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Purification and Characterization of a Low-Molecular-Weight Xylanase Produced by *Acrophialophora nainiana*

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Abstract. A low-molecular-weight xylanase activity (XynI) was isolated from the fungus *Acrophialophora nainiana* after growth in a solid medium containing wheat bran. XynI was purified to apparent homogeneity by ultrafiltration and gel filtration chromatography. The purified enzyme had a molecular weight value of approx. 17 kDa, as determined by SDS-PAGE. This enzyme was most active at 50°C and pH 6.0. At 50°C the half-life was 150 min. The apparent K_m value for birchwood xylan was much lower than the K_m value for oat spelt xylan. XynI was activated by L-cysteine, DTE, β-mercaptoethanol, and L-tryptophan. XynI did not show significant sequence homology with other xylanases. The analysis of hydrolysis products of xylans and wood pulps showed that XynI was able to release xylooligomers ranging from X2 to X3 and X2 to X6, respectively. The enzyme was not active against acetylated xylan. A small amount of xylose was released from deacetylated, birchwood, and oat spelt xylans. The results obtained with enzymatic treatment of Kraft pulp indicated a reduction in the amount of chlorine compounds required for the process and enhanced brightness gain.

The complete breakdown of native xylan is only effected by the synergistic action of a multienzymic system comprising main-chain (endo-β-1,4-xylanases and β-xylosidases) and side-chain (acetylxylan esterases, α-glucuronidases, ferulic acid esterases, and α-arabinofuranosidases) enzymes [15, 16].

The potential and use of xylanases as an alternative approach in pulp bleaching was first reported by Finnish researchers [22]. The most significant effects of xylanases on pulp bleaching were described to be improvement in the viscosity of cellulosic fiber, higher brightness ceilings, reduction in the requirement of elemental chlorine, and decrease in the amount of organochlorine compounds in bleach plant effluents [22]. To our knowledge, this is the first paper that reports the purification, some properties, and applications in the pulp and paper industry of a small-molecular-weight xylanase from *Acrophialophora nainiana* when grown on a solid medium containing wheat bran.

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Materials and Methods

Growth conditions. The fungus was isolated from a hot water fountain in Brazil (Caldas Novas-Go) and identified as *Acrophialophora nainiana* by Wilma R.C. Ribeiro (Departamento de Fitopatologia, Universidade de Brasília, Brasil). The procedure for growth conditions in a solid-state medium at 40°C was as described before [9, 24].

Assays. Xylanase and cellulase activities were determined as described before [14, 24]. Protein concentration was quantified by the Bradford method [4] with bovine serum albumin as the standard. Optimum pH value was determined by monitoring each activity at 50°C at various pH values between 3.0 and 8.0. All buffers were adjusted to the same ionic strength with NaCl or KCl. The stability of purified XynI was determined by preincubating the enzyme solution at 50°C for different time periods (1–24 h) and in the pH range 5.0–6.0. Aliquots were withdrawn and assayed for xylanase activity. The optimum temperature for xylanase activity was determined by carrying out standard assays at various temperatures between 30° and 80°C in 100 mM sodium acetate buffer, pH 5.0. Account was taken of the variation in pH as a function of temperature. The effect of some reagents (L-cysteine, β-mercaptoethanol, dithioerythritol (DTE), and L-tryptophan, 5 mM final concentration) in the xylanase activity was determined by performing the assay under the same conditions as described elsewhere [24]. For the kinetic experiments, the affinity of XynI for the substrates was examined from a

Michaelis-Menten equation with a non-linear regression data analysis program [12]. Soluble oat spelt and birchwood xylans were used at the concentration range of 0.05–10 mg/ml.

Enzyme purification. All purification steps were carried out at 4°C unless otherwise specified. The crude extract of *A. nainiana* was concentrated approx. 10-fold by ultrafiltration in an Amicon system (Stirred Cells, Series 8000, Amicon Inc., USA) with a 30-kDa cut-off point membrane (PM 30). The resulting ultrafiltrate was fractionated by gel filtration chromatography (Sephacryl S-100, 2.5 × 80 cm). The column was equilibrated with 50 mM sodium acetate buffer, pH 5.0. XynI was eluted with a flow rate and fraction size of 12 ml/h and 4 ml, respectively.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12.5% acrylamide gel [11]. Protein bands were revealed by silver staining [3]. Low-molecular-weight standards from Sigma (St. Louis, MO, USA) were used as size markers.

Hydrolysis products. Reaction mixtures containing 100 µl (1.40 µg) of enzyme solution and 500 µl of 0.2% xylan in distilled water were incubated for 16 h at 28°C with shaking at 100 rev/min. The hydrolysis of kraft and sulfite pulp was carried out in a reaction mixture of 5.0 ml at 40°C with shaking at 80 rev/min for 18 h. The reaction was stopped by placing the mixture in boiling water for 5 min, and then the mixture was centrifuged at 3000 *g* for 10 min. The determination of products of hydrolysis was made by high-performance anion exchange chromatography coupled with pulsed amperometric detection (Dionex Corp., USA) [17].

Amino acid sequence. Native XynI was subjected to protein sequence by the Edman degradation method [8] with an Applied Biosystems 477A/120A automated protein sequencer. The amino acid sequence of XynI was used for homology search against the SWISS-PROT database using the BLAST program [1].

Kraft pulp treatment by XynI. The Kraft pulp, obtained from a composition of *Eucalyptus grandis* (40%), *E. saligna* (30%) and *E. urophylla* (30%), was previously treated with oxygen. It had an initial kappa number, consistency, viscosity, and brightness (ISO) of 9.7, 34%, 29.8 mPa.s and 47.7%, respectively. XynI was incubated with 10–20 g of pulp at 50°C, for 2 h. The pulp consistency was 4%. Xylanase activity was expressed as the amount of enzyme to catalyze the transformation of 1 mol of substrate per second under specified conditions (katal). The charge of enzyme activity used in the process varied from 30 to 100 nkat/g. The pH and pulp consistency were adjusted to 5.0 and 4%, respectively. A xylanase commercial preparation (Ecopulp T-100) was used for comparative purposes. A charge of 11–55 nkat/g of enzyme activity was incubated with 20 g of pulp at temperature, pH and, consistency of 50°C, 7.5 and 10%, respectively. Brightness and viscosity tests with xylanase activity were performed according to the recommendations of the Technical Association of the Pulp and Paper Industry: Tappi T452 om-92 and Tappi T230 om-94, respectively. The kappa number (Tappi T 236 cm-85) was expressed as the amount (ml) of a 0.1 *N* $KMnO_4$ solution consumed by 1 g of moisture-free pulp. Enzyme (X), chlorine dioxide (D1 and D2) and alkaline peroxide delignification (E) steps were used in the following treatment sequences: X-D1-E-D2 or D1-E-D2.

Chemicals. Kraft and sulfite pulps used in the hydrolysis products experiments were from Modobirch, Sweden and Zellstoffabrik, Germany, respectively. Deacetylated and acetylated xylans were obtained by dimethylsulfoxide (DMSO) extraction of beechwood and wheat straw hollocelluloses, respectively [17, 18]. Xylan extracted by HCl from the seaweed *Palmaria palmata* was a gift from Maria G. Tuohy (University College Galway, Ireland). Ecopulp T-100 was from ICI Forest Products, Canada.

Table 1. Summary of the purification of xylanase (XynI) from *Acrophialophora nainiana*

Steps	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (-fold)	Recovery (%)
Crude extract	166.12	401.97	2.42	1.00	100.00
Ultrafiltration					
Retentate	155.56	239.60	1.54	0.64	59.60
Ultrafiltrate	11.53	152.43	13.21	5.45	37.92
Sephacryl S-100	2.10	28.70	13.61	5.62	7.14

Results and Discussion

The two-step purification procedure of a xylanase activity from *Acrophialophora nainiana* is described in Table 1. Aliquots of the freeze-dried ultrafiltrate were fractionated by gel filtration chromatography on Sephacryl S-100 column. A protein peak containing XynI was obtained and used for further enzyme characterization. The above purification procedure provided an apparently homogeneous preparation of XynI. Since other forms of xylanase were detected in the crude extract, and these enzymes may act synergistically with XynI to effect xylan breakdown, the fold-purification and recovery values were probably underestimated [23]. The molecular weight of the enzyme was determined to be 17 kDa, as estimated by SDS-PAGE (Fig. 1). This result is in agreement with the literature, in which most of the fungal and bacterial xylanases are described to have molecular weight values in the range of 8.5–85 kDa [7, 15].

The pH and temperature optimum values of XynI were within the range reported for many xylanases [7]. At the routine assay temperature of 50°C, the purified enzyme was most active at pH 6.0. The optimum temperature value was 50°C at pH 5.0. The enzyme activity was reduced to 47% after 24 h of incubation at 50°C, pH 5.0. The enzyme showed a half life of 150 min at pH 6.0, 50°C.

The apparent K_m values at 50°C on oat spelt and birchwood xylans as substrates were 0.731 mg/ml and 0.343 mg/ml, respectively. The K_m values of the purified XynI was lower than that observed for xylanases from *Fusarium oxsporum*, *Cephalosporium* sp. strain RYM-202 and *Aureobasidium pullulans* [6, 10, 13].

XynI was activated by some thiol-containing reagents (L-cysteine, DTE, and β-mercaptoethanol) and L-tryptophan. These effects may indicate the presence of cysteine and tryptophan residues at the active site.

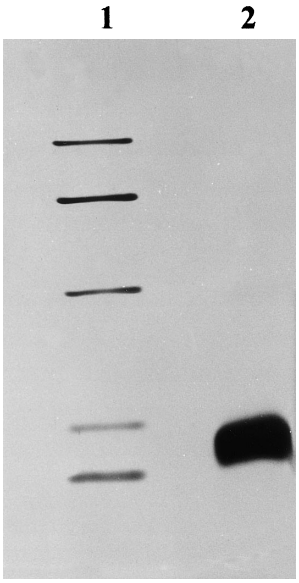

Fig. 1. SDS-PAGE of purified β-xylanase (XynI) from *Acrophialophora nainiana*. Lane 1, molecular weight standards (from the top): bovine serum albumin (66 kDa); ovalbumin (42 kDa); trypsinogen (24 kDa); lactoglobulin (18.4 kDa); lysozyme (14.3 kDa). Lane 2, β-xylanase from *Acrophialophora nainiana*.

1	5	10	15	20	25
A D V X Y D V G Y D X X X D S L T Q V A X S D G T					
	30	35	40	45	50
N G L M V S Y F Q N V T M D M X G F P Y I G X A E					

Fig. 2. The sequence of the first 50 amino acids of the purified β-xylanase (XynI) from *Acrophialophora nainiana*. A, D, V, X, Y, G, S, L, T, Q, N, M, F, P, I, and E mean alanine, aspartic acid, valine, nonidentified amino acid residue, tyrosine, glycine, serine, leucine, threonine, glutamine, asparagine, methionine, phenylalanine, proline, isoleucine, and glutamic acid, respectively.

Involvement of cysteine and tryptophan residues in the maintenance of the tertiary structure of the active site and the ability to permeate an ultrafiltration membrane was reported for a small xylanase from *Myrothecium verrucaria* [10]. The sequence of the 50 first residues of XynI is described in Fig. 2. It did not reveal significant homology to those from other published xylanase sequences.

A comparison was made of the hydrolysis of xylans and pulps by the purified XynI. The purified enzyme was able to release a small amount of xylose from xylans, suggesting an exo-acting mechanism. Xylose-liberating xylanases have been detected in *Microtetraspora flexuosa* SIIX, *Trichoderma harzianum*, and *T. viride* [2, 20, 21]. The enzyme did not show an exo-activity on kraft and sulfite pulps. Xylobiose and xylotriose were liberated from deacetylated, oat spelt, and birchwood xylans. The enzyme had no action against acetylated xylan, indicating that the acetyl groups were steric obstacles that limited the enzyme activity. Xylan from seaweed (*Palmaria palmata*) presents in its structure unbranched chains of about 80% and 20% of β-1,4 and β-1,3 linkages, respectively [9]. In this case, XynI showed an endo-acting mechanism, releasing xylobiose and xylotriose. The enzyme activity against kraft and sulfite pulps produced xylooligomers ranging from xylobiose to xylohexaose. The purified enzyme was also able to release higher xylooligomers from xylans and pulps.

The pulp treatment with XynI activity reduced the amount of chlorine compounds required for the process and enhanced brightness gain. In the pulp treatment without enzyme stage, a brightness of 84.3% and a viscosity of 19.7 mPa.s was obtained. On the other hand, the use of XynI in the early stage of pulp bleaching reached a brightness of 87.60–90.1% with a viscosity of 19.51–16.3 mPa.s. The gain in brightness and reduction of the amount of chlorine compounds after xylanase treatment have been previously reported in the literature [5, 19]. However, a loss of viscosity when a 100 nkat/g charge of XynI was used may be for some reason other than cellulose-degrading enzyme activity. The enzyme preparation did not present activity when assayed for cellulase. In comparison to the results obtained for pulp treatments with control (without enzyme) and Ecopulp T-100, the amount of chlorine residues detected after pulp treatment with XynI was higher than the two former procedures. Furthermore, it seems likely that the low molecular weight of XynI enhances its access into pulp.

In conclusion, these findings represent the first report on the purification and characterization of a low molecular weight xylanase from *A. nainiana*. The purified enzyme was able to convert efficiently xylans and pulps and showed a good stability at 50°C. The kraft pulp treatment with XynI indicated its potential use as an alternative approach in pulp bleaching.

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