European Journal of Plant Pathology **101**: 291–299, 1995.

Purification and partial characterization of xylanase from the fungal maize pathogen *Helminthosporium turcicum* (Pass)

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Accepted 10 November 1994

Key words: cell wall degrading enzymes, *Helminthosporium turcicum*, xylanase

Abstract

The fungal pathogen *Helminthosporium turcicum* was found to secret xylanase when grown on minimal medium containing xylans, wheat straw or isolated maize cell walls. The highest xylanase activity occurred when the fungus was grown on maize cell walls. When glucose was added to this medium xylanase activity was suppressed. The xylanase enzyme was purified from the culture filtrate by subsequent anion exchange chromatography, cation exchange chromatography and gel filtration. The purified xylanase gave a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis corresponding to an apparent molecular weight of 22.5 kDa. It is determined to have a pI of 7.4, specific activity of 11300 nanokatals mg^{-1}, pH optimum between pH 5.5 and 6.5 and optimal temperature between 50 °C and 60 °C. The half-life of the enzyme at pH 6.0 and 50 °C was found to be 35 min. For primary structure comparison with other xylanases, the protein was digested with trypsin and the resulting peptides were separated by reversed phase chromatography and selected peptides were sequenced. The determined amino acid sequence showed high homology with xylanase from *Cochliobolus carbonum* and three other fungal xylanases.

Introduction

The cell walls of the plants are important barriers for pathogen invasion. For many pathogens to infect a plant they have to dissolve these potential barriers and obtain their nutrients. Cellulose, hemicellulose and lignin are the predominant polymeric components of plant cell walls and hemicelluloses are major constituents of the primary and secondary wall regions of plants. Xylan, which is the major hemicellulose, make up about 40% of the primary walls of monocots [McNeil *et al.*, 1984; Cooper *et al.*, 1988]. A detailed analysis of maize (*Zea mays*) cell wall polysacharides [Kato and Nevins, 1984] showed that arabinoxylan is the predominant polymer in the primary walls. Complete hydrolysis of xylan is achieved mainly by endo-1,4-β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) [Erikkson *et al.*, 1990].

Several cereal fungal pathogens are known to produce xylanase activity using cereal cell walls as carbon source [Anderson, 1978; Cooper *et al.*, 1988; Holden and Walton, 1992; Southerton *et al.*, 1993]. Some studies also showed that increasing levels of xylanase activity is associated with developing lesions in fungus infected cereal hosts [Cooper *et al.*, 1988; Southerton *et al.*, 1993]. Moreover, xylanase from a commercial preparation of *Trichoderma viride* has been found to be toxic to rice cell cultures [Ishii, 1988].

On the other hand fungal xylanases activate

host defense responses such as tissue necrosis and electrolyte leakage [Bailey *et al.*, 1990], ethylene biosynthesis [Dean and Anderson, 1991] and synthesis of pathogenesis related proteins [Lotan and Fluhr, 1990].

The above evidences suggest that xylanases may play an important role in plant pathogenesis but the efforts made to determine the direct role of xylanases by molecular genetic techniques is very limited. The number of xylanases purified from plant pathogens is also quite limited. Only one xylanase gene is cloned from a plant pathogenic fungus at present, namely *Cochliobolus carbonum* [Apel *et al.*, 1993]. The fungus is reported to produce as many as three xylanases [Holden and Walton, 1992] and the disruption of the gene encoding the major xylanase (*XYL1*) did not affect the pathogenicity of the mutant which lacked *XYL1* [Apel *et al.*, 1993]. However, no valid conclusion was drawn since the mutant retained an appreciable amount of xylanase activity originating from another functional xylanase gene. In addition, there is no evidence for an assessment to detect variation in virulence. At this juncture, it is worth noting the inconsistent reports about the results of cutinase gene disruption mutant of *Fusarium solani* f. sp. *pisi* [Stahl and Schäfer, 1992; Rogers *et al.*, 1994]. By detailed analysis of virulence using multiple spore levels and microscopic observation of the progress of lesion development, Rogers *et al.* [1994] showed significant difference in virulence between the cutinase gene disrupted mutant and the wild type strain.

From the information available at present, the role of xylanase in pathogenesis is still not clearly known and needs further investigation. We have found that the maize pathogen *Helminthosporium turcicum* produces very high level of xylanase activity when grown on a medium containing maize cell wall. As an important step for a detailed analysis of the role of this activity in pathogenesis by molecular techniques, we report the purification and partial characterization of the enzyme from the fungus.

Materials and methods

Source of isolate

The fungus, *H. turcicum*, used in this study was isolated from an infected maize leaf and routinely maintained on Czapeck's Dox Agar in 20% glycerol at –20 °C.

Media and culture conditions

The fungus was grown in 250 ml Erlenmeyer flasks in 100 ml of minimal salt medium containing: $NaNO_3$ (2.0 g l^{-1}); KCl (0.5 g l^{-1}); K_2HPO_4 (1.0 g l^{-1}); $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}); $FeSO_4 \cdot 7H_2O$ (0.01 g l^{-1}); $ZnSO_4 \cdot 7H_2O$ (0.01 g l^{-1}) and $CuSO_4 \cdot 5H_2O$ (0.0025 g l^{-1}). The carbon sources added as a supplement to the minimal basal medium were 1.5% (w/v) of wheat straw, maize cell wall, glucose, xylan from oat spelt (Sigma) or xylan from birchwood (Sigma).

The carbon sources, wheat straw and maize cell wall, were prepared as follows. Wheat straws were washed thoroughly with tap water to make them dust free, air dried and ground into small pieces. For preparation of maize cell wall, leaves from three-week old maize (variety Alamura Yellow) were collected and frozen at –20 °C. Extraction of cell walls was carried out according to Bateman *et al.* [1973] with slight modifications. Leaves were ground in a prechilled mortar in liquid nitrogen. The ground leaves were washed with 1% (w/v) of sodium deoxycholate (3 ml g^{-1} of leaves) and vacuum filtered through Whatmann 3 MM filter paper in a Buchner funnel. The retained residue was again washed with ice cold chloroform:methanol (1:1) and ice cold acetone (2 ml g^{-1}). The resulting cell wall was further washed once with ice cold diethyl ether (2 ml g^{-1}), air dried and either used immediately or stored in a capped bottle at room temperature. The sterilized media (100 ml) containing the different carbon sources were seeded with conidial suspension (approximately 10^6 conidia ml^{-1}) prepared from three-week old culture of *H. turcicum* (isolate H-2) grown on Czapeck's agar. The cultures were incubated at 28 °C in darkness in an orbital shaker at 180 rpm up to 15 days, filtered through one layer of Miracloth (Calbiochem) and centrifuged at 10000 × g for 10 min to remove fungal fragments, spores and other debris. The clarified

culture filtrates were either used immediately or frozen at –70 °C until needed.

Enzyme activity assays

During cultivation of *H. turcicum* xylanase activity was assayed according to Poutanen and Puls [1988] with slight modifications. Xylan from oat spelt (1%) suspended by stirring and warming in 0.05 M sodium-citrate phosphate buffer pH 5.3 was used as a substrate. The samples (40 µl) from the culture media were incubated with 360 µl of substrate solution in a water bath at 50 °C for exactly 5 minutes. The reaction was terminated by adding 600 µl dinitrosalisylic acid (DNS) reagent [Miller, 1959]. After heating for 5 min in boiling water, cooling and centrifugation, the absorbance of the supernatant was measured at 540 nm using a Labsystems Multiscan MCC/340 (Labsystems Ltd) microtiter plate spectrophotometer. Xylose (Merck) was used as a standard and xylanase activities were expressed in nanokatals.

Activity measurements during purification of xylanase were performed as above except that 1% (w/v) birchwood xylan (Roth 7500) was used as the substrate.

Purification of xylanase

Clarified culture medium, stored at –70 °C, was thawed and centrifuged at 20000 × g (Sorvall SS-34 rotor). The clear supernatant was passed in 2.5 ml portions through a 1.0 × 8.0 cm Q-Sepharose Fast Flow (Pharmacia) column, equilibrated with 20 mM sodium acetate, pH 4.8, using a flow rate of 1.0 ml min^{-1} and monitoring at 280 nm. The column was washed between the runs with 2.0 ml of 1 M sodium chloride and equilibrated to the buffer above. The xylanase activity containing fractions (about 4 ml) from four runs were combined and diluted (1:2) with distilled water to reduce the buffer ionic strength to 10 mM. Cation exchange chromatography was performed on a Mono S HR5/5 (Pharmacia) column equilibrated with 10 mM sodium acetate, pH 4.8, using a linear gradient of sodium chloride (0–0.5 M in 30 min) in the equilibration buffer and a flow rate of 1.0 ml min^{-1}. Xylanase active fractions from cation exchange chromatography were combined, concentrated to about 1/4 of the original volume in a vacuum centrifuge and subjected in 250 µl portions to gel filtration in a Superdex S-75 HR10/30 (Pharmacia) column equilibrated with 20 mM sodium acetate, pH 4.8, 0.1 M sodium chloride. Chromatography was performed with a flow rate of 0.5 ml min^{-1} and detection at 280 nm. Xylanase active fractions were pooled and used for further studies. For calculation of specific activities during purification, the protein content of different fractions was determined using the Bio-Rad protein assay reagent and γ-globulin (Sigma) as standard.

SDS-PAGE (12.5%) was carried out according to Laemmli [1970].

Tryptic cleavage and peptide separation

About 1 nmol of the purified xylanase from gel filtration was concentrated to 100 µl and passed through a 0.5 × 5 cm Bio-Gel P6 (Bio-Rad) column equilibrated with 10 mM ammonium bicarbonate. The xylanase active flow through fraction was dried in a vacuum centrifuge and dissolved in 20 µl 8 M urea, 0.4 M ammonium bicarbonate. Dithiothreitol (2 µl of 112 mM) was added and the sample incubated at 50 °C for 30 min. To alkylate the cystein residues, 4-vinylpyridine (2 µl, diluted 1:50 with water) was added and the sample incubated at room temperature for 15 min. Water (200 µl) and 1 µg trypsin (modified trypsin, sequencing grade, Promega, V5111) were added and the solution was incubated in the thermostated (37 °C) autosampler compartment of an Applied Biosystems 270A-HT Capillary Electrophoresis System. Digestion was monitored by injecting samples (4 nl) every 30 min followed by peptide electrophoresis at 25 kV in a 50 µm fused silica (52 cm effective length) capillary in 20 mM sodium citrate buffer, pH 2.5. Electrophoresis was monitored at 200 nm. After completion of the enzymatic cleavage, the reaction mixture was acidified with 1 µl trifluoroacetic acid. Peptides were separated by narrow bore reversed phrase HPLC (Applied Biosystems 140B, 785A) on a 2.1 × 150 mm Rexchrom C8 (300 Å, 5 µm, Regis Chemical Company) column using a linear gradient of acetonitrile (3–60% in 60 min) in 0.1% trifluoroacetic acid and detection at 214 nm. Manually collected peptides were concentrated to about 30 µl in a vacuum centrifuge.

Protein and peptide sequencing
Protein and peptide sequencing was performed on a gas-pulsed-liquid-phase sequencer using on-line PTH-amino acid analysis [Kalkkinen and Tilgmann, 1988].

pI determination
Capillary isoelectric focusing was performed using an Applied Biosystems Model 270A-HT Capillary Electrophoresis System according to the manufacturers instructions (ABI 270A-HT user bulletin No. 5). The system was calibrated using proteins with known PI's as standards. PhastGel (Pharmacia) isoelectric focusing in polyacrylamide gel (pH 3–9) was performed according to the manufacturers instructions (Pharmacia Phast-System Separation Technique File No. 100), using Pharmacia pI 3–10 standard mixture.

pH-optimum and temperature dependence
For pH-optimum determination of purified xylanase, the substrate (birchwood xylan) was dissolved in 50 mM sodium citrate phosphate buffer of different pH values between pH 3.2 and 8.8. Activity measurements were performed at 50 °C. Temperature dependence determination of the purified xylanase was performed at defined temperatures for 5 min pH of 6.2. However, at temperatures above 40 °C the incubation times were reduced to 2 min.

Thermal stability of purified xylanase
Two samples of the purified xylanases (12 µg each) were incubated at 50 °C in 0.125 M citric acid phosphate buffer pH 6.0 or 7.0 respectively, containing 200 µg ml^{-1} bovine serum albumin, 10 µg ml^{-1} pepsatin A (Sigma) and 1 mM PMSF (phenylmethylsulphonyl fluoride; Sigma). Samples were withdrawn at defined time points, and assayed for residual xylanase activity at pH 6.2 and 50 °C as above.

Results

The effect of carbon sources on xylanase production
The production of xylanase activity using five different substrates as carbon sources is shown in Table 1. High xylanase activity was detected when wheat straw and maize cell wall were used. Use of oatspelt xylan and xylan from birchwood as a carbon source result in low production of xylanasese activity. No xylanase activity could be detected when the readily metabolizable substrate, glucose, was used as a sole carbon source. Addition of 0.5% and 1% glucose to the maize cell wall containing medium reduced the xylanase activity by about 45% and 75% respectively (not shown). Moreover, the appearance of xylanase activity was delayed to 13 days from the date of inoculation when 1% glucose was added to the medium containing maize cell wall.

Table 1. The effect of different carbon sources on the production of xylanase by *Helminthosporium turcicum*. Xylanase activity was measured by the DNS method. Shown are ± standard error of three replications

Carbon source	Xylanase activity (nkat ml^{-1}	Day of maximum xylanase activity
Glucose (1%)	0 ± 0.0	–
Oatspelt xylan	21 ± 1.5	5
Birchwood xylan	58 ± 2.0	6
Maize cell wall	335 ± 10	7
Wheat straw	182 ± 5	9

Time course of xylanase activity
The time course of production of the enzyme was followed by growing *H. turcicum* in a medium containing maize cell wall. Xylanase activity could be detected after three days of incubation following accumulation of mycelial mats of the fungus and continued to increase until the seventh day, after which it declined sharply (Fig. 1). The time for maximum xylanase activity was different for all carbon sources used in the study (Table 1).

Purification of xylanase
The clarified culture filtrate, stored at –70 °C was after thawing centrifuged to remove materials precipitated during freezing and thawing. The specific activity of this centrifuged supernatant was 1460 nkat mg^{-1} protein which is essentially the same as measured directly from the fresh culture filtrate. This indicates that no xylanase activity was lost during freezing, thawing or centrifugation. The clarified culture filtrate was green but the colour was completely adsorbed to the first purification

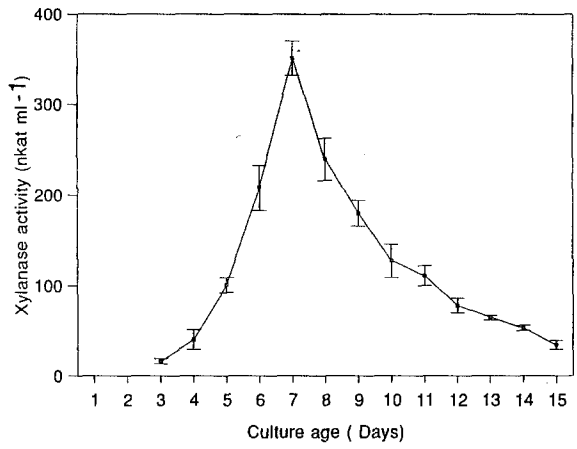

Fig. 1. Production of xylanase by *Helminthosporium turcicum* during a 15-day cultivation. Xylanase activity was assayed as described in Materials and methods using 1% oatspelt xylan in 0.05 M sodium citrate buffer pH 5.3 as the substrate.

column, Q-Sepharose, from which the xylanase activity was eluted as one peak in the flow-through (not shown). The specific activity of the collected xylanase active flow-through peak was 2020 nkat mg^{-1} protein. No additional xylanase activity could be washed from the column with 1 M sodium chloride. The xylanase activity containing flow-through fraction was then diluted (1:2) with water to lower the buffer ionic strength to 10 mM in which the buffer xylanase could be adsorbed to the following cation exchange column, Mono S. Elution of this column with an increasing linear gradient of sodium chloride (0–0.5 M in 30 min) resulted in elution of xylanase and the specific activity of the collected xylanase active peak was 5740 nkat mg^{-1} corresponding to a 4-fold purification as compared to the crude culture filtrate. The concentrated xylanase fraction was subjected to gel filtration with a result as shown in Fig. 2A. The specific activity of the collected xylanase active fraction was 11300 nkat mg^{-1} protein corresponding to a 7.7-fold purification from the crude culture filtrate. The purified xylanase from the gel filtration gave a single band on SDS-PAGE corresponding to an apparent molecular weight of 22.5 kDa as shown in Fig. 2B, which also shows corresponding SDS-PAGE results from the previous purification steps and from the crude culture filtrate.

Characterization of the purified xylanase

For further characterization, the purified xylanase was subjected to N-terminal sequence analysis. No amino acid derivatives were however, released in Edman degradation indicating that its N-terminus is blocked. In order to obtain sequence data from the enzyme, we tried to digest it with trypsin. The native protein was however resistant to digestion except after denaturation with 8 M urea, reduction with dithiothreitol and alkylation with 4-vinylpyridine. The whole reaction mixture was diluted 20-fold in water since it was impossible to desalt the protein after denaturation, reduction and reversed phase chromatography. Trypsin was then added and digestion was performed in the thermostated (37 °C) autosampler compartment of a capillary electrophoresis system. Appearance of peptides was followed by capillary electrophoretic analysis (1 analysis per 30 min) of the cleavage mixture (result not shown). After about 4 h the generated peptide pattern remained constant indicating completion of the tryptic cleavage. The resulted eight peptides were separated by narrow bore reversed phase chromatography (not shown). Individual peptides were collected and subjected to sequence analysis with the results as shown in Table 2.

For determination of the pI, the purified xylanase was subjected to capillary electrophoretic isoelectric focusing in which the enzyme migrated to a position corresponding to a pI of 7.4. In Polyacrylamide gel isoelectric focusing, the enzyme gave repeatedly a slightly diffuse band corresponding to a pI of about 7.4.

The pH optimum of the purified xylanase was between pH 5.5 and 6.5 (Fig. 3A) and its temperature dependence showed a maximum between 50 °C and 60 °C (Fig. 3B). Thermal stability studies (Fig. 3C) showed that the half-life of the enzyme at pH 6.0 and temperature 50 °C was 35 min.

Amino acid sequence comparison

Primary structure data were obtained from the tryptic peptides (100 residues altogether) corresponding to about 50% of the suggested total primary structure as estimated from the molecular weight. The amino acid sequence comparison with four fungal xylanases is shown in Fig. 4. Striking amino acid homology was found with that of *C. carbonum* [Apel *et al.*, 1993] where 94 out of the 100 amino acids were found in identical positions.

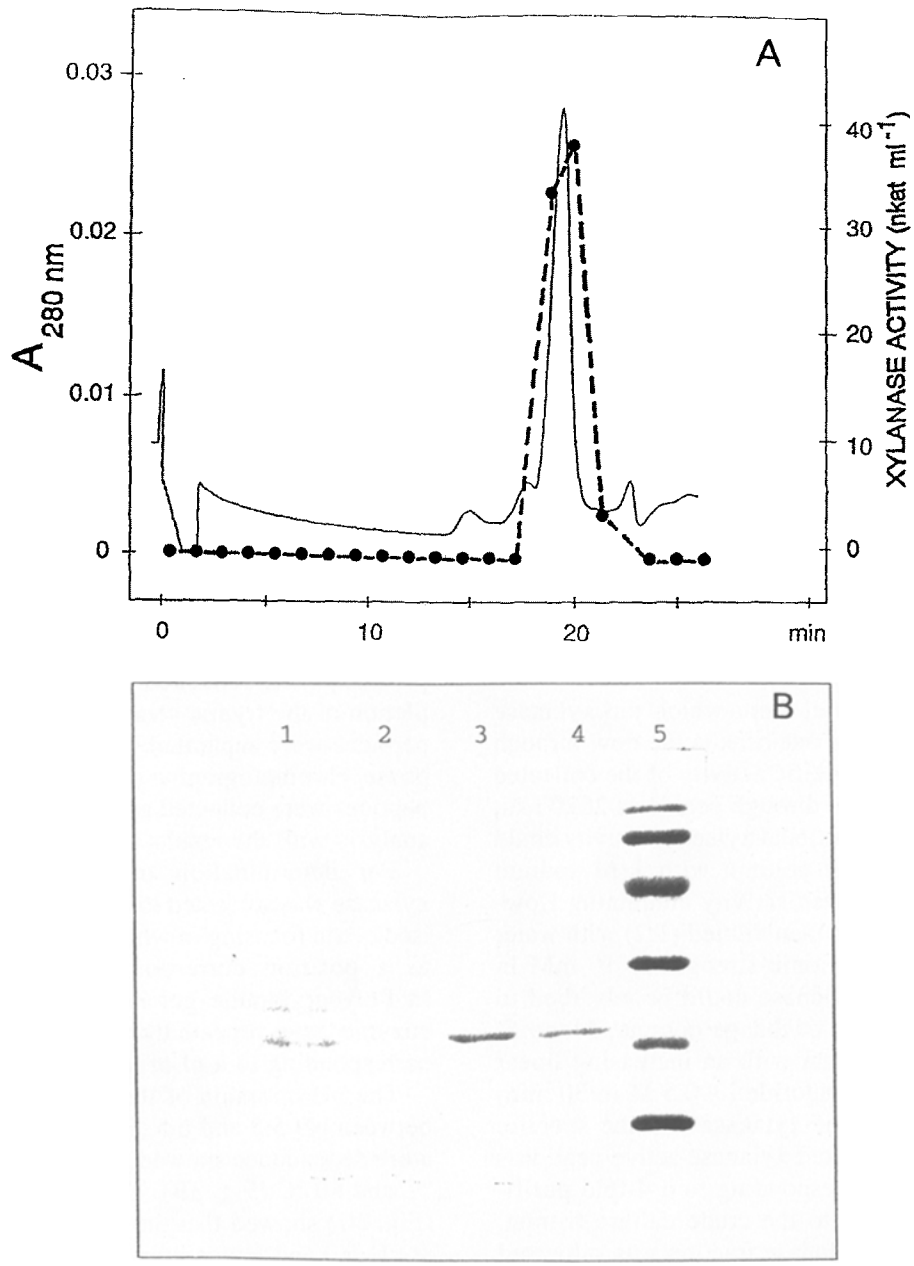

Fig. 2. (A) Chromatogram of gel filtration of xylanase active fractions from Mono S on a Superdex HR10/30 column in 20 mM sodium acetate, pH 4.8, 0.1 M sodium chloride. Fractions were collected and their xylanase activity was measured with results as shown with broken lines. (B) 12.5% SDS-polyacrylamide gel electrophoresis. (Lane 1) clarified culture filtrate; (lane 2) xylanase active Q-Sepharose pool; (lane 3) xylanase active Mono S fraction; (lane 4) xylanase active Superdex 75 fraction; (lane 5) Molecular weight markers of 94, 68, 43, 30, 20.1 and 14.4 kDa.

Schizophyllum commune [Paice *et al.*, 1978], *Trichoderma harzianum* [Yaguchi *et al.*, 1992] and *Trichoderma viride* [Ujiie and Yaguchi, 1991] showed 63%, 71% and 70% identity respectively.

Discussion

Xylanases have been purified from many microorganisms mostly for industrial applications [Dekker

Table 2. Determined amino acid sequences of tryptic peptides derived from *Helminthosporium turcicum* xylanase. The numbering of the peptides is according to their appearance in the reverse phase chromatography

Peptide No.	Amino Acid Sequence
1	Gly-Thr-Val-Thr-Ser-Asp-Gly-Ser-Ser-Tyr-Lys
2	Gly-Asn-Gln-Pro-Ser-Ile-Asp-Gly-Thr-Arg
3	Gly-Trp-Asn-Pro-Gly-Thr-Ala-Arg
4	Thr-His-Phe-Asp-Ala-Trp-Ala-Ser-Lys
5	Ala-Thr-Tyr-Thr-Asn-Gly
6	Thr-Phe-Gln-Gln-Tyr-Trp-Ser-Val-Arg
7	No sequence result
8	Thr-Ile-Thr-Tyr-Ser-Gly-Gln-Tyr-Asn-Pro-Asn-Gly-Asn-Ser-Tyr-Leu-Ala-Ile-Tyr-Gly-Trp-Thr-Arg
9	Asn-Pro-Leu-Val-Glu-Tyr-Tyr-Val-Val-Glu-Asn Phe-Gly-Thr-Tyr-Asp-Pro-Ser-Ser-Gln-Ala-Gln-Asn-Lys

and Richards, 1976; Eriksson *et al.*, 1990]. From the increasing interest in their potential role in plant pathogenesis in cereal fungal pathogens, some xylanases have also been purified and characterized [Holden and Walton, 1992; Southerton *et al.*, 1993]. In this study we purified and partially characterized xylanase from the maize pathogen *H. turcicum*.

H. turcicum produced very high amount of xylanase activity when grown in the medium containing maize cell wall as carbon source. To stimulate growth and xylanase production some studies reported the use of supplemental carbon source or pregrowing the fungus in a rich medium [Ghosh and Deb, 1988; Holden and Walton, 1992]. *H. turcicum* grew abundantly in the minimal medium containing maize cell wall as sole carbon source and produce very high xylanase activity. The high degree of variation in xylanase activity on different carbon sources observed in this study is in agreement with other findings [Ghosh and Deb, 1988; Cavazzoni *et al.*, 1989; Royer and Nakas, 1989; Holden and Walton, 1992; Ito *et al.*, 1992].

Like other fungal xylanases [Royer and Nakas, 1989; Ito *et al.*, 1992], and pectinases [Dean and Timberlake, 1989], the xylanase from *H. turcicum* is repressed by glucose. The implication of this phenomenon in xylanase production during host parasite interaction is not known. But a study on pectic enzymes [Holz and Knox-Davies, 1986] showed that accumulation of high concentration of free sugars such as glucose, fructose and sucrose in onion bulb tissues suppressed enzyme synthesis and reduced pathogenicity of *F. oxysporum f. sp. cepae.*

The apparent molecular weight of the purified xylanase from *H. turcicum* as determined by SDS-PAGE was 22.5 kDa. This is consistent with the characteristically low molecular weight feature of microbial xylanases [Eriksson *et al.*, 1990], and it is about the same as the apparent molecular weights of xylanases from *Trichoderma reesei, T. harzianum* and *C. carbonum* [Lappalainen, 1986; Wong *et al.*, 1986; Holden and Walton, 1992].

The xylanases purified from plant pathogenic fungi [Holden and Walton, 1992; Southerton *et al.*, 1993] have alkaline pI of at least 9.3. But the xylanase from *H. turcicum* has an apparent pI of 7.4 indicating that it has no conserved pI relationship with these fungal xylanases. There is striking amino acid sequence similarity between xylanases from *H. turcicum* and that of *C. carbonum*. This is not uncommon among such

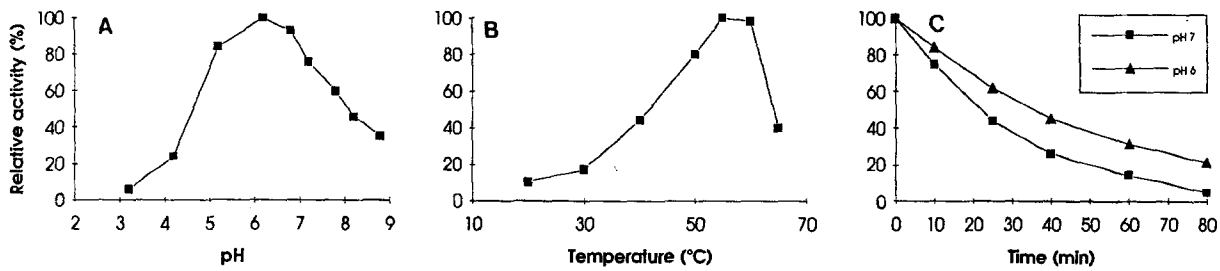

Fig. 3. (A) pH optimum, (B) temperature dependence (C) thermal inactivation of purified *Helminthosporium turcicum* xylanase. Optimal pH was determined as described in the Materials and methods by incubating the enzyme at 50 °C for 5 min. Activity at pH 6.2 corresponds to 19 nkat. Temperature dependence was determined at pH 6.2 and activity at 55 °C corresponds to 47 nkat. For thermal inactivation studies two samples were incubated as described in the Materials and methods. After the times indicated, samples were withdrawn and assayed for residual xylanase activity. Maximum activity corresponds to 43 nkat.

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Sc          SGTPSSTGTDGGYYYSWWTDGAGDATYQNNGGGSYTLTWSGNNGNLVGGK
Th          QTIGPGTGYSNGYFYYYWNDGHAGVTYTNGGGGSFSVNW-SNSGNFVGGK
Tv          QTIGPGTGFNNGYFYSYWNDGHGGVTYTNGPGGQFSVNW-SNSGNFVGGK
Cc          QNTPNGEGTHNGCFWSWWSDGGARATYTNGAGGSYSVSW-GSGGNLVGGK
Ht          ----------------------ATYTNG--------------------

Sc          GWNPGAASRSISYSGTYQPNGNSYLSVYGWTRSSLIEYYIVESYGSYDPS
Th          GWQPGTKNKVINFSGSYNPNGNSYLSVYGWSRNPLIEYYIVENFGTYNPS
Tv          GWQPGTKNKVINFSGSYNPNGNSYLSVYGWSRNPLIEYYIVENFGTYNPS
Cc          GWNPGT-ARTITYSGTYNYNGNSYLAVYGWTRNPLVEYYVVENFGTYDPS
Ht          GWNPGT-ARTITYSGQYNPNGNSYLAIYGWTRNPLVEYYVVENFGTYDPS

Sc          SAASHKGSVTCNGATYDILSTWRYNAPSIDGTQTFEQFWSVRNPKKAPGG
Th          TGATKLGEVTSDGSVYDIYRTQRVNQPSIDGTATFYQYWSVRRNHRS---
Tv          TGATKLGEVTSDGSVYDIYRTQRVNQPSIDGTATFYQYWSVRRTHRS---
Cc          SQSQNKGTVTSDGSSYKIAQSTRTNQPSIDGTRTFQQYWSVRQNKRS---
Ht          SQAQNKGTVTSDGSSY------KGNQPSIDGTRTFQQYWSVR--------

Sc          SISGTVDVQCHFDAWKGLGMNLGSEHNYQIVATEGYQSSGTATITV---
Th          --SGSVNTANHFNAWASHGLTLGT-MDYQIVAVEGYFSSGSASITV---
Tv          --SGSVNTANHFNAWAQQGLTLGT-MDYQIVAVEGYFSSGSASITV---
Cc          --SGSVNMKTHFDAWASKGMNLGQ-HYYQIVATEGYFSTGNAQITVNCP
Ht          ---------THFDAWASK-----------------------------
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Fig. 4. Comparison of the amino acid sequence of xylanase from *Helminthosporium turcicum* (*Ht*) with those of other fungal xylanases. *Sc* = *Schizophyllum commune*, *Th* = *Trichoderma harzianum*, *Tv* = *Trichoderma viride* and *Cc* = *Cochliobolus carbonum*. The totally conserved sequences are shaded. Dashed lines are spacers for alignment.

closely related fungal species since an almost similar degree of amino acid sequence identity is observed between *T. viride* and *T. harzianum* as can be seen from the sequence alignment (Fig. 4).

The apparent lack of xylanase multiplicity in *H. turcicum* is very interesting. Multiple xylanases have often been reported from many microorganisms including pathogenic fungi [Wong *et al.*, 1988; Holden and Walton, 1992; Southerton *et al.*, 1993]. Our work showed a single peak of xylanase activity in this fungus. But the possibility that the production and/or purification procedure(s) might have favored the most abundantly secreted xylanase is something which can not be ruled out.

This study provides very useful information for isolating the gene encoding xylanase from *H. turcicum*. The strain is highly virulent on maize and this study showed that it has apparently one xylanase gene. The genetic transformation and cloning of the xylanase gene is in progress. Thus by a transformation mediated gene disruption, it is possible to determine the role of this xylanase in pathogenesis.

Acknowledgements

We thank Keijo Virta for excellent technical assistance, Lars Paulin for comparison of xylanase amino acid sequences and Peter Stephensen Lübeck for valuable discussion and critical reading of the manuscript. One of the authors (Y. D) is supported by the Finnish International Development Agency (FINNIDA).

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