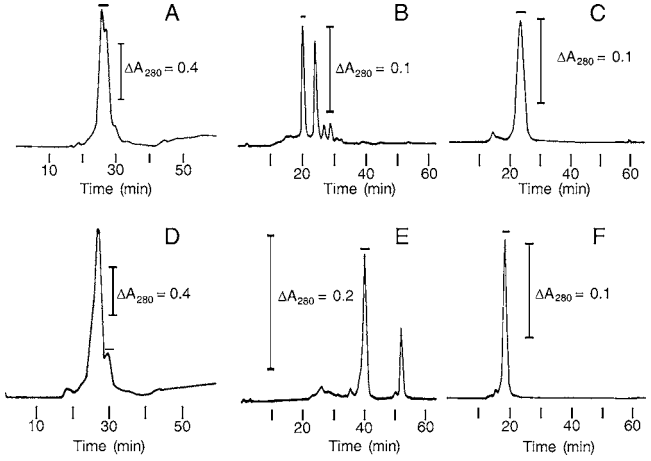

Figure 2. Purification of xylanases 1 (A–C) and 2 (D–F) by gel filtration, HPLC cation exchange, and HR-phenyl-Sepharose hydrophobic interaction chromatography. In all panels the active fractions are indicated by the horizontal bars. (A, D) The concentrated sample was separated on a Superdex-75 column. (B, E) Combined active fractions from the gel filtration column were injected into the Shodex IEC SP-825 cation exchange column and eluted at a flow rate of 1 mL/min. (C, F) The active fractions from the HPLC cation exchange chromatography were separated on a HR-phenyl-Sepharose column.

undigested Xyl2 gave two major peaks at masses of 20974 and 20846 Da. This difference in mass of 128 Da is equivalent to

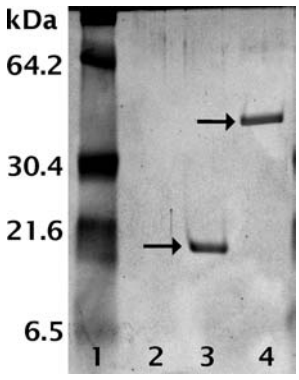

Figure 3. SDS-PAGE of the purified xylanases. Purified xylanases were separated on a 12:3 polyacrylamide gel as described by Schägger and Von Jagow,[35] electrophoresed for 1 h, and stained with colloidal Coomassie blue. Lanes: 1, Bio-Rad Kaleidoscope standards; 2, empty; 3, xylanase 2; 4, xylanase 1.

the mass of lysine or asparagine, suggesting that proteolysis had occurred during the purification even though protease inhibitors were present throughout the purification process. Given below are the first 40 amino acids of FG0_3456. The signal sequence is predicted to be cleaved between A19 and A20. Assuming our conjecture that the amino acid being cleaved between the two forms is a lysine or an arginine, we should obtain the measured molecular weight by digesting the protein to an arginine or lysine. Doing so with the carboxyl terminal resulted in a protein that is smaller than measured. If the N-terminal amino acids up to K37 are removed, the measured molecular weight (20794.91) is obtained, and removal of K37 gives the second molecular weight measured.

MVSFTYLLAA VSAVTGAVAA PNPTKVDAQP PSGLLEKRTS

Fusarium sp. are known to produce large amounts of proteases when grown on cereals.[13,36] During development of the purification method, Xyl2 was not observed until the addition of high concentrations of protease inhibitors, suggesting that this enzyme is more sensitive to proteolysis. During ion exchange chromatograpy, the protease inhibitor concentration had to be lowered by dilution so that the proteins would bind to the ion exchange column. Protease inhibitors were added to the samples after each chromatographic step.

Table 3 shows the purification of the enzymes after each chromatographic step. Overall, the specific activity increased from 0.62 to 77.8 U/mL for Xyl1 and to 166.6 U/mL for Xyl2. This represents 125- and 268-fold purifications of Xyl1 and Xyl2 with recoveries of 4.2 and 2.6% of the initial activity, respectively.

Characterization of the Enzymes. Identification of the xylanases was accomplished through trypsin digestion followed by LC-MS/MS. The fragments sequenced were consistent with the identification of Xyl1 as the product of the gene FGSG_6445 (GenBank gene id 2788192) and Xyl2 of the gene FGSG_3624 (GenBank accession no. AJ863566). The MS data provided coverages of 61% (20 different peptides) for Xyl1 and 51% (8 different peptides) for Xyl2. A search of the literature showed that the enzyme encoded by FGSG_3624 had been previously cloned and characterized,[30] but the enzyme encoded by FGSG_6445 had not been previously studied. Because the previous work was from a cloned enzyme, we felt that characterization and comparison of the isolated enzyme to the cloned enzyme would be useful.

This is the first reported purification of two endoxylanases from *F. graminearum* cultures. To date, four xylanases have

been cloned and purified,[30,31] and none of the 11 putative xylanases or related genes (*F. graminearum* Genome Database) have been purified from the fungal source. In proteomic experiments, six of the xylanases have been reported.[20,21] In experiments using quantitative PCR, transcripts of four of the xylanases were identified.[19] The two xylanases purified here were identified in all three studies. In the work by Hatsch et al.,[19] the level of mRNA for the gene FGSG_3624 was the highest of the enzymes reported, and the level of RNA from FGSG_6445 was the fourth highest. This suggests that these are the predominant xylanases produced during growth on cell walls. In support of these results, we found wheat bran to be the best inducer of xylanase activity. Bran comprises the pericarp, seed coat, and aleurone tissues and is composed largely of the cell wall polysaccharides arabinoxylan and cellulose.

The pH dependence of the two xylanases is shown in Figure 4A,C. Xyl1 (panel A) was active over a wide pH range, from pH

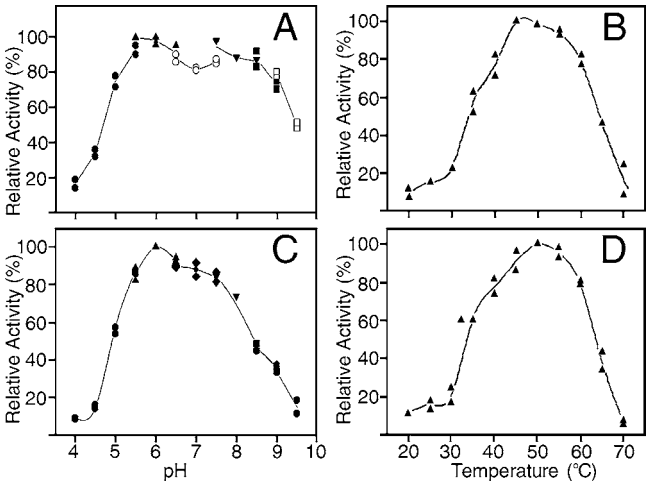

Figure 4. pH and temperature dependences of xylanase 1 (A, B) and 2 (C, D) activities. (A, C) The enzymes were diluted into 100 mM of buffer at the pH indicated and incubated at 40 °C for 10 min. The buffers used were (●) sodium acetate, (▲) MES, (○) MOPS, (▼) Tricine, (■) Bicine, (□) CHES. (B, D) Activities were measured in 100 mM of MES buffer, pH 6.0, at the indicated temperature and incubated with the substrate.

5 to 9, with the activity dropping steeply outside these pH values. The optimal pH for the activity was pH 6, but it varied by only approximately 20% over pH 5.5−9. Xyl2 (panel C) was most active over a narrow pH range, from 5.5 to 7.5, with an optimal pH of 6. The activity decreased rapidly below pH 5.5, but decreased more slowly above pH 7.5. The pH stability of the enzymes was tested at the edges of the active range, pH 5.5 and 8.5. At these pH values, both enzymes were relatively stable, losing only approximately 20% of their activity over 5 h (data not shown).

The temperature dependence of these enzymes is shown in panels B (Xyl1) and D (Xyl2) of Figure 4. For both enzymes, the highest activity was between 45 and 55 °C, with each showing a dramatic increase in activity (20−80%) between 30 and 40 °C. The activity for both enzymes decreased above 55 °C, with only 10−20% present at 70 °C.

Figure 5 presents the temperature stability of the two xylanases. Xyl2 showed little to no loss in activity when incubated at 35 °C for 1 h. At 55 °C, the loss was 40% over 60 min. The curve indicates that the rest of the activity decreased

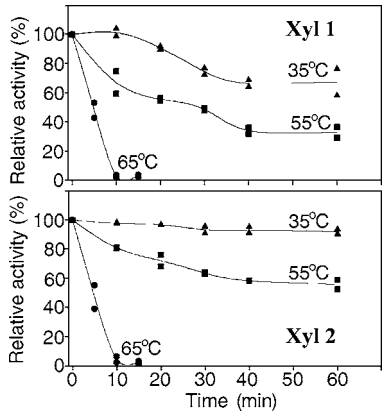

Figure 5. Temperature stability of the xylanase activities. The enzymes were added to 100 mM MES, pH 6.0, temperature compensated, and incubated at the indicated temperature and duration. The samples were then cooled to 40 °C, and the activity was measured.

very slowly after the initial 60 min. At 65 °C, the activity was completely absent after 10 min, with half the activity being lost after 5 min. Xyl1 was more temperature-sensitive, losing 25−30% of the activity after 60 min at 35 °C. The loss in activity occurred over the first 40 min, and the remaining activity appeared to be stable. At 55 °C, the activity decreased to only 30−35% of the original activity after 60 min. As in the case of Xyl2, the activity of Xyl1 was completely absent after 10 min at 65 °C.

Xyl2 purified here is the same as XylB of Beliën et al.[30] They found that the *Escherichia coli*-expressed enzyme had a temperature optimum of 30 °C and a pH optimum of 7. The pH optimum was narrow, with 40% of the activity remaining at pH 5 and 8.5. The results obtained here for this enzyme purified from cultures show a much higher temperature optimum of 50 °C and a lower pH optimum of 5.5.

Four xylanases have been purified from *Fusarium oxysporum* F3 with molecular masses of 60, 38, 23.5, and 20.8 kDa[37−40]. The 20.8, 23.5, and 38 kDa xylanases were basic proteins (p*I* values from 8.5 to 9.5) and were in the F10 and G11 families of xylanases. The optimum temperature for the 20.8 and 23.5 kDa xylanases was 45 °C, whereas that for the 38 kDa protein was 55 °C. The pH optimum of both the 20.8 and 23.5 kDa xylanases was 6.0. Beliën et al.[30] used 250 mM buffer in their experiments, whereas in this study and those on *F. oxysporum* F3, measurements were made at 100 mM buffer. In preliminary experiments, we noticed a significant change in the activity at pH 6 as the ionic strength of the buffer was changed by the addition of various salts ($MgCl_2$, NaCl, KCl) (unpublished results). Therefore, the difference in the buffer concentration between the studies could explain differences in the observed pH and temperature profiles.

The substrate specificity was studied using both beechwood xylan and high-viscosity arabinoxylan as substrates. The K_m and V_{max} values obtained with xylan and arabinoxylan are given in Table 4 along with reported values from several species. The V_{max} values obtained for Xyl1 and Xyl2 are within the reported values, whereas the K_m values are lower than most. The low K_m values suggest that these enzymes have a relatively high affinity for xylan and bind to it tightly.

Lower K_m values were observed for both xylanases when xylan was used as a substrate, suggesting it as the preferred substrate. These results were not unexpected as substitution of

Table 4. K_m and V_{max} Values for Xylanases from Various Organisms

organism	enzyme	substrate	K_m (mg/mL)	V_{max} (μmol/min-mg protein)
F. graminearum	Xyl1	xylan	0.86	15.9
		arabinoxylan	1.7	12.4
F. graminearum	Xyl2	xylan	0.44	18.4
		arabinoxylan	5.23	61.7
Aspergillus veriscolor[41]		xylan	2.3	1.44
Penicillium capsulatum[42]		xylan	20.18	157
Rhizopus oryzae[43]		xylan	18.5	90
Aspergillus niveus[44]		xylan	2.5	26

the xylose backbone with arabinose is known to cause steric hindrance of endoxylanases.[45] In addition, the outer tissues of cereal grains (e.g., pericarp, lemma, and palea) show a lesser degree of substitution with arabinose than the inner tissues (e.g., aleurone or endosperm cell walls).[25] Nevertheless, endoxylanases are key components in the degradation of arabinoxylans. Complete degradation of xylans to monosaccharides requires additional enzymes, such as arabinofuranosidase and xylobiase.

Inhibition of the xylanases by TAXI and XIP was evaluated by the Laboratory of Food Chemistry and Biochemistry at the Catholic University Leuven (Belgium) by Beliën et al. using previously reported methods.[30] Their work confirmed that Xyl2 is inhibited 90% by TAXI, but is not significantly inhibited by XIP (Jan Delcour, personal communication). Xyl1 was tested only with XIP and was not inhibited by this protein. These results are consistent with those reported by Pollet et al., with the family 11 xylanases A and B being inhibited by TAXI and the family 10 xylanases C and D inhibited by XIP.[31] TLXI has been shown to be effective against some family 11 xylanases by Fierens et al., but it was not tested against xylanases from *F. graminearum*.[46]

The results of this study clearly demonstrated that the growth of *F. graminearum* induced the production of significant amounts of the two xylanases purified here. *F. graminearum* is an important disease of wheat and barley, and the isolation of these enzymes from wheat bran indicates their importance in the colonization process. As the lemma, palea, and pericarp of both wheat and barley are rich in arabinoxylans, production of these xylanases during penetration and colonization would be anticipated. The significant susceptibility of Xyl2 to proteolysis was not expected and may have an impact on its role in fungal growth on plants because proteases are secreted simultaneously with the xylanases. The proteins isolated in this study can be used to prepare antibodies to aid in the histological investigation of the role that xylanases play in penetration.

AUTHOR INFORMATION

Corresponding Author

*E-mail: Steven.Meinhardt@ndsu.edu.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Nganje, W. E.; Bangsund, D. A.; Leistritz, F. L.; Wilson, W. W.; Tiapo, N. M. Regional economic impacts of *Fusarium* head blight in wheat and barley. *Rev. Agric. Econ.* **2004**, *26*, 332−347.

(2) McMullen, M. P.; Jones, R.; Gallenberg, D. Scab of wheat and barley: a re-emerging disease of devastating impact. *Plant Dis.* **1997**, *81*, 1340−1348.

(3) Rudd, J. C.; Horsley, R. D.; McKendry, A. L.; Elias, E. M. Host plant resistance genes for fusarium head blight: sources, mechanisms, and utility in conventional breeding systems. *Crop Sci.* **2001**, *41*, 620−627.

(4) Bushnell, W. R.; Hazen, B. E.; Pritsch, C. Histology and physiology of *Fusarium* head blight. In *Fusarium Head Blight of Wheat and Barley*; Leonard, K. J., Bushnell, W. R., Eds.; American Phytopathological Society Press: St. Paul, MN, 2003; pp 44−83.

(5) Shaner, G. Epidemiology of fusarium head blight of small grain cereals in North America. In *Fusarium Head Blight of Wheat and Barley*; Leonard, K. J., Bushnell, W. R., Eds.; American Phytopathological Society Press: St. Paul, MN, 2003; pp 84−119.

(6) Wanyoike, M. W.; Walker, F.; Buchenauer, H. Relationship between virulence, fungal biomass and mycotoxin production by *Fusarium graminearum* in winter wheat head blight. *J. Plant Dis. Prot.* **2002**, *109*, 589−600.

(7) Urbanek, H. The role of cutinase and cell wall degrading enzymes produced by *Fusaria* in pathogenesis. In *Fusarium. Mycotoxins, Taxonomy and Pathogenicity.*; Chelkowski, J., Ed.; Elsevier: New York, 1989; pp 243−256.

(8) Beliën, T.; Van Campenhout, S.; Robben, J.; Volckaert, G. Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems. *Mol. Plant−Microbe Interact.* **2006**, *10*, 1072−1081.

(9) Pritsch, C.; Muehlbauer, G. J.; Bushnell, W. R.; Sommers, D. A.; Vance, C. P. Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. *Mol. Plant−Microbe Interact.* **2000**, *13*, 159−169.

(10) Wanjiru, W. M.; Kang, Z.; Buchenauer, H. Importance of cell wall degrading enzymes produced by *Fusanrium graminearum* during infection of wheat heads. *Eur. J. Plant Pathol.* **2002**, *108*, 803−810.

(11) Carpita, N.; McCann, M. The cell wall. In *Biochemistry and Molecular Biology of Plants*; Buchanan, B. B., Gruissem, W., Jones, R. L., Eds.; American Society of Plant Physiologists: Rockville, MD, 2002; pp 52−108.

(12) Klechkovskaya, E. A.; Adamovskaya, V. G.; Wolf, G. A.; Vovchuk, S. V. The role of hydrolases and trypsin inhibitor in development of winter wheat resistance to fusarium infection. *Russian J. Plant Physiol.* **1998**, *45*, 728−735.

(13) Schwarz, P. B.; Jones, B. L.; Steffenson, B. J. Enzymes associated with *Fusarium* infection of barley. *J. Am. Soc. Brew. Chem.* **2002**, *60*, 130−134.

(14) Kang, Z.; Buchenauer, H. Studies on the infection process of *Fusarium culmorum* in wheat spikes: degradation of host cell wall components and localization of trichothecene toxins in infected tissue. *Eur. J. Plant Pathol.* **2002**, *108*, 653−660.

(15) Kang, Z.; Buchenauer, H. Ultrastructural and cytochemical studies on cellulose, xylan and pectin degradation in wheat spikes infected by *Fusarium culmorum*. *J. Phytopathol.* **2000**, *148*, 263−275.

(16) Apel-Birkhold, P. C.; Walton, J. D. Cloning, disruption, and expression of two endo-β-1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. *Appl. Environ. Microbiol.* **1996**, *62*, 4129−4135.

(17) Bowen, J. K.; Templeton, M. D.; Sharrock, K. R.; Crowhurst, R. N.; Rikkerink, E. H. A. Gene inactivation of the plant pathogen *Glomerella cingulata*: three strategies for the disruption of the pectin lyase gene *pnl*A. *Mol. Gen. Genet.* **1995**, *246*, 196−205.

(18) Wu, S. C.; Ham, K. S.; Darvill, A. G.; Albersheim, P. Deletion of the two endo-β-1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. *Mol. Plant−Microbe. Interact.* **1997**, *10*, 700−708.
(19) Hatsch, D.; Phalip, V.; Petkovski, E.; Jeltsch, J. M. *Fusarium graminearum* on plant cell wall: no fewer than 30 xylanase genes transcribed. *Biochem. Biophys. Res. Commun.* **2006**, *345* (3), 959−966.
(20) Paper, J. M.; Scott-Craig, J. S.; Adhikari, N. D.; Cuomo, C. A.; Walton, J. D. Comparative proteomics of extracellular proteins *in vitro* and *in planta* from the pathogenic fungus *Fusarium graminearum*. *Proteomics* **2007**, *17*, 3171−3183.
(21) Phalip, V.; Delalande, F.; Carapito, C.; Goubet, F.; Hatsch, D.; Leize-Wagner, E.; Dupree, P.; Van Dorsselaer, A.; Jeltsch, J. M. Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall. *Curr. Genet.* **2005**, *48*, 366−379.
(22) Jenczmionka, N. J.; Schafer, W. The Gpmk1MAP kinase of *Fusarium graminearum* regulates the induction of specific secreted enzymes. *Curr. Genet.* **2005**, *47*, 29−36.
(23) Jenczmionka, N. J.; Maier, F. J.; Losch, A. P.; Schafer, W. Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase gpmk1. *Curr. Genet.* **2003**, *43*, 87−95.
(24) Urban, M.; Mott, E.; Farley, T.; Hammond-Kosack, K. The *Fusarium graminearum* MAP1 gene is essential for pathogenicity and development of perithecia. *Mol. Plant Pathol.* **2003**, *4*, 347−359.
(25) Elliott, G. O.; McLauchlan, W. R.; Williamson, G.; Kroon, P. A. A wheat xylanase inhibitor protein (XIP-I) accumulates in the grain and has homologues in other cereals. *J. Cereal Sci.* **2003**, *37*, 187−194.
(26) Goesaert, H.; Gebruers, K.; Brijs, K.; Courtin, C. M.; Delcour, J. A. XIP-type endoxylanase inhibitors in different cereals. *J. Cereal Sci.* **2003**, *38*, 317−324.
(27) Igawa, T.; Ochiai-Fukuda, T.; Takahashi-Ando, N.; Ohsato, S.; Shibata, T.; Yamaguchi, I.; Kimura, M. New TAXI-type xylanase inhibitor genes are inducible by pathogens and wounding in hexaploid wheat. *Plant Cell Physiol.* **2004**, *45* (10), 1347−1360.
(28) Dornez, E.; Croes, E.; Gebruers, K.; Carpentier, S.; Swennen, R.; Laukens, K.; Witters, E.; Urban, M.; Delcour, J. A.; Courtin, C. M. 2-D DIGE reveals changes in wheat xylanase inhibitor protein families due to *Fusarium graminearum* ΔTri5 infection and grain development. *Proteomics* **2010**, *10*, 2303−2319.
(29) Gebruers, K.; Dornez, E.; Bedo, A.; Rakszegi, M.; Courtin, C. M.; Delcour, J. Variability in xylanase and xylanase inhibition activities in different cereals in the HEALTHGRAIN diversity screen and contribution of environment and genotype to this variability in common wheat. *J. Agric. Food Chem.* **2010**, *58*, 9362−9371.
(30) Beliën, T.; Van Campenhout, S.; Van Acker, M.; Volckaert, G. Cloning and characterization of two endoxylanases from the cereal pathogen *Fusarium geminearum* and their inhibition profile against endoxylanase inhibitors from wheat. *Biochem. Biophys. Res. Commun.* **2005**, *327*, 407−414.
(31) Pollet, A.; Beliën, T.; Fierens, K.; Delcour, J. A.; Courtin, C. M. *Fusarium graminearum* xylanases show different functional stabilities, substrate specificities and inhibition sensitivities. *Enzyme Microb. Technol.* **2009**, *44*, 189−195.
(32) Lowry, O. H.; Rosebrough, N. J.; Farr, L. A.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(33) Peterson, G. L. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **1977**, *83*, 346−356.
(34) Bailey, M. J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **1992**, *23*, 257−270.
(35) Schägger, H.; Von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368−379.
(36) Pekkarinen, A. I.; Jones, B. L. Trypsin-like proteinase produced by *Fusarium culmorum* grown on grain proteins. *J. Agric. Food Chem.* **2002**, *50*, 3849−3855.
(37) Christakopoulos, P.; Kekos, D.; Macris, B. J.; Claeyssens, M.; Bhat, K. M. Purification and characterization of a major xylanase with cellulase and transferase activities from *Fusarium oxysporum*. *Carbohydr. Res.* **1996**, *289*, 91−104.
(38) Christakopoulos, P.; Nerinckx, W.; Kekos, D.; Macris, B.; Claeyssens, M. Purification and characterization of two low molecular mass xylanases from *Fusarium oxysporum* F3. *J. Biotechnol.* **1996**, *51*, 181−191.
(39) Christakopoulos, P.; Nerinckx, W.; Kekos, D.; Macris, B.; Claeyssens, M. The alkaline xylanases III from *Fusarium oxysporum* F3 belongs to family F/10. *Carbohydr. Res.* **1997**, *302*, 191−195.
(40) Egi, A.; Speers, R. A.; Schwarz, P. B. Arabinoxylans and their behavior during malting and brewing. *Tech. Q. Master Brew. Assoc. Am.* **2004**, *41*, 248−267.
(41) Carmona, E. C.; Fialho, M. B; Buchgnani, É. B.; Coelho, G. D.; Brocheto-Braga, M. R.; Jorge, J. A. Production, purification and characterization of a minor form of xylanase from *Aspergillus versicolor*. *Process Biochem.* **2005**, *40*, 359−364.
(42) Ryan, S. E.; Nolan, K.; Thompson, R.; Gubitz, G. M.; Savage, A. V.; Tuohy, M. G. Purification and characterization of a new low molecular weight endoxylanase from *Penicillium capsulatum*. *Enzyme Microb. Technol.* **2003**, *33*, 775−785.
(43) Bakir, U.; Yavascaoglu, S.; Guvenc, F.; Ersayin, A. An endo-β-1,4-xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization. *Enzyme Microb. Technol.* **2001**, *29*, 328−334.
(44) Sudan, R.; Bajaj, B. K. Production and biochemical characterization of xylanase from an alkalitolerant novel species *Aspergillus niveus* RS2. *World J. Microbiol. Biotechnol.* **2007**, *23*, 491−500.45.
(45) Viëtor, R. J.; Angelino, S. A. G. F.; Voragen, A. G. J. Arabinoxylans in barley, malt and wort. *Proceedings of the European Brewing Convention*; IRL Press: Oxford, U.K., 1991; pp 139−146.
(46) Fierens, E.; Rombouts, S.; Gebruers, K.; Goesaert, H.; Brijs, K.; Beaugrand, J.; Volckaert, G.; Van Campenhout, S.; Proost, P.; Courtin, C. M.; Delcour, J. A. TLXI, a novel type of xylanase inhibitor from wheat (*Triticum aestivum*) belonging to the thaumatin family. *Biochem. J.* **2007**, *403*, 583−591.