This is to certify that the

thesis entitled

PURIFICATION AND CHARACTERIZATION OF XYLANASE FROM THE
FUNGAL MAIZE PATHOGEN, *COCHLIOBOLUS CARBONUM*

presented by

FRANK ROBERTSON HOLDEN

has been accepted towards fulfillment
of the requirements for

M.S. degree in Botany and Plant Pathology

Jonathan Walton
Major professor

Date August 6, 1991

O-7639

PURIFICATION AND CHARACTERIZATION OF XYLANASE FROM THE FUNGAL MAIZE PATHOGEN, *COCHLIOBOLUS CARBONUM*

by

Frank Robertson Holden

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1991

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF XYLANASE FROM THE FUNGAL MAIZE PATHOGEN, *COCHLIOBOLUS CARBONUM*

by

Frank Robertson Holden

Xylans are the most abundant hemicellulose and are found in all land plants. They are a major component of plant cell walls, making up to 40% by mass of the primary cell wall of monocots and a significant portion of the secondary cell walls of dicots. There is a considerable amount of indirect evidence that enzymes that degrade xylan (xylanases) are important in pathogenesis.

Three xylanases and a ß-xylosidase were purified from cultures of the maize pathogen, *Cochliobolus carbonum* grown on xylan or corn cell walls. The major xylanase was found to have a molecular weight of 24,000 by SDS-PAGE and 8,500 by gel filtration chromatography and a high isoelectric point (pI greater than 9.5). The other xylanases had similar properties and the ß-xylosidase was shown to have a molecular weight of 41,000 by SDS-PAGE and 31,500 by gel filtration chromatography. The major xylanase was purified to homogeneity and was analyzed for amino acid composition. It was also digested with trypsin and the resulting peptides were purified and sequenced. Using the tryptic peptide sequences, two degenerate oligonucleotides were synthesized and used as probes against a genomic DNA library made in lambda. Approximately 50 lambda clones were picked from the library.

ACKNOWLEDGEMENTS

I wish to thank Jonathan Walton for all his help in preparing this thesis and too many other things to mention. I would also like to thank Joseph Leykam for the help in preparing the peptides and the amino acid sequencing. I thank Ray Hammerschmidt for his help by being on my committee and his support.

Finally, I wish to thank Laura for just being herself.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
ß	beta
C	Celsius
CM	carboxy methyl
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	(ethylenedinitrilo)tetraacetic acid
gm	gram(s)
HFBA	hexafluorobutyric acid
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
LB	Luria broth
M	molar
mm	millimeter(s)
mM	millimolar
mg	milligram(s)
µCi	microCurie(s)
µl	microliter(s)
µm	micromolar

ml	milliliter(s)
mw	molecular weight
N	normal
ng	nanogram(s)
nm	nanometer(s)
OD	optical density
%	percent
p	*para*
pI	isoelectric point
PTH	phenylthiohydantoin
RVU	relative viscometric units
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
TLC	thin layer chromotagraphy
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume

INTRODUCTION

Many plant pathogens need the ability to penetrate the plant cell wall before successful infection can occur. Penetration of the plant cell wall can occur by mechanical force or by enzymatic degradation. Research on the role of plant cell wall degrading enzymes in infection is considerable and most bacterial and fungal plant pathogens are known to produce a battery of plant cell wall degrading enzymes (Cooper, 1984). However, few of these enzymes have been studied for a role in pathogenesis.

Nearly all plant cell wall degrading enzymes directly implicated in pathogenesis are from bacterial pathogens. A few cell wall degrading enzymes which when selectively mutated in bacterial pathogens result in reduced virulence, directly implicating the enzymes in pathogenesis. The best evidence showing involvement of a cell wall degrading enzyme in pathogenesis comes from transposon mutagenesis of the pectin methyl esterase gene of *Erwinia chrysanthemi* 3937. The mutant pathogen became noninvasive in *Saintpaulia* plants (Boccara *et al.,* 1989). Other evidence suggests that certain cell wall degrading enzymes are, in some cases, important but not essential in bacterial pathogenesis. The endoglucanase gene in *Pseudomonas solanacearum* was disrupted by transposon mutagenesis causing a 200-fold decrease in endoglucanase activity and significantly less virulence on tomato (Roberts *et al.*, 1988). The major endoglucanase activity in *Xanthomonas campestris* pv. *campestris* was disrupted by transposon insertion and pathogenicity tests of these mutants demonstrated that this enzyme plays only a minor role in of pathogenesis on turnip and radish (Glough *et al.*, 1988). All four pectate lyase genes in *E. chrysanthemi* were deleted causing a severely reduced ability to degrade pectin, but the bacterium could still survive on pectin as a carbon source and caused significant maceration of potato, carrot and pepper tissue (Payne *et al.*, 1987; Reid and Collmer, 1988).

In fungi, no polysaccharide-degrading enzymes have been directly shown to be important in pathogenesis. It was shown by targeted gene disruption that endopolygalacturonase is not essential for infection of maize by *Cochliobolus carbonum* (Scott-Craig *et al.*, 1990).

Although for the majority of diseases direct evidence is lacking, there is much indirect evidence that cell wall degrading enzymes are involved in pathogenesis: a) Polysaccharide degrading enzymes are produced by plant pathogens when grown on plant cell walls or purified components of plant cell walls as a carbon source (Bateman *et al.*, 1973; Mullen and Bateman, 1975; Bateman *et al.*, 1969; Cooper *et al.*, 1988; Anderson, 1978). b) The timing and extent of production of a particular cell wall degrading enzyme in culture often reflects the importance of that enzyme's substrate in the host cell wall (Cooper *et al.*, 1988; Cooper, 1984; Anderson, 1978; English *et al.*, 1971). c) Polysaccharide degrading enzymes are frequently present in infected plant tissue (Mullen and Bateman, 1975; Cooper *et al.*, 1988; Bateman *et al.*, 1969). d) Certain plant cell wall degrading enzymes are capable of eliciting host defense responses. Endo-polygalacturonase (EC 3.2.1.15) produces short oligogalacturonides that act as elicitors of plant defense responses such as phytoalexin accumulation in soybean (Davis *et al.,* 1984) or necrosis in *Vigna unguiculata* (Cervone *et al.*,1987). Pectin lyase can cause the formation of superoxide radical in rice protoplasts (Ishii, 1988).

Most if not all plant pathogens produce xylanase (ß-1,4-endoxylanase, EC 2.3.1.8). Although xylanase has not been studied directly for a role in any plant disease, either by traditional mutagenesis or by recombinant DNA methods, indirect evidence suggests that they might be important. The same indirect evidence used to implicate cell wall degrading enzymes in general can be used for xylanase: a) Xylans are present in all land plants. ß-1,4-xylan is the major constituent of monocot primary cell walls, making up to 40% or more by mass (Burke *et al.,* 1974; Darvill *et al.*, 1980a; Cooper, 1984). Since xylan is the most abundant component of the primary cell wall of monocots, xylanases may play a significant role in the pathogenesis of monocots. Xylans account for approximately 5% of the primary cell wall and a more significant percentage of the secondary cell wall of dicots (Darvill *et al.*, 1980b) b) Xylanase is produced by plant pathogens when grown on plant cell walls. c) Xylanase is sometimes produced earlier than other enzymes when monocot pathogens are grown on plant cell walls (Mullen and Bateman, 1975; Bateman *et al.,* 1969). d) Xylanase is present in infected plant tissue (Cooper *et al.*, 1988; Mullen and Bateman, 1975; Bateman *et al.,* 1969). e) Xylanase from *Trichoderma viride* can either directly or indirectly elicit plant defense responses (Dean and Anderson, 1991). Xylanase from *Trichoderma viride* can elicit pathogenesis-related proteins (Lotan and Fluhr, 1990), electrolyte leakage (Bailey, *et al.*, 1990) and ethylene biosynthesis (Fuchs, *et al.*, 1989) in tobacco. Xylanase can cause the formation of superoxide radical in rice protoplasts (Ishii, 1988).

A great deal of research has focused on the bacterial pathogens of dicots and this has demonstrated the importance of pectin degrading enzymes in these systems. However, due to the different composition of monocot and dicot cell walls, pectin degrading enzymes may not play such a significant role in the pathogenesis of monocots. Instead, enzymes such as xylanase, glucanase and arabanase may be more important. Some researchers believe that the importance of xylanase in pathogenesis, especially in monocots, has been underestimated (Cooper, 1984).

The purpose of this research was to prepare the groundwork to test the hypothesis that xylanase is important in plant pathogenesis. Targeted gene disruption of xylanase in *C. carbonum* and other pathogenic fungi is be possible as a result of this research. This will directly establish whether xylanase is important in these systems.

MATERIALS AND METHODS

Fungal Growth Conditions

Conidia of *Cochliobolus carbonum* race 1, isolate SB111 (HC-toxin producing), or race 2, isolate 1309 (HC-toxin nonproducing), from stocks stored at -80° C, were used to inoculate V-8 juice agar plates (Walton, 1987; Walton and Holden, 1988). Isolate SB111 was provided by S. P. Briggs, Pioneer Hi-Bred International, Inc., Johnston, Iowa. Isolate SB111 was used for all experiments unless otherwise noted. Isolate 1309 was supplied by K. J. Leonard, North Carolina State University. Plates were grown at room temperature (21-23° C) under fluorescent lights and 0.5 cm^2 squares were cut out and used to inoculate one-liter Erlenmeyer flasks containing 125 ml of liquid media. Liquid media was either modified Fries' medium (Pringle and Braun, 1957) or mineral salts medium (Table 1) (Bateman *et al.*, 1973) with 1 or 2 gm/liter of sucrose (unless otherwise noted). 0.4% or 0.8% (w/v) oat-spelt xylan (Fluka Chemie AG, Switzerland) or 0.8% (w/v) washed corn cell walls was added to flasks individually. *C. carbonum* was grown for 7 or 9 days on an open laboratory bench (21-23° C). Washed corn cell walls were prepared by chopping whole mature corn plants into small pieces and from this a fine powder was prepared using a Waring blender with liquid nitrogen.

Table 1. Mineral salts medium.

	per liter
$MgSO_4$	0.18 gm
KCl	0.15 gm
$(NH_4)_2SO_4$	1 gm
KH_2PO_4	0.65 gm
Sucrose	1 or 2 gm (unless otherwise noted)
trace element stock	1 ml
yeast extract	2 gm

Trace element stock (1000x)

$MnSO_4 \cdot 4H_2O$	0.1 gm
H_3BO_3	0.1 gm
$CuSO_4$	0.01 gm
$ZnSO_4 \cdot 7H_2O$	0.01 gm
$FeSO_4$	2.0 gm

This powdered corn plant preparation was suspended in 0.1 M KH_2PO_4, pH 7.0 buffer and stirred for one hour, strained through cheese cloth and then resuspended in chloroform, this was repeated twice with methanol after which the washed corn cell wall preparation was allowed to dry.

Xylanase production *versus* time was determined by harvesting the culture filtrate of *C. carbonum* grown on modified Fries' medium or mineral salts medium with 2 gm/liter of sucrose and 0.8% (w/v) xylan at varying times and assaying for xylanase activity. Inhibition of xylanase production by sucrose was tested by growing *Cochliobolus carbonum* for 7 days in modified Fries' medium containing 0, 1, 2, 4, 8 or 12 gm/liter sucrose and 0.8% (w/v) xylan. Xylanase activities and the dry weights of the fungal mats were measured.

Enzyme Assays

Viscometric assays for xylanase activity were done by measuring the decrease in relative viscosity of 1.5% (w/v) solution of oat-spelt xylan (Fluka) in 50 mM sodium acetate, pH 5.0, at 30° C.

Reducing end groups were assayed using *p*-hydroxybenzoic acid hydrazide (H-9882, Sigma) (Lever, 1972). Aliquots of a 0.6% (w/v) solution of oat-spelt xylan (Fluka) were tested at different times to measure the free reducing ends liberated by xylanase activity in 50 mM sodium acetate, pH 5.0. The *p*-hydroxybenzoic acid hydrazide working solution was prepared from a 5% (w/v) stock solution in 0.5 N HCl by diluting 1:4 in 0.5 N NaOH. 10 or 20 µl of xylan solution was added to 1.5 ml of the *p*-hydroxybenzoic acid hydrazide working solution and this was heated at 100° C for 10 min after which absorbance at 410 nm was measured. Xylose was used as a standard to quantify the amount of end reducing groups liberated in the digestions.

ß-xylosidase activity was determined by measuring the amount of *p*-nitrophenol released from the synthetic substrate *p*-nitrophenyl-ß-D-xylopyranoside (N-2132, Sigma). The method used was adapted from Montreuil *et al.*, 1986. 0.1 ml of 10 mM *p*-nitrophenyl-ß-D-xylopyranoside was added to 0.1 ml 200 mM sodium acetate, pH 5.0, and 0.2 ml of each fraction of a cation exchange HPLC run of semipurified culture filtrate of *C. carbonum* grown on corn cell walls. After incubating for 20 min, 0.6 ml of 1 M sodium carbonate was added and the absorbance at 400 nm was measured.

ß-glucosidase activity was assayed by the same method except *p*-nitrophenyl-ß-D-glucopyranoside (N-7002, Sigma) was used as the substrate.

Purification by Conventional Liquid Chromatography

Culture filtrate was poured through cheesecloth and filtered through Whatman #1 filter paper by vacuum. The culture filtrate was then diluted two-fold with 25 mM sodium acetate buffer, pH 5.0, and loaded onto DEAE-cellulose (D-3764, Sigma) with a bed volume of 80 ml packed in two 60 ml-syringes in series. Following the DEAE-cellulose was a column of CM-cellulose (C-4146, Sigma), in series, with a bed volume of 60 ml. Both columns were washed with two bed volumes of 25 mM sodium acetate, pH 5.0. The CM-cellulose was eluted with a 250 ml gradient going from 25 mM sodium acetate, pH 5.0, to 25 mM sodium acetate plus 0.8 M KCl, pH 5.0. In some experiments the CM-cellulose was eluted stepwise with 25 mM sodium acetate plus 0.4 M KCl, pH 5.0. In earlier purifications the culture filtrate was not diluted and loaded directly. Instead, the culture filtrate was concentrated by rotary evaporation under reduced pressure at 37° C (Büchi model 011) and then dialyzed (Spectrum Medical Industries, Inc., Spectrapor, 6,000 to 8,000 mw cutoff, 32 mm diameter) overnight in 25 mM sodium acetate before loading onto the DEAE and CM-cellulose columns.

The eluate from the CM-cellulose column was dialyzed (same membrane as above) overnight against 12.5 mM sodium acetate, pH 5.0. In earlier purifications the CM-cellulose eluate was desalted by membrane filtration (Amicon, 10,000 mw cutoff).

Purification by High Performance Liquid Chromatography (HPLC)

Further protein fractionation was done by HPLC on a Waters system with a model 680 automated gradient controller, two model 501 pumps and a model 440 UV detector (280 nm). Aqueous solvents were filtered through a 0.45 µm nitrocellulose membrane by vacuum and organic solvents were filtered through a 0.45 µm nylon membrane by vacuum.

Ion exchange HPLC was done on a 200 x 4.6 mm polysulfoethyl aspartamide cation exchange column (The Nest Group Inc., Southboro, Massachusetts). Flow rate was 1 ml/min with a linear gradient from 0 to 100% B in 40 min. Buffer A was 25 mM sodium acetate, pH 5.0, and buffer B was 25 mM sodium acetate and 0.4 M KCl, pH 5.0. Fractions were collected at one min intervals. In some ion exchange runs 0.5 mM EDTA was added to buffers A and B. Also, phosphate buffer at pH 6.5 was used in one run. Buffer A was 12.5 mM KH_2PO_4, pH 6.5 and buffer B was 12.5 mM KH_2PO_4 and 0.4 M KCl, pH 6.5 the same gradient described above was used.

Gel filtration was done on a 600 x 7.5 mm TSK-3000SW column with a 75 x 7.5 mm guard column (Beckman). The mobile phase was 100 mM KH_2PO_4, pH 6.5, and the flow rate was 0.5 ml/min. Molecular weight standards to calibrate the gel filtration column were ovalbumin, mw 44,000 (Sigma A-7641), myoglobin, mw 17,000 (Sigma M-0630) and vitamin B-12, mw 1,350 (Sigma V-2876).

Reverse phase HPLC was done on a 250 x 4.6 mm C-18 column (Beckman) and a 250 x 4.6 mm C-4 column (Vydac). A linear gradient from 0 to 100% B was run in 30 min at a flow rate of 1 ml/min. Solvent A was Milli-Q water plus 0.1% TFA and solvent B was acetonitrile plus 0.1% TFA or acetonitrile/isopropanol (1:1 v/v) plus 0.1% TFA. No xylanase was recovered from either column under either solvent condition.

Hydrophobic interaction was on a 75 x 7.5 mm TSK-Phenyl-5-PW column (Bio-Rad). A 30 min linear gradient was run from 0 to 100% B. Solvent A was 100 mM KH_2PO_4 plus 1.5 M $(NH_4)_2SO_4$, pH 6.5, and solvent B was Milli-Q water and the flow rate was 1 ml/min.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was done using the method described by Hames and Rickwood (1981) with 12 or 15% acrylamide (Table 2). These gels were run on a Hoeffler Mini Protean II unit at 120 volts until the bromphenol blue tracking dye had just run off the bottom of the gel. Also, SDS-PAGE was done on a Pharmacia PhastSystem with 8-25% acrylamide

Table 2. Recipe used for SDS-PAGE.

Running gel (10 ml)	12%	15%	
Acrylamide (30:0.8%)	4.0	5.0	ml
1.75 M Tris, pH 8.8	2.0	2.0	ml
10% SDS	0.1	0.1	ml
Water	3.8	2.8	ml
TEMED	10	10	µl
10% Ammonium persulfate	60	60	µl

Stacking gel (6 ml)	
Acrylamide (30:0.8%)	1.0 ml
1.25 M Tris, pH 6.8	0.6 ml
10% SDS	60 µl
Water	4.3 ml
TEMED	5 µl
10% Ammonium persulfate	50 µl

Acrylamide (30:0.8%)
30% acrylamide
0.8% Bis-acrylamide
filtered through Whatman #1 filter paper

1.75 M Tris, pH 8.8
1.75 M Tris base, titrated with HCl

1.25 M Tris, pH 6.8
1.25 M Tris base, titrated with HCl

Running buffer	per liter
Tris base	3 gm
Glycine	14.4 gm
SDS	1 gm

Sample buffer (4x) (save at -20°C in 0.5 ml aliquots)	
1.25 M Tris, pH 6.8	5 ml
DTT	0.15 gm
SDS	10 ml 20%
Sucrose	10 gm
Bromphenol blue	1 mg
Water	to 25 ml

gradient gels by the method described in the Pharmacia PhastSystem manual.

The size markers used were Bio-Rad low molecular weight SDS-PAGE standards. These were rabbit muscle phosphorylase b, 97,400 mw; bovine serum albumin, 66,200 mw; hen egg white ovalbumin, 42,699 mw; bovine carbonic anhydrase, 31,000 mw; soybean trypsin inhibitor, 21,500 mw; and hen egg white lysozyme, 14,400 mw. Standards were loaded at concentrations of 0.5 or 1.0 µg of each protein per lane in the Hoeffler mini-gel unit and 10 ng of each protein per lane for the Pharmacia PhastSystem.

Hoffler mini-gels were stained with coomassie blue R-250 (Eastman Kodak Co.) 0.1% in methanol:water:acetic acid (5:5:2), and destained by several washes in 7% acetic acid heated to 90°C. Pharmacia PhastSystem gels were silver stained according to the manual except the concentration of formaldehyde used in the development step was decreased by one half.

Isoelectric Focusing (IEF)

Isoelectric focusing was done on an LKB Ampholine PAGplate, pH 3.5 to 9.5. The gel was run for 2 hours at 4°C at a current of 10 mA. The gel was fixed in 10% (w/v) trichloroacetic acid plus 3% (w/v) sulfosalicylic acid for 1 hour and stained with Coomassie blue R-250

(Eastman Kodak Co.) in methanol, water and acetic acid (5:5:2) and destained in the same solvent minus the dye. Isoelectric focusing was also done on a Pharmacia PhastSystem using a pH 3 to 9 IEF gel and silver stained according to the Pharmacia PhastSystem manual. Pharmacia isoelectric focusing markers were used to calibrate the resulting pH profile.

Mesurement of Xylanase Activity *versus* pH and Temperature

Xylanase I, II and III activity was measured in 30 min assays using 50 mM KH_2PO_4 adjusted to pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 or 8.0. Activity *versus* temperature of xylanase I was measured in a 30 min reaction at 30, 37, 45, 54 and 65° C.

Thin Layer Chromatography of Xylanase Reaction Products

Thin layer chromatography (TLC) was done with ethanol soluble (67% v/v) products of an overnight digestion of oat-spelt xylan (Fluka). The solvent was butanol, water and acetic acid (4:1:5) and the plates used were cellulose on polyester (T-6890, Sigma). Free xylose was added to some samples prior to ethanol precipitation to determine whether components of the reaction mixture interfered with the migration of xylose.

Plates were developed by the method of Trevelyan *et al.*, 1950, which is specific for end reducing groups. 0.1 ml of saturated $AgNO_3$ was added to 20 ml of acetone and water was added until the $AgNO_3$ had dissolved. The $AgNO_3$ in acetone was sprayed on the plates and allowed to dry. 0.5 N NaOH in ethanol was prepared by adding 20 M NaOH to 95% ethanol and water was added until the NaOH dissolved. The plates were sprayed with this and then submerged in 6 N NH_4OH for less than 1 min and washed in water for 1 hour.

Standards used were xylose (X-1500, Sigma), arabinose (Uninted States Biochemical), glucuronic acid (ICN Biochemicals), glucose (Mallinckrodt) and xylobiose (X-1501, Sigma).

Enzymatic Digestion of Xylanase I

The xylanase sample was desalted by Centricon membrane filtration (3,000 mw cutoff) (Amicon) and then dried by vacuum centrifugation (Savant). The trypsin digestion followed the procedure by Stone *et al.* (1989). 50 µl 8 M urea, 0.4 M NH_4HCO_3 was added to the dried protein. The pH was checked by spotting 1 µl on pH paper and was determined to be between 7.5 and 8.5. 5 µl were removed for amino acid analysis. 5 µl of 45 mM dithriothreitol (DTT) were added and the sample was incubated at 50° C for 15 min to denature it. After cooling to room temperature 5 µl of 100 mM iodoacetamide was added to alkylate the cysteine residues so the xylanase became permanently denatured. The sample was incubated at room temperature for 15 min to allow the iodoacetamide to react with the cysteine residues. 140 µl of water was added and trypsin was added equal to 1/25th the amount of xylanase as determined by a coomassie blue R-250 (Eastman Kodak Co.) stained SDS-polyacrylamide gel. Trypsin (TPCK-treated, Worthington Enzymes) was prepared as a 0.2 mg/ml solution in 0.001 N HCl and stored at -20° C in 0.5 ml aliquots. The reaction was incubated at 37° C for 4 hours and then frozen at -80° C until the peptides were separated by reverse phase HPLC. Other attempts were made with digestions 24 hours in length but these resulted in very short peptides. Additionally, alkylation of cysteines by 4-vinylpryidine was tried unsuccessfully.

Enzymatic digestion by endoproteinase lys-C (Boehringer Mannheim Biochemica) was attempted by the method of Stone *et al.* (1989) but the details were not sufficiently worked out to get the enzyme to cleave the substrate.

Separation of Tryptic Peptides by Reverse Phase HPLC

The first attempt at fractionation of peptides from a 24 hour trypsin digestion by reverse phase HPLC was done on a Beckman system with a model 421 controller, two model 114 pumps and a model

163 UV detector (215 nm). The column used was a 250 x 4.6 mm Ultrasphere C-18 (Beckman). A linear gradient was run from 0 to 100% B in 60 min. Solvent A was water (Burdick and Jackson) and solvent B was acetonitrile (Burdick and Jackson), both solvents contained 0.1% trifloroacetic acid (TFA) (Pierce Chemical Co.). Peaks were collected manually and were either sequenced or repurified by microbore HPLC. Microbore HPLC was used to further resolve peptides purified on the Beckman system and to fractionate tryptic peptides generated from 4 hour digestions. Microbore HPLC was performed on an Applied Biosystems model 130 microbore HPLC system with a Spectroflow 783 detector (Kratos Analytical Instruments) set at 214 nm using a 200 nl flow cell. The column used was a 250 x 1 mm C-8 (Applied Biosystems). Peaks were collected manually. Data collection was by Beckman System Gold computer software. Gradients were linear going from water to 90% acetonitrile in 60 min. Water contained 0.1% TFA and the 90% acetonitrile slightly less TFA so the absorbance at 214 nm was identical for both solvents. Peaks that were not homogenous as determined by the computer software were run again using 0.1% hexaflorobutaric acid (HFBA) instead of TFA in the solvents. Peptides were dried by vacuum centrifugation (Savant) and stored at -80°C until they could be sequenced.

Preparation of Samples for N-Terminal Sequencing

Four separate attempts to sequence the N-terminus of xylanase I and one attempt to sequence the N-terminus of ß-xylosidase were made. Two of the attempts made with xylanase I used cation exchange and gel filtration purified samples. A third xylanase I sample was purified by cation exchange HPLC, run on a preparatory gel (SDS-PAGE) and then transferred to immobilon membrane (Millipore) using a semi-dry electrophoresis transfer unit (LKB-Produkter AB, Sweeden). After transfer the immobilon membrane was stained with 0.2% (w/v) ponceau-S (P-3504, Sigma) in 1% (v/v) acetic acid for 1 min and destained by washing in water for several min. The xylanase band was cut out and direct N-terminal sequencing of the xylanase bound to the membrane was attempted. The fourth attempt to sequence the N-terminus of xylanase I was made using a preparation that was purified by cation exchange HPLC and hydrophobic interaction HPLC. ß-xylosidase was purified by cation exchange HPLC and gel filtration HPLC before N-terminal sequencing was attempted. Salts were removed from all samples, except the immobilon membrane sample, by centricon membrane filtration (3,000 mw cutoff) (Amicon). Samples were dried by vacuum centrifugation (Savant) and stored at -80° C until they could be sequenced.

Amino Acid Sequencing

Amino acid sequencing was performed by Joseph Leykam, Macro-Molecular Facility, Michigan State University, using Edman degradation on an Applied Biosystems model 477A protein sequencer using an Applied Biosystems model 120A analyzer.

Amino Acid Composition

A sample of xylanase was hydrolyzed with acid and the free amino acids were derivatized with phenylthiohydantoin (PTH) and separated by HPLC. HPLC was on a Waters system with a model 680 automated gradient controller, two model 510 pumps and a model 440 UV detector (254 nm). The column used was a Waters 150 x 3.9 mm C-18 and data collection and analysis was by Beckman System Gold computer software. The system was calibrated using PTH derivatized amino acid standards.

Oligonucleotide Synthesis

Two peptides, 13 and 22, were chosen to make degenerate oligonucleotides, 1 and 2, respectively (Table 3). Both oligonucleotides were 17 bases long. Oligonucleotide 1 was 32-fold degenerate and in the complementary orientation and oligonucleotide 2 was 128-fold

Table 3. Peptide 13 and 22 Sequences and the Oligonucleotides Made From Them

PEPTIDE	SEQUENCE
13	THFDAWA
22	NPLVEYYVVENFGTYDP(P/S)SQWQNK

PEPTIDE	SEQUENCE FOR OLIGO		OLIGO SEQUENCE	DIRECTION
13	HFDAWA	oligo 1	GCCCANGC(A/G)TC(A/G)AA(A/G)TG	complementary
22	VEYYVV	oligo 2	GTNGA(G/A)TA(T/C)TA(T/C)GTNGT	normal

OLIGO	LENGTH	DEGENERACY
1	17	32
2	17	128

degenerate and in the normal orientation (Table 3).

Oligonucleotide synthesis was performed on an Applied Biosystems model 380B DNA synthesizer. The oligonucleotides were HPLC purified on a Waters system with a model 680 automated gradient controller, two model 510 pumps and a Spectroflow 783 UV monitor (Kratos Analytical Instruments) set at 260 nm. The column used was a DuPont oligo column. The gradient was linear from 10 to 65% B in 40 min. Buffer A was 20 mM NaH_2PO_4, pH 7.0, with 20% (v/v) acetonitrile. Buffer B was the same plus 2 M NaCl. Data collection was by a Waters model 730 datamodule. HPLC purified oligonucleotides were dried by vacuum centrifugation (Savant) and redissolved in water. A C-18 SEP-PAK cartridge (Waters) was prepared by washing with 10 ml acetonitrile and then 10 ml water after which the oligonucleotide sample was loaded. The cartridge was washed with 5 ml of water and the oligonucleotide was eluted with 60% methanol 1 ml at a time. Absorbance at 260 nm was measured of a 1:50 dilution to determine the concentration of the samples.

^{32}P Labeling of the Oligonucleotides

Oligonucleotides were labeled 250 ng at a time with T4 polynucleotide kinase (Uninted States Biochemicals) at 10 units per reaction and 75 to 150 µCi of gamma ^{32}P labeled ATP (NEG 035C, New

England Nuclear). Reactions were run for 15 min at 37°C in a volume of 50 µl and then stopped by adding 5 µl of 0.2 M EDTA. The reaction was diluted to 100 µl and passed over a Sephadex G-25 (G25-80, Sigma) spin column made from a 1 ml disposable syringe (60 x 4.5 mm) to remove unincorporated ^{32}P label. 100 µl of spin column buffer was used to follow the labeling reaction through the spin column after the first spin. Spin column buffer was 100 mM NaCl, 10 mM Tris and 1 mM EDTA adjusted to pH 8.0.

Probing Southern Blots of Genomic DNA from *C. carbonum*

C. carbonum genomic DNA was digested with either *Bam* HI, *Eco* RI or *Hind* III for 2 hours with 3 units of restriction enzyme per µg of DNA. Lambda cut with *Bst* EII was used as size standards. The cut DNA was run on a 0.7% agarose gel and then transferred to a Zeta-Probe membrane (Bio-Rad). Prior to transfer the gel was washed twice in 0.25 M HCl, 8 min each time and then twice in 1.0 M NaCl, 0.5 M NaOH, 15 min each time and then once in 1.0 M Tris, 0.6 M NaCl at pH 7.0. DNA was transferred overnight by capillary action using 0.6 M NaCl and 60 mM sodium citrate adjusted to pH 7.0 with 1 N HCl. The Zeta-Probe membrane was dried in a 80°C vacuum oven for 1 hour.

Prehybridization and hybridization were done at 41 and 47°C initially for both oligonucleotides and then later at 51°C for oligonucleotide 1 and 47° C for oligonucleotide 2. Prehybridization was done in 5 x SSPE, 7% SDS, 10 x Denhardt's solution and denatured sheared herring sperm DNA (100 µg/ml) for 2 to 24 hours (Table 4). For hybridization, the same solution was used from the prehybridization step plus 5 to 20 µCi of labeled oligonucleotide as a probe. Hybridizations were carried out overnight at the same temperatures used for prehybridization. The Zeta-Probe membrane was washed in 1 x SSPE and 1% SDS at the same temperatures used for the hybridizations three times for 30 min each time. The membrane was wrapped in plastic wrap while still damp and exposed to X-ray film overnight using an intensifying screen.

Screening a *C. carbonum* Genomic DNA Library Made in Lambda EMBL3

NM539 cells were grown from a single colony in LB (Table 4) and 0.2% maltose to an absorbance at 600 nm of 0.5 to 2 and spun down and resuspended to an absorbance of 0.5 to 1.0 in 10 mM $MgSO_4$. 300 µl of cells were added to 120,000 plaque forming units of EMBL3 (titer was 3×10^4 plaque forming units per µl) diluted in 600 µl of SM (Table 4) and allowed to adsorb for 20 minutes at 37° C. 150 µl of this was added to each of six 9 ml portions of top LB agarose (Table 4), that had been cooled to 50° C. The top agarose was then immediately poured over each

Table 4. Recipies used for molecular biology.

All recipies are from Maniatis *et al.*, 1982.

20 x SSPE

3.6 M NaCl
0.2 M Na_2HPO_4
0.02 M EDTA (disodium salt)
Adjust to pH 7.7 with NaOH (approximately 1 gm/liter)

100 x Denhardt's Solotion

2% Ficoll, type 400 (F-4375, Sigma)
2% Polyvinylpyrrolidone (PVP-40, Sigma)
2% Bovine Serum Albumin (B-2518, Sigma)

LB

	per liter
Tryptone (Difco)	10 gm
Yeast Extract (Difco)	5 gm
NaCl	5 gm

For LB agar add 15 gm/liter agar
For LB agarose add 7.5 gm/liter agarose

SM

	per liter
NaCl	5.8 gm
$MgSO_4 \cdot H_20$	2 gm
1.0 M Tris, pH 7.5	50 ml
2% gelatin	5 ml

of six 150 mm plates containing LB agar (Table 4) that had been dried and preheated to 37° C. Plates were incubated overnight at 37° C after which there were confluent plaques. Plaque lifts were done using nitrocellulose filters (HATF, Millipore). Each lift was treated for 5 min by placing on Whatman #1 paper wetted with each of the following solutions: 0.4 M NaOH; 1.5 M Tris, pH 7.4; and 70% ethanol. The plaque lifts were then baked in a vacuum oven at 80° C for 2 hours. Prehybridizations and hybridizations were done using the same method used for the Southern blot described earlier except fresh solution was used for the hybridization. Three plaque lifts were probed with oligonucleotide 1 and the remaining three probed with oligonucleotide 2. The plaque lifts were then used to expose X-ray film overnight using an intensifying screen. 50 plaques were picked, some from each probe, and stored in SM. 10 plaques that hybridized to oligonucleotide probe 1 were replated and double lifts were made. The lifts were treated as before and each set of lifts was probed with oligonucleotide 1 or 2.

RESULTS

Effects of Different Media on the Production of Xylanase

C. carbonum made xylanase when grown on modified Fries' or mineral salts media with either xylan or corn cell walls as a carbon source. Xylanase production was delayed with higher sucrose concentrations. Mineral salts medium was chosen for the production of xylanase for purification because it was easy to make and did not differ greatly in the amount of xylanase produced compared to modified Fries' medium. Corn cell walls were used to induce *C. carbonum* to make xylanase for purification because the corn cell walls were insoluble and did not contribute to the viscosity of the culture filtrate. The corn cell walls could be removed from the culture filtrate by filtering through Whatman #1 filter paper. This made running the low pressure chromatography columns easier because they would not clog. Xylan would go through the Whatman #1 filter paper but very slowly and would then clog the DEAE-cellulose columns.

Inhibition of Xylanase Production by Sucrose

C. carbonum was grown for 7 days in modified Fries' media containing xylan and different concentrations of sucrose as described.

It was found that the maximum amount of xylanase was produced at 2 gm/liter sucrose and no xylanase was produced after 7 days at sucrose concentrations of 8 gm/liter or higher (Figure 1).

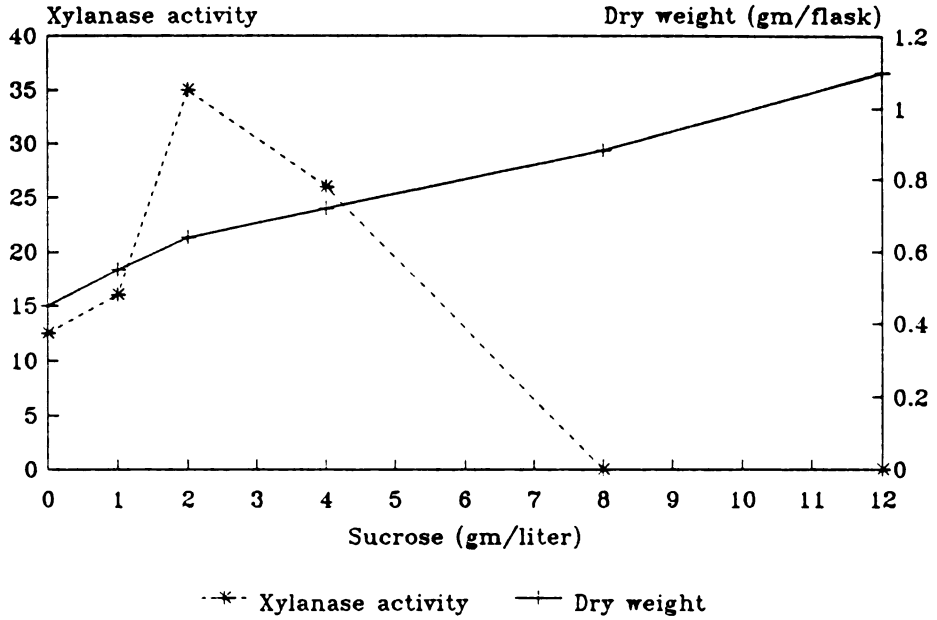
Comarison of Xylanase Production in Toxin Producing and Nonproducing Strains

Both isolates, SB111 (race 1) and 1309 (race 2), are capable of producing xylanase. It appeared that isolate 1309 produced more but this was probably due to a greater number of conidia used to inoculate the flasks of liquid media.

Purification of Xylanase

The final method used to purify xylanase was DEAE-cellulose followed by CM-cellulose in series, dialysis, cation exchange HPLC and then hydrophobic interaction HPLC. This method resulted in a overall recovery of 18% and a 28-fold increase in specific activity (Table 5). The hydrophobic interaction HPLC step was able to separate proteins that had the same molecular weight as xylanase. A preparation of xylanase from the cation exchange HPLC column that appeared homogenous by SDS-PAGE could be separated into 5 or 6 distinct peaks by hydrophobic interaction HPLC (Figure 2). When reverse

Figure 1. Effect of sucroce on xylanase activity and fungal dry weight.



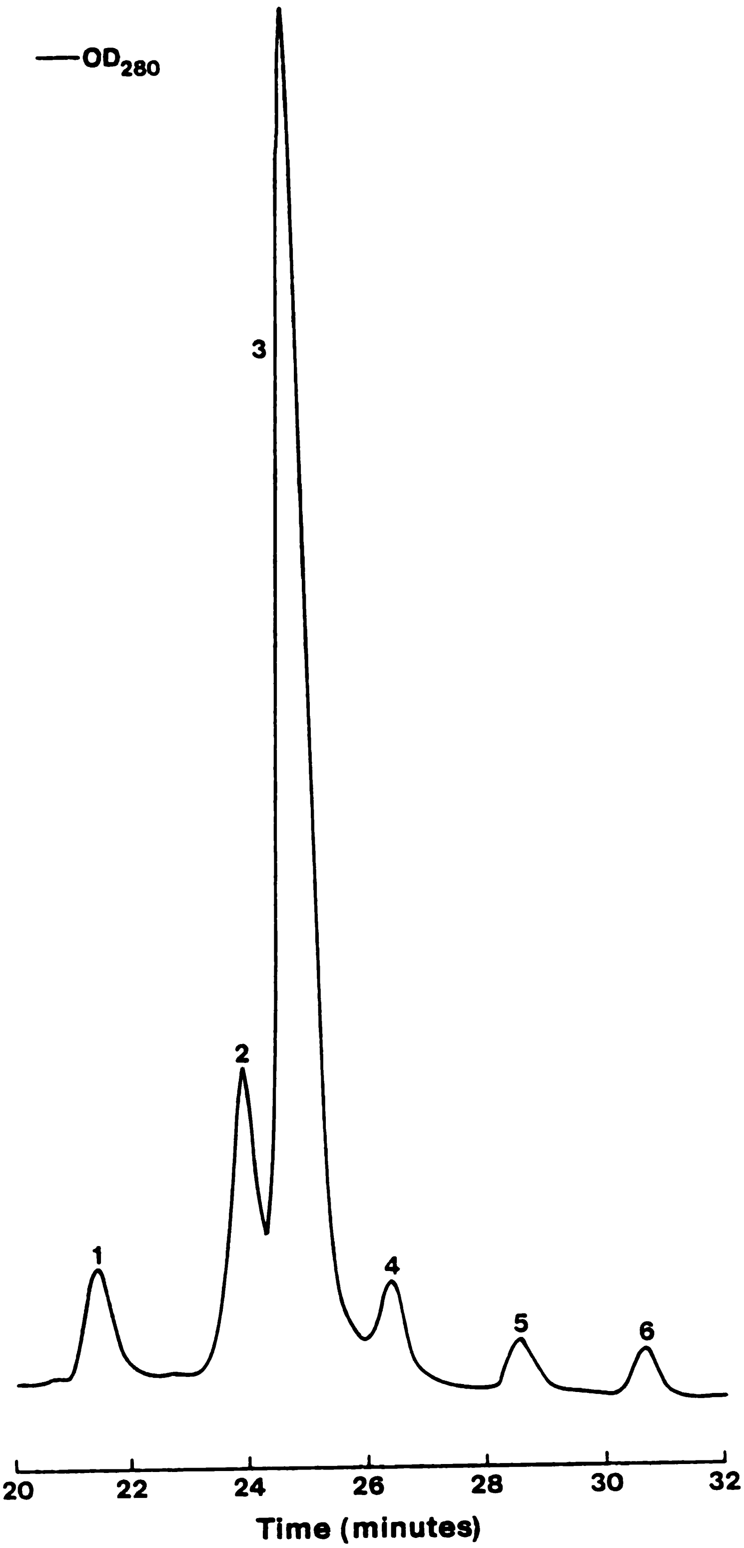
Xylanase activity is expressed in relative viscometric units (RVU).

Table 5. Purification table for xylanase.

	Total protein (mg)	Total xylanase (reducing units)	Specific activity (reducing units/μg)	Yield (%)	Purification (fold)
1. Crude filtrate[a]	27	750	0.28	100	1.0
2. DEAE and CM-cellulose[b]	2.0	516	2.5	69	8.9
3. Dialysis	3.4	666	2.0	89	7.0
4. Cation exchange HPLC	0.30	120	4.0	16	14.3
5. Hydrophobic interaction HPLC	0.17	132	7.9	18	28.3

[a] 260ml was diluted with 25mM sodium acetate to 500ml and filtered.
[b] Columns were run in series, only CM-cellulose was eluted with salt.

Figure 2. Hydrophobic interaction HPLC.



Peak 3 is the major xylanase activity. Hydrophobic interaction was on a TSK-Phenyl-5-PW column (Bio-Rad). A 30 minute linear gradient was run from 0 to 100% B. Solvent A was 100 mM KH_2PO_4 plus 1.5 M ammonium sulfate, pH 6.5, and solvent B was water. The flow rate was 1 ml/min.

phase HPLC was attempted no xylanase activity was recovered from either the C-18 or the C-4 column. Peaks at 280 nm were collected and analyzed by SDS-PAGE stained with Coomassie blue but none these peaks appeared to be protein.

Determination of the Number of Xylanases

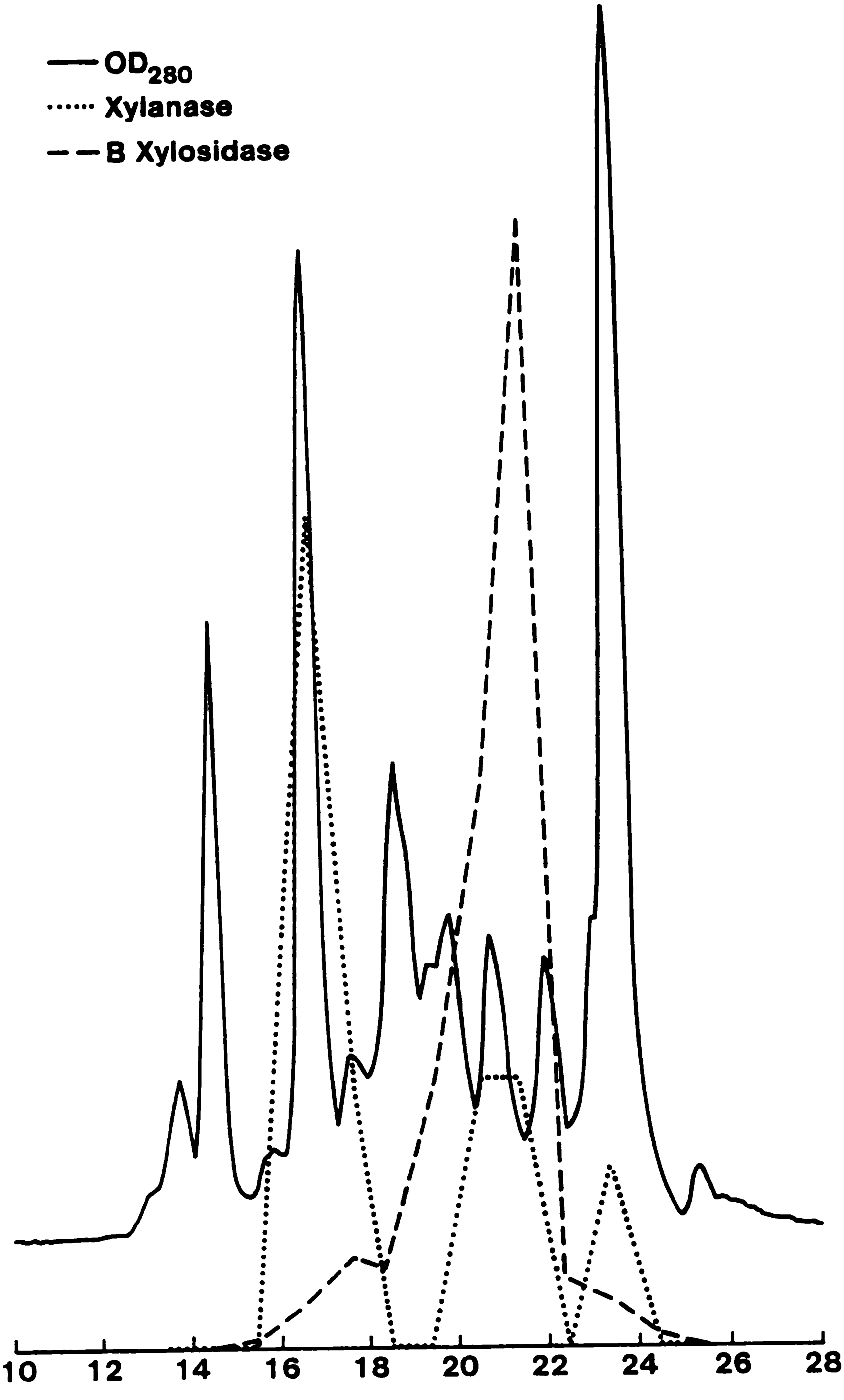
Three xylanases were found one time when the CM-cellulose eluate was fractionated by cation exchange HPLC (Figure 3). Other times this was done one or two xylanase activities were found (Figure 4).

ß-xylosidase and ß-glucosidase

A single ß-xylosidase was found when the CM-cellulose eluate was fractionated by cation exchange HPLC (Figure 3). It was later separated from the xylanase activity in that fraction by gel filtration HPLC.

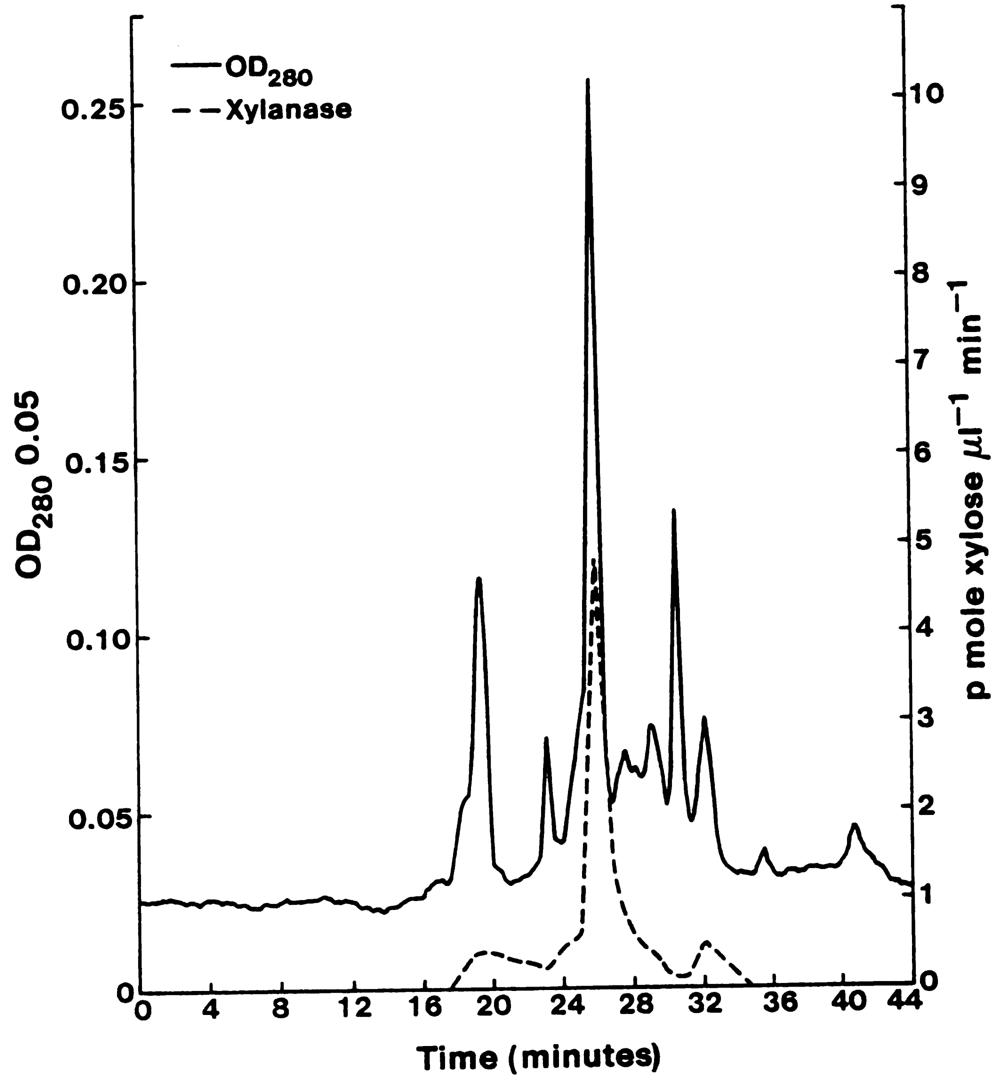
No ß-glucosidase activity was found in the same samples tested for ß-xylosidase activity.

Figure 3. Cation exchange HPLC showing three xylanase activities and a ß-xylosidase activity.



Cation exchange HPLC was done on a polysulfoethyl aspartamide column (The Nest Group) Flow rate was 1 ml/min with a linear gradient from 0 to 100% B in 40 minutes. Buffer A was 25 mM sodium acetate, pH 5.0, and buffer B was the same with 0.4 M KCl.

Figure 4. Cation exchange HPLC showing a single xylanase activity.



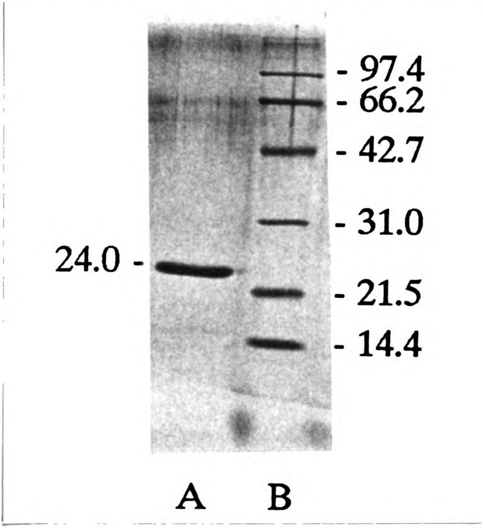
Cation exchange HPLC was done on a polysulfoethyl aspartamide column (The Nest Group). Flow rate was 1 ml/min with a linear gradient from 0 to 100% B in 40 min. Buffer A was 25 mM sodium acetate, pH 5.0, and buffer B was the same with 0.4 M KCl.

Determination of Molecular Weights by SDS-PAGE and Gel Filtration

The molecular weights of xylanase I, II and III were determined to be 24,000, 22,000 and either 22,000 or 33,000, respectively, by SDS-PAGE (Figures 5 and 6). The molecular weight of ß-xylosidase by SDS-PAGE was determined to be 42,000 (Figure 6). Xylanase I was run on 12 and 15% polyacrylamide gels and a 8 to 25% gradient gel and this did not alter its apparent molecular weight. Xylanase I and II and ß-xylosidase had molecular weights of 8,500, 7,300 and 31,500, respectively, by gel filtration HPLC (Figures 7 and 8). The molecular weight of xylanase III was not determined by gel filtration.

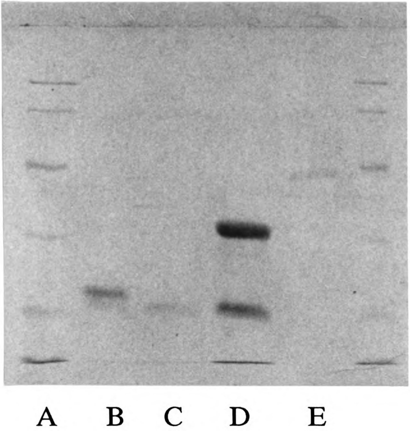
It should be noted that the xylanases appeared small by membrane filtration. Several attempts were made to concentrate or remove salts from xylanase preparations by Centricon membrane filtration (Amicon) with a molecular weight cutoff of 10,000. It was determined by SDS-PAGE of the filtrate that the xylanase passed through the membrane. The concentration of xylanase in the filtrate was nearly equal to that in the retentate. Xylanase did not pass through a Centricon with a molecular weight cutoff of 3,000. This lends support to the apparent molecular weight determined by gel filtration.

Figure 5. SDS-PAGE of xylanase I prior to digestion with trypsin.



Lane A is xylanase I from phenyl hydrophobic interaction HPLC. Lane B is Bio-Rad low molecular weight standards for SDS-PAGE. Values are in kDa.

Figure 6. SDS-PAGE showing three xylanases and a ß-xylosidase.



Lane A is Bio-Rad low molecular weight markers for SDS-PAGE, B is xylanase I, C is xylanase II, D is xylanase III and E is ß-xylosidase. The xylanases and ß-xylosidase were separated by cation exchange HPLC (Figure 3).

Figure 7. Gel filtration HPLC showing the molecular weight of xylanase I.

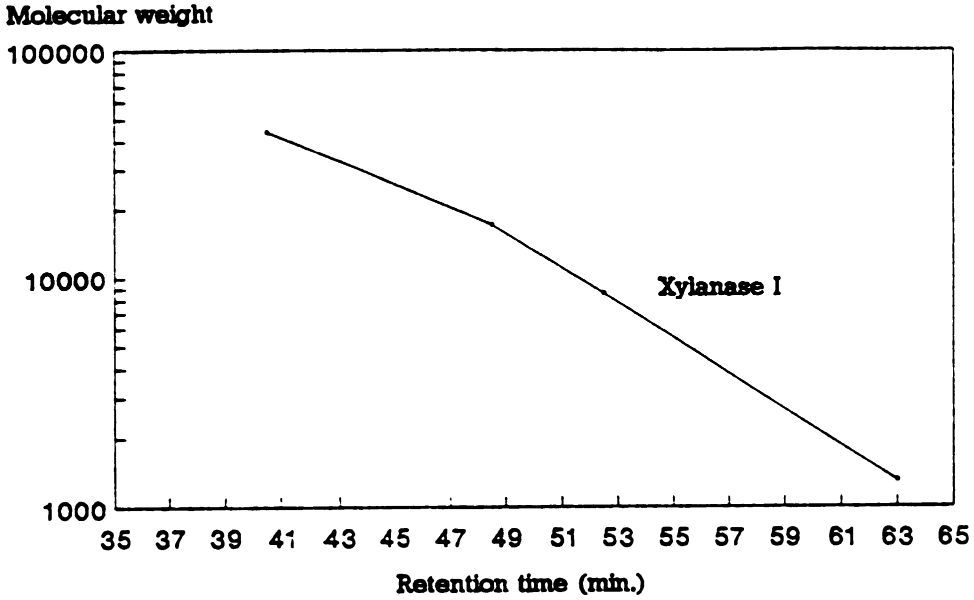
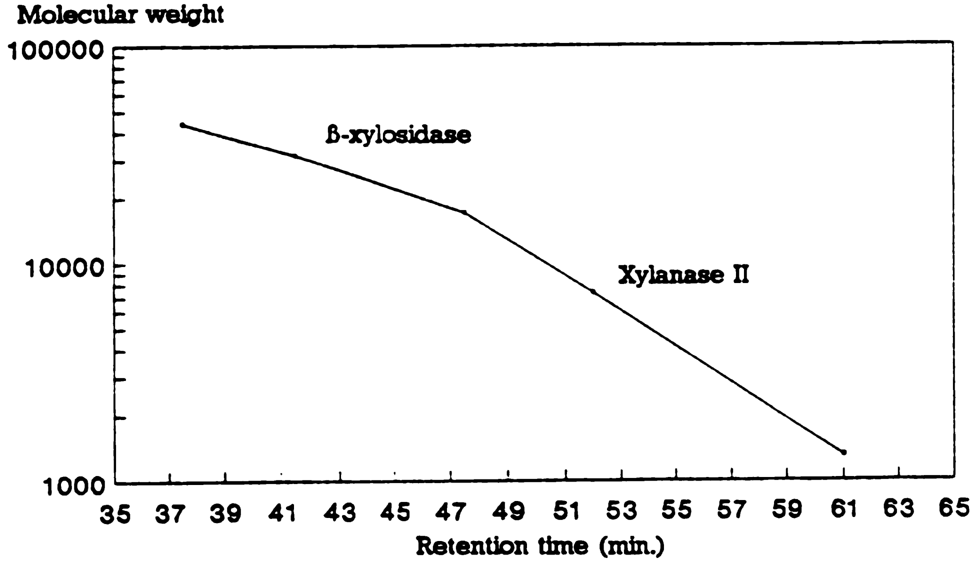


Figure 8. Gel filtration HPLC showing the molecular weight of xylanase II and ß-xylosidase.



Gel filtration HPLC was on a TSK-3000SW column (Beckman). The mobile phase was 100 mM KH_2PO_4, pH 6.5, and the flow rate was 0.5 ml/min. Ovalbumin, myoglobin and vitamin B-12 were used to calibrate the column.

Isoelectric Focusing

In all instances where xylanase I and II were run on isoelectric focusing gels they appeared to migrate to a position very close to the cathode. An accurate measurement of the isoelectric points could not be made because they were higher than the limits of the gels used. The isoelectric points of xylanase I and II are higher than 9.5.

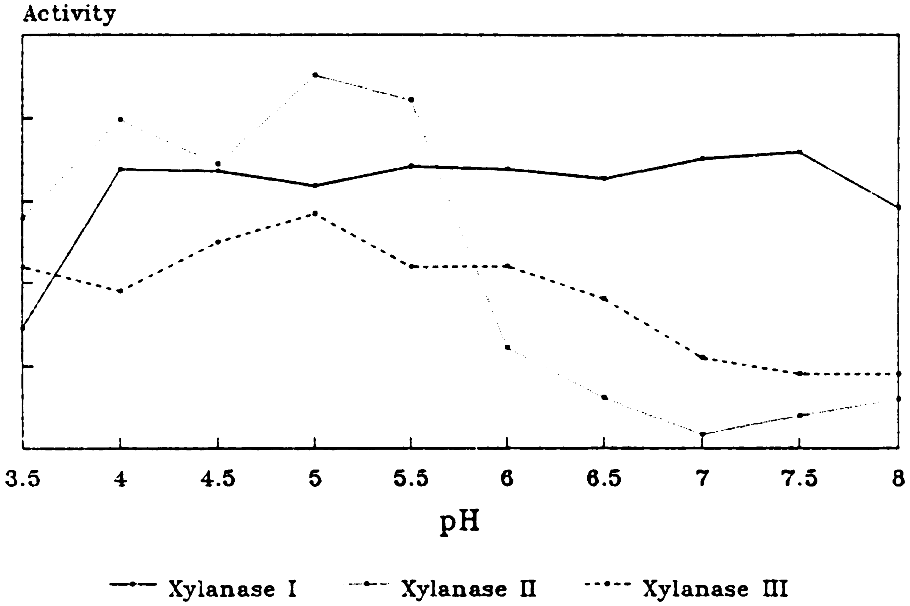
pH and Temperature Profiles of Xylanase Activities

Xylanase I activity was nearly constant from pH 4.0 to 8.0 and xylanase II and III had maximal activity at pH 5.0 (Figure 9). Xylanase I had the greatest activity at 45°C, less at 54°C and almost none at 65°C in a 30 min assay (Table 6). The activities of xylanase II and III were not measured with respect to temperature.

TLC of Xylanase Products

The products of xylanase using oat-spelt xylan as a substrate were not identified. However, these products were determined not to be free xylose by adding xylose to some of the reactions prior to ethanol precipitation. The xylose added to the reactions migrated the same distance as xylose alone and there was nothing detected that migrated

Figure 9. Effect of pH on activity of xylanases I, II and III.



Reactions were carried out at 30°C for 30 minutes in 50 mM potassium phosphate.

Table 6. Effect of temperature on xylanase activity.

Temperature (°C)	Activity (% of activity at 30°C)
30	100
37	129
45	209
54	119
65	22

Assay was for 30 minutes in 50 mM sodium acetate, pH 5.0.

the same distance as xylose in the reactions where xylose was not added. It was concluded by the absence of xylose monomers in the reaction products that xylanase acts to cleave the xylan internally and is an endo enzyme.

Enzymatic Digestion of Xylanase I and Peptide Sequences

The 24 hour digestion of xylanase I with trypsin resulted in very short peptides (Table 7). Apparently this digestion was too long and the xylanase was being cleaved nonspecifically due to minor contaminating activities present in the trypsin. These peptides were separated on the Beckman HPLC system and some were repurified by microbore HPLC prior to sequencing. The next trypsin digestion was monitored by SDS-PAGE to determine the length of time required for the xylanase to disappear. Nearly all xylanase had disappeared after one hour. This digestion was allowed to react for four hours and the peptides were separated by microbore HPLC using TFA in the buffers. The peaks at 214 nm were judged to be impure and run again using HFBA in the buffers instead of TFA (Table 7). The subsequent digestion was done at the same ratio of 1 to 25 (trypsin to xylanase, w/w) for two hours. The method used to purify the xylanase used in this digestion differed by a new purification step, hydrophobic interaction HPLC (phenyl). The hydrophobic interaction HPLC resolved several peaks from a xylanase

Table 7. Peptide sequences.

Peptide	Source	Amount	Sequence
X13	A	166pmol	GINPGTA
X17	A	88pmol	LAV(Y)A
X18-5	B	68pmol	QQY(P)(V)
X21-6	B	33pmol	ATYT
4C	C	1pmol	NPLVEY_(V)
5B	C	58pmol	QQYWSVR
6B	C	83pmol	THFDAWA(S)(A/K)
7A	C	24pmol	ATYTNGAGGSY(S)VG
8A	C	46pmol	TFQQYWSVR
9	D	1053pmol	G(I/F)NPGTAR
13	D	184pmol	THFDAWA
16	D	1816pmol	ATYTNGAGGSYSVSWGSGGNNV
22	D	262pmol	NPLVEYYVVENFGTYDP(P/S)SQWQNK

Source A peptides are from an overnight digestion purified by C-18 reverse phase HPLC. Source B peptides were from the same experiment as source B peptides except they were repurified by microbore HPLC. Source C peptides are from a four hour digestion, purified by microbore HPLC using TFA and repurified by microbore HPLC using HFBA. Source D peptides are from a xylanase preparation that was purified by hydrophobic interaction HPLC prior to a four hour digestion. Source D peptides were purified by microbore HPLC with TFA.

sample that was previously thought to be homogeneous. The microbore HPLC peaks at 214 nm were determined to be homogeneous by visual inspection of their shape using the Beckman System Gold software. These peptides were sequenced without further purification and yielded the longest sequences (Table 7).

Peptide Homology Search

Eight xylanase DNA sequences were obtained from Genebank. These files were converted into amino acid sequence and compared to each peptide in Table 7. Four bacterial xylanase sequences matched peptide 8A in eight out of nine amino acids, differing by a threonine or lysine instead of a glutamine position 3 (Table 8).

Amino Acid Composition of Xylanase I

Amino acid composition analysis of xylanase I showed that it was rich in glycine, alanine, leucine and phenylalanine (Table 9). Cysteine and tryptophan residues were not looked at and the acid hydrolysis of the protein deaminated the glutamine and asparagine causing them to appear as glutamate and aspartate so the true number of acidic residues was not determined.

Table 8. Homology search of xylanase sequences.

Peptide sequence	Match	Percentage	Source
TFTQYWSVR	8/9	88.9%	*Bacillus circulans*
TFKQYWSVR	8/9	88.9%	*Bacillus pumilus*
TFTQYWSVR	8/9	88.9%	*Bacillus subtilis*
TFKQYWSVR	8/9	88.9%	*Clostridium acetobutylicum*

Target sequence	Source
TFQQYWSVR	Tryptic peptide from *C. carbonum* xylanase (peptide 8A)

Organism	Genebank entry name
Bacillus circulans	BCIXYLAG
Bacillus pumilus	BPUXYNA
Bacillus subtilis	BSUXYNA
Clostridium acetobutylicum	CLOXYNB

Table 9. Amino acid composition of xylanase I.

Amino acid	Residues per molecule
Asx	24
Glx	21
Ser	11
Gly	20
His	6
Arg	9
Thr	12
Ala	15
Pro	13
Tyr	9
Val	12
Met	5
Cys	n.d.*
Ile	9
Leu	21
Phe	15
Lys	11
Trp	n.d.*

*n.d.=not determined

Oligonucleotide Synthesis

Peptides 13 and 22 were chosen to make oligonucleotides because the sequences had appeared from more than one digestion and the degeneracy of the resulting oligonucleotides was low, 32 and 128-fold, respectively. Peptides containing glutamine residues were not considered because the iodoacetamide used to alkylate the cysteine residues causes the cysteine residues to appear as glutamine when the Edman degradation products are analyzed. The portion of peptide 22 used to make oligonucleotides was near the first residues because this sequence is more reliable. Oligonucleotide 1 was synthesized in the reverse (complementary) orientation so that it could be used with the other oligonucleotide as primers for polymerase chain reaction (PCR). PCR was not done because the orientation of the peptides relative to one another was not known and the probing of the lambda clones was successful.

Establishing Hybridization Conditions for Probing with Labeled Oligonucleotides

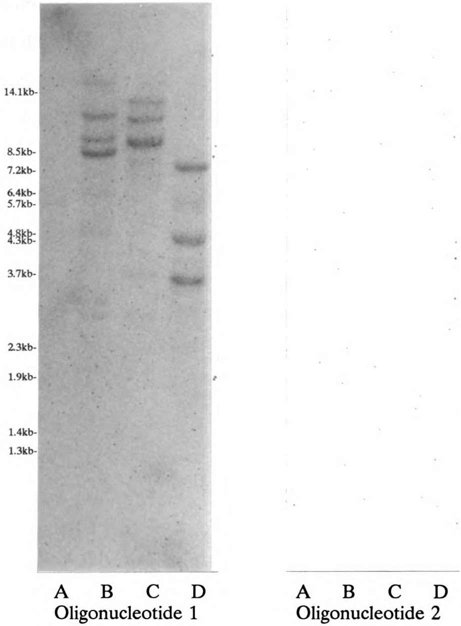
Oligonucleotide 1 showed strong hybridization to many bands, including the lambda standards, at 41 or 47°C. Oligonucleotide 2 hybridized faintly to several bands of genomic DNA at 41 and 47°C

(Figure 10). When the hybridization temperature was raised to 51°C for oligonucleotide 1 it hybridized to three bands in each lane of genomic DNA cut with a different restriction enzyme (Figure 10). It also hybridized faintly to several other bands of genomic DNA.

Probing the Genomic DNA Library Made in Lambda

Plaque lifts were hybridized to oligonucleotide 1 and 2 at 51° and 47°C, respectively. From 150 mm plates containing confluent plaques oligonucleotide 1 hybridized to about 35 plaques on three plates and oligonucleotide 2 hybridized to about 15 plaques on three plates. These 50 plaques were picked and 10 that hybridized to oligonucleotide 1 were replated and probed with both oligonucleotides. One of these 10 lambda clones hybridized to both probes.

Figure 10. Southern blot of *C. carbonum* genomic DNA probed with oligonucleotide 1 or 2.



Lane A is lambda DNA cut with *Bst*EII. Lanes B,C and D are *C. carbonum* genomic DNA cut with *Bam*HI, *Eco*RI and *Hind*III, respectively. Hybridizations were done in 5x SSPE at 51° C and 47° C for oligonucleotides 1 and 2, respectively.

DISCUSSION

Most if not all bacterial and fungal plant pathogens produce plant cell wall degrading enzymes. Much attention has been focused on pectin degrading enzymes and their involvement in pathogenesis of dicotyledonous plants. There is increasing evidence that the importance of hemicellulose degrading enzymes, particularly xylanase, has been underestimated (Cooper, 1984). In this research, xylanases from *C. carbonum* have been purified and characterized and putative clones of the corresponding genes identified. *In vitro C. carbonum* produces at least two, perhaps three, xylanases and a ß-xylosidase. Preliminary data suggests there are three xylanase genes (Figure 10).

The major xylanase from *C. carbonum* (xylanase I) was to be quite stable. All manipulations, with the exception of dialysis, were carried out at room temperature with no significant loss of activity. Also, the molecular weight of xylanase I by SDS-PAGE remained unaltered after incubating in the presence of trypsin (trypsin to xylanase ratio of 1:50, w/w) for 4 hours at 37° C in the absence of urea. The other xylanases (II and III) may not be as stable as xylanase I and this might explain why they were not always observed. The relationship between activity and pH indicates that xylanase I is stable over a greater range than xylanases II and III.

There was only a 16% recovery of xylanase activity following purification by cation exchange HPLC. The heterogeneous nature of the xylan used in the xylanase assay could account for this. Xylans usually exist as ß-1,4-D-xylopyranose residues to which some or all of three other saccharide residues, L-arabinose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid, may be attached as side chains (Adams, 1965; Darvill *et al.*, 1980a). *C. carbonum* may make enzymes that act in concert with xylanase to cleave off these three other saccharide side chains. It is not unreasonable to expect that these other enzymes could contribute to the number of reducing ends liberated as well as making more of the xylan accessible to xylanase as proposed by Darvill *et al.* (1980b). When these enzymes are separated from each other the sum of their individual activities may be less than their activity when acting in concert.

How do xylanases I, II and III of *C. carbonum* differ? Xylanases I and II differ in size by SDS-PAGE (24,000 and 22,000, respectively) and gel filtration (8,500 and 7,300, respectively). The molecular weight of xylanase III by SDS-PAGE was either 22,000 or 33,000. The preparation of xylanase III was not run on the gel filtration column and appeared as two bands by SDS-PAGE. It is not known which band is xylanase III (Figure 6). All three xylanases were separated by cation exchange HPLC so, presumably, there is a difference in their net charge. There are several possible reasons that could explain these differences. There could be three genes encoding three xylanases. The Southern blot of genomic DNA probed with oligonucleotide 1 supports this (Figure 10). This would mean that oligonucleotide 2 may be hybridizing in a non specific manner (Figure 10). Out of the ten lambda clones picked that hybridized to oligonucleotide 1, one also hybridized to oligonucleotide 2. It is possible that this one lambda clone represents the xylanase I gene and oligonucleotide 1 is hybridizing to common motifs of two other xylanase genes in the Southern blot (Figure 10) and the plaque lifts. An alternative explanation is one gene that encodes three xylanases. This could occur by different glycosylation patterns or different mRNA processing as is the case with glucoamylases from *Aspergillus niger* (Boel *et al.*, 1984). Glycosylation has not been looked at in the xylanases from *C. carbonum* but xylanases from *Trichoderma viride* (Dean and Anderson, 1991).

A number of xylanases from pathogenic and nonpathogenic bacteria and fungi have been identified. Some of these xylanases have been purified and characterized (Table 10). Several of the nonpathogenic bacterial xylanase genes have been cloned and sequenced.

Xylanases I and II from *C. carbonum* resembles xylanases from *Trichoderma* in molecular weight by gel filtration chromatography and SDS-PAGE and in isoelectric point and pH optima (Dean and Anderson, 1991; John and Schmidt, 1988; Fuchs and Anderson, 1987; Hashimoto *et al.*, 1971; Baker *et al.*, 1977). They have small apparent molecular weights by gel filtration (less than 10,000), their molecular weights by

Table 10. Physical properties of some xylanases.

Organism	mw by SDS-PAGE	pI	pH opt.	Temp. opt.	Reference
Trichoderma pseudokoningii	15-22kDa	9.6	5.0	-	Baker *et al.* (1977)
Trichoderma lignorum (2)[a]	20kDa	8.7	6.5	-	John and Schmidt (1988)
"	21kDa	5.1	3.5	-	"
Trichoderma viride (2)[a]	-	-	3.5	-	Hashimoto and Muramatsu (1971)
"	-	-	4.5	-	"
Trichoderma viride	22kDa (9.2kDa[b])	9.4	-	-	Dean and Anderson (1991)
Trichoderma viride (several)[a]	-	6.7-9.8	-	-	Fuchs and Anderson (1987)
Schizophyllium commune	21kDa	4.5	5.0	50°C	Jurasek and Paice (1988)
Aspergillus niger (4)[a]	-	4.1	-	-	Hrmovcà *et al.* (1989)
"	-	7.1	-	-	"
"	-	7.6	-	-	"
"	-	8.0	-	-	"
Aspergillus niger (4)[a]	26.5-28kDa[c]	3.75	-	-	Reilly (1981)
"	12kDa[c]	4.5	-	-	"
"	12kDa[c]	8.5	-	-	"
"	12kDa[c]	9.0	-	-	"
Helminthosporium maydis race T	-	-	4-6	-	Bateman *et al.* (1973)
Fusarium roseum	58.9kDa[b]	-	6-7	-	Mullen and Bateman (1975)
Thermomonospora fusca	-	-	5-8	70°C	McCarthy *et al.* (1985)
Thermomonospora curvata	-	-	5-8	60°C	"
Thermomonospora chromogena	-	-	5-8	75°C	"
Saccharomonospora viridis	-	-	5-8	60°C	"
Bacillus pumilus	24kDa	-	6.5	-	Okada and Shinmyo (1988)
Cryptococcus albidus	48kDa (26kDa[b])	5.0	5.4	-	Biely *et al.* (1988)

[a] Indicates the number of xylanases observed
[b] Molecular weight by gel filtration chromatography
[c] Method of molecular weight determination not stated

SDS-PAGE are about 22,000 to 24,000 and their pH optima are near 5.0. Comparison of the amino acid composition, however, reveals differences. The xylanases from *C. carbonum* resemble two of four xylanases from *Fusarium* in isoelectric points (Reilly, 1988). Some, but not all, other xylanases are approximately the same size by SDS-PAGE (Jurasek and Paice, 1988; Okada and Shinmyo, 1988), while some are larger (Biely *et al.*, 1988; Mullen and Bateman, 1975). No xylanases were found in the literature with pH optima greater than 6.5, but xylanase I from *C. carbonum* had nearly equal activity from pH 4.0 to 8.0 (Figure 9). Certain thermophilic actinomycetes have xylanases that exhibit maximal activity at temperatures between 60 and 75°C (Table 10) (McCarthy *et al.*, 1985), at least 15° higher than the maximum for xylanase from *C. carbonum* (Table 6).

The homology search of known xylanase sequences revealed an eight out of nine amino acid match between peptide 8A (Table 8) and four bacterial xylanase sequences. This would appear to be a conserved sequence similar to that shown by comparison of fungal, bacterial and plant polygalacturonase amino acid sequences (Scott-Craig *et al.,* 1990).

It is interesting that these xylanases have small apparent molecular weights, less than 10,000, by gel filtration and membrane filtration. This may allow xylanases to migrate through the smallest cell wall pores to degrade xylan, thus depolymerizing the cell walls from the inside. Also of interest is the ability of xylanase from *Trichoderma*, which resembles those of *C. carbonum*, to elicit defense responses in tobacco (Bailey *et al.*, 1990; Lotan and Fluhr, 1990; Fuchs and Anderson, 1987) and superoxide radical in rice protoplasts (Ishii, 1988). This evidence suggests that the ability to recognize xylanases is important to the plant and, therefore, that xylanases are important in pathogenesis.

There is the possibility that xylanases may only be important in the saprophytic phase of *C. carbonum* since the toxin itself made by race 1 can mimic disease symptoms. Race 2, however, is a weak pathogen on corn that is not susceptible to race 1. Xylanases may be important in this interaction or could contribute to the fitness of race 1 on susceptible corn by making xylan available as a carbon source. The best way to test this is to disrupt the xylanase genes in race 1 and 2 and observe the effect on corn and measure the number of spores produced in each interaction. Since targeted gene disruption is possible in *C. carbonum* (Scott-Craig *et al.*, 1990) xylanase activity could be disrupted to test this. In this research potential clones of a xylanase gene have been isolated and await further testing to determine how many genes encode xylanases. This research will provide a method of testing the importance of xylanases in infection of corn by *C. carbonum* and in other plant-pathogen interactions.

BIBLIOGRAPHY

Adams GA (1965) Xylans. Methods in Carbohydrate Chemistry vol. V. Whilstler RL (ed). Academic Press, Inc.

Anderson AJ (1978) Extra cellular enzymes produced by *Collectotrichum lindemunthianum* and *Helminthosporium maydis* during growth on isolated bean and corn cell walls. Phytopath. 68:1585-1589

Bailey BA, Dean JFD and Anderson JD (1990) An ehtylene biosynthesis-inducing endoxylanase elicits electrolyte leakage and necrosis in *Nicotiana tabacum* cv. xanthi leaves. Plant Physiol. 94:1849-1854

Baker CJ, Whalen CH and Bateman DF (1977) Xylanase from *Trichoderma pseudokoningii*: Purification, Chacterization and effects on isolated plant cell walls. Phytopathology 67:1250-1258

Bateman DF, Van Etten HD, English PD, Nevins DJ and Albersheim P (1969) Susceptibility to enzymatic degradation of cell walls from bean plants resistant and susceptible to *Rhizoctonia solani* Kuhn. Plant Physiol. 44:641-648

Bateman DF, Jones TM and Yoder OC (1973) Degradation of corn cell walls by extracellular enzymes produced by *Helminthosporium maydis* race T. Phytopath. 63:1523-1529

Biely P and Vršanaská M (1988) Xylanase of *Cryptococcus albidus*. Methods in Enzymology 160:638-648

Boccara M and Chatain V (1989) Regulation and role in pathogenicity of *Erwinia chrysanthemi* 3937 pectin methylesterase. J. Bacteriol. 171:4085-4087

Boel E, Hjort I, Svensson B, Norris F, Norris KE and Fiil NP (1984) Glucoamylases G1 andG2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs. The EMBO Journal 5:1097-1102

Burke D, Kaufman P, McNeil M and Albersheim P (1974) A survey of the walls of suspension-cultured monocots. Plant Physiol. 54:109-115

Cervone F, De Lorenzo G, Degrà L and Salvi G (1987) Elicitation of necrosis in *Vigna unguiculata* Walp. by homogeneous *Aspergillus niger* endo-polygalacturonase and by α-D-galacturonate oligomers. Plant Physiology 85:626-630

Cooper RM (1984) The role of cell wall-degrading enzymes in infection and damage. Plant Diseases: infection, damage and loss. Wood RKS and Jellis GJ eds. Blackwell Scientific Publications, Oxford.

Cooper RM, Longman D, Campbell A, Henry M and Lees PE (1988) Enzymic adaption of cereal pathogens to the monocotyledonus primary cell wall. Physiol. Mol. Plant Path. 32:33-47

Darvill A, McNeil M, Albersheim P and Delmer DP (1980a) The primary cell wall of flowering plants. The biochemistry of plants, vol. I. Tolbert NE, ed. Academic Press, Inc.

Darvill JE, McNeil M, Darvill AG and Albersheim P (1980b) Glucuronoarabinoxylan, a second hemicellulose in the primary cell walls of suspension-cultured sycamore cells. Plant Physiol. 66:1135-1139

Dean JFD and Anderson JD (1991) Ethylene biosynthesis-inducing xylanase. Plant Physiol. 95:316-323

English PD, Jurale JB and Albersheim P (1971) Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemunthianum* grown in culture. Plant Physiol. 47:1-6

Fuchs Y and Anderson JD (1987) Purification and chacterization of ethylene inducing proteins from cellulysin. Plant Physiology 84:732-736

Fuchs Y, Saxena A, Gamble HR and Anderson JD (1989) Ethylene biosynthesis-inducing protein from cellulysis is an endoxylanase. Plant Physiol. 89:138-143

Glough CL, Dow JM, Barber CE and Daniels MJ (1988) Cloning of two endogluvanase genes of *Xanthomonas campestris* pv. *campestris*: Analysis of the role of the major endoglucanase in pathogenesis. Molecular Plant-Microbe Interactions 1:275-281

Hames BD and Rickwood D (Eds.) (1981) Gel Electrophoresis of Proteins: A Practical Approach. IRL Press

Hashimoto S, Muramatsu T and Funatsu M (1971) Studies on xylanase from *Trichoderma viride.* Agr. Biol. Chem. 35:501-508

Hrmovcá M, Biely P and Vršanská M (1989) Cellulose- and xylan-degrading enzymes of *Aspergillus terreus* and *Aspergillus niger.* Enzyme Microb. Technol. 11:610-616

Ishii S (1988) Factors influencing protoplast viability of suspension-cultured rice cells during isolation process. Plant Physiology 88:26-29

John M and Schmidt J (1988) Xylanases and ß-xylosidase of *Trichoderma lignorum.* Methods in Enzymology 160:662-671 Academic Press, Inc.

Jurasek L and Paice MG (1988) Xylanase A of *Schizophyllum commune.* Methods in Enzymology 160:659-662

Lotan T and Fluhr R (1990) Xylanase, a novel elicitor of pathogenesis related proteins in tobacco, uses a non-ethylene pathway for induction. Plant Physiol. 93:811-817

Maniatis T, Fritsch EF and Sambrook J (1982) Molecular Cloning (A Laboratory Manual) Cold Spring Harbor Laboratory

McCarthy AJ, Peace E and Broda P (1985) Studies on the extracellular xylanase activity of some thermophilic actinomycetes. Appl. Microbiol. Biotechnol. 21:238-244

Montreuil J, Bouquelet S, Debray H, Fournet B, Spik G and Strecker G (1986) Carbohydrate Analysis: A Practical Approach (Chaplin MF and Kennedy JF eds.) IRL Press

Mullen JM and Bateman DF (1975) Polysaccharide degrading enzymes produced by *Fusarium roseum* "Avenaceum" in culture and during pathogenesis. Physiol. Plant Path. 6:233-246

Okada H and Shinmyo A (1988) Xylanase of *Bacillus pumulis.* Methods in Enzymology 160:632-637

Payne JH, Schoedel C, Keen NT and Collmer A (1987) Multiplication and virulence in plant tissue of *Escherichia coli* clones producing pectate lyase isozymes PLb and PLe at high levels and of an *Erwinia chrysanthemi* mutant deficient in PLe. Appl. Env. Microbiol 53:2315-2320

Pringle and Braun (1957) The isolation of the toxin of *Helminthosporium victoria.* Phytopathology 47:369-371

Reid JL and Collmer A (1988) Construction of an *Erwinia chrysanthemi* mutant with directed deletions in all pectate lyase structural genes. Molecular Plant-Microbe Interactions 1:32-38

Reilly PJ (1981) Xylanases: Structure and function. Trends in the Biology of Fermentations for Fuels and Chemicals. Premium Press

Roberts DP, Denny TP and Schell MA (1988) Cloning of the *egl* gene of *Pseudomonas solanacearum* and analysis of its role in pathogenicity. J. Bacteriol. 170:1445-1451

Scott-Craig JS, Panaccione DG, Cervone F and Walton JD (1990) Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell 2:1191-1200

Stone KL, LoPresti MB, Crawford JM, DeAngelis R and Williams KR (1989) Enzymatic digestion of proteins and HPLC peptide isolation. A Practical Guide to Protein and Peptide Purification for Microsequencing. Academic Press, Inc.

Trevelyan WE, Proctor DP and Harrison JS (1950) Detection of sugars on paper chromatograms. Nature 166:444-445

Walton JD (1987) Two enzymes involved in the biosynthesis of the host-selective phytoxin HC-toxin. Proc. Natl. Acad. Sci. USA 84:8444-8447

Walton JD and Holden FR (1988) Properties of two enzymes involved in the biosynthesis of the fungal pathogenicity factor HC-toxin. Molecular Plant-Microbe Interactions 1:128-134