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Ethylene Biosynthesis-Inducing Xylanase[1]

III. Product Characterization

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ABSTRACT

Induction of ethylene biosynthesis in tobacco (*Nicotiana tabacum* cv Xanthi) leaf discs by the ethylene biosynthesis-inducing xylanase (EIX) isolated from Cellulysin or xylan-grown cultures of *Trichoderma viride* was dependent upon the concentration of xylanase applied and upon the length of incubation. Arrhenius activation energies of 9,100 and 10,500 calories for the Cellulysin and *T. viride* EIX xylanase activities, respectively, were derived from the K_m and V_{max} values determined for each enzyme at several temperatures. The two xylanases digested xylan in a strictly *endo* fashion, releasing neither xylobiose nor free xylose, and no debranching activity was associated with either enzyme. The xylanases released polysaccharides from ground corn cobs, but little or no carbohydrate was released from tobacco mesophyll cell walls incubated with EIX. No heat-stable products capable of inducing ethylene biosynthesis in tobacco leaf discs were found in EIX digests of purified xylans.

A protein purified from a commercial preparation (Cellulysin)[3] of plant cell wall-digesting enzymes secreted by the saprophytic fungus *Trichoderma viride* is a potent inducer of ethylene biosynthesis in tobacco (*Nicotiana tabacum* cv Xanthi) leaf discs (12). The protein was identified as an endoxylanase (14) whose secretion by *T. viride* can be induced in culture by growth on D-xylose, β-1,4-D-xylan, or unfractionated plant materials (7). The enzyme purified from these induced fungal cultures was shown to be a glycoprotein having a number of unusual physical characteristics (8). Production of immunologically related EIXs[4] can also be induced in some pathogenic fungi (7). *T. viride* EIX has been shown to stimulate production of pathogenesis-related proteins in tobacco, even in the presence of ethylene biosynthesis and ethylene action inhibitors (17). More recently, EIX introduced into the vascular system of tobacco plants was shown to be translocated to leaf mesophyll tissue where it induced ethylene biosynthesis, electrolyte leakage, and tissue necrosis, effects often associated with the hypersensitive defense response (1). The induction of defense responses by fungal xylanases of unknown relationship to EIX have also been demonstrated in other plant systems (6, 11, 16, 26).

Oligosaccharides released from plant cell walls by fungal hydrolases are capable of inducing ethylene biosynthesis in plants (3, 24) and, in some cases, may cause rapid death of plant cells (6). However, polypeptide products of pathogenic fungi harboring no apparent enzymatic activities also stimulate ethylene biosynthesis (25) and cause tissue necrosis in plants (9). This paper examines the endoxylanase and ethylene biosynthesis-inducing activities of two EIX proteins in parallel in an effort to identify enzymatic products that might be responsible for inducing defense responses in tobacco tissue.

MATERIALS AND METHODS

Reagents and Biologicals

EIX was purified from either xylan-grown liquid shake cultures of *Trichoderma viride* T-1 (ATCC 52438) or Cellulysin (Calbiochem) as previously described (8). Birchwood β-D-xylan (lot No. 1691175) was obtained from Atomergic Chemetals Corp. (Farmingdale, NY); oat-spelt β-D-xylan (lot No. 116F-0240) was from Sigma Chemical Co.; and ground corn cobs were from The Andersons (Maumee, OH). Tobacco (*Nicotiana tabacum* cv Xanthi) mesophyll cell walls were prepared as previously described (7). Blue dextran (Pharmacia) and low molecular weight malto-oligosaccharides (Pfanstiehl Laboratories, Inc., Waukegan, IL) were used as calibration standards for gel filtration chromatography of xylanase products. Standards for methylation analysis of xylan glycosyl linkages were kindly provided by Andrew Mort, Biochemistry Department, Oklahoma State University. Sep-Pak C_{18} cartridges were a product of Millipore. All other reagents were obtained at the highest purity available and used without further purification.

Assays

Purified EIX protein was quantified by measuring the A_{280} of solutions containing the enzyme (8). Ethylene biosynthesis-inducing activity was assayed in triplicate with leaf discs from

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[3] The mention of specific instruments, trade names, or manufacturers is for the purpose of identification and does not imply any endorsement by the United States government.

[4] Abbreviation: EIX, ethylene biosynthesis-inducing endoxylanase.

N. tabacum cv Xanthi as described previously (12). Ethylene biosynthesis-inducing activity is expressed as nL C_2H_4 evolved/h · g fresh weight of tobacco tissue. The endoxylanase assay was modified from previous work (7) as follows. EIX was appropriately diluted in 200 mM sodium acetate (pH 5.0), and 100 μL of this enzyme solution was mixed with 100 μL of 1.0% birchwood xylan on ice. The reaction mixture was incubated at 50°C for 10 min, and the reaction was terminated by boiling for 1 min, which eliminated all enzyme activity. After diluting the reaction mixture 100-fold, a 500-μL aliquot was treated as previously described (7). Units of endoxylanase activity are expressed as μmol of reducing sugar equivalents released/min as compared to a standard curve for D-xylose. For kinetic analyses, assay mixtures containing increasing concentrations of xylan (0.67, 0.8, 1.0, 1.3, 2.0, 4.0 mg/mL) were reacted with increasing amounts of EIX (50, 100, 150, 200, 250 ng) at 20, 30, and 40°C. All xylanase assays were performed in duplicate.

GC-MS Analysis of Xylan Substrates and Reaction Products

Birchwood xylan, oat-spelt xylan, ground corn cobs, and tobacco cell walls (10 mg), each suspended in 200 mM sodium acetate (pH 5.0) containing 0.01% sodium azide (5 mL), were stirred with *T. viride* EIX (500 μg) for 3 d at 30°C. Control digests were incubated without the addition of EIX. Insoluble material from corn cobs and tobacco cell walls was removed by centrifugation, and aliquots (1 mL) were applied to a Bio-Gel P2 (Bio-Rad) gel filtration chromatography column (1.6 × 30 cm) equilibrated with 200 mM pyridine acetate (pH 5.0) buffer. Total carbohydrate in fractions (1 mL) eluted from the column with equilibration buffer at room temperature was quantified by the phenol-sulfuric acid assay (19) against a D-xylose standard; selected fractions were lyophilized prior to glycosyl-linkage analysis. Maltooligosaccharide standards were chromatographed after fractionation of each digest to verify that the materials in a given column fraction had corresponding mol wt. The elution volumes of standards varied by no more than 0.4 mL between any two column runs.

For glycosyl-linkage analysis, samples were per-*O*-methylated according to Hakamori (15) using butyl-lithium in place of potassium-methyl sulfinyl carbanion as described by Blakeney and Stone (4). Methylated polysaccharides were purified on a Sep-Pak C_{18} cartridge (18), then reduced and acetylated by a procedure similar to that of Blakeney *et al.* (5). Partially methylated, partially acetylated derivatives were identified by relative retention time on a 30 m Restek Rt_x-5 dimethyl 5% diphenyl polysiloxane cross-bonded SE-54 capillary column (0.25 mm i.d.; 1 μm film thickness) and subsequent mass spectral analysis of fragmentation patterns after electron impact mass spectrometry. The GC-MS system consisted of a Hewlett-Packard 5890 gas chromatograph coupled to a 5970A mass selective detector and a series 9000–236 computer system. The GC conditions were as follows: injection volume, 1 μL; injection port temperature, 250°C; flow rate, He at 1 mL/min; detector temperature, 250°C. The oven temperature was programmed from 140 to 200°C at 1°C/min, and then to 230°C at 10°C/min, where it was held for 10 min. Quantitation was done with a Hewlett-Packard 5880 GC-flame ionization detector based on the effective carbon response of derivatized glycosyl residues (23). Chromatographic conditions and column were similar to those described for GC-MS analysis.

Glycosyl linkages were deduced from GC-MS of partially methylated, partially acetylated alditol acetates. Nomenclature denotes the carbons participating in the glycosyl linkage. For example, 4-xylose represents a C-1 to C-4 glycosyl linkage, with the actual alditol acetate derivative being 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl xylitol. Nonreducing terminal xylosyl residues are designated *t*-xylose.

RESULTS

Kinetic Parameters of Ethylene Biosynthesis Induction by EIX

Induction of ethylene biosynthesis by EIX purified from either Cellulysin or cultures of xylan-grown *T. viride* was optimal at pH 6.2 in incubation medium containing a minimal sorbitol concentration of 250 mM. Under these conditions (incubation at 25°C in 1 mL of 10 mM sodium-Mes, pH 6.2, containing 250 mM sorbitol), the accumulation of ethylene from six EIX-treated 1-cm leaf discs (approximately 85 mg fresh weight), excised from *N. tabacum* cv Xanthi, was nearly linear from 1 to 4 h, and leveled off after 8 h (Fig. 1A). An incubation time of 4 h was used as a standard condition in

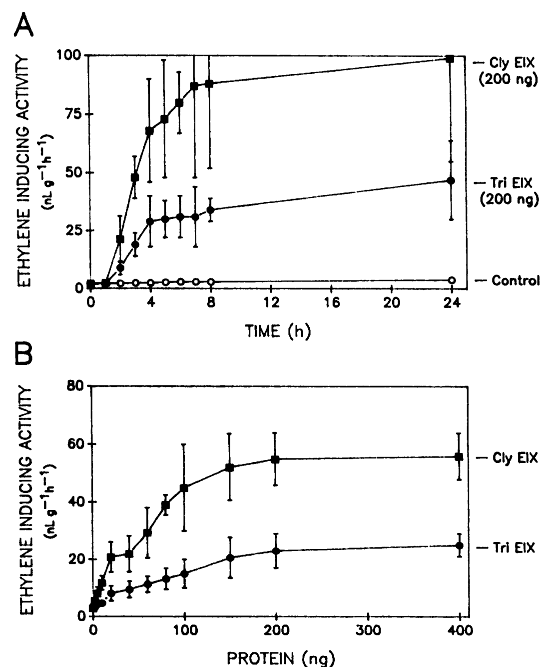

Figure 1. Induction of ethylene biosynthesis by EIX as a function of protein concentration and time. A, Ethylene, accumulated from tobacco leaf discs treated with Cellulysin or *T. viride* EIX (200 ng), was measured after the indicated incubation times. B, Total ethylene accumulated after 4 h of treatment with increasing amounts of Cellulysin or *T. viride* EIX.

Table I. *Endoxylanase Kinetic Constants Determined for the Ethylene Biosynthesis-Inducing Xylanases Purified from Cellulysin and T. viride Culture Filtrates*

Temperature	Cellulysin[a]		*Trichoderma*[b]	
	K_m	V_{max}	K_m	V_{max}
°C	mg/mL	μmol/min·mg	mg/mL	μmol/min·mg
20	3.3 ± 1.0	811 ± 106	3.2 ± 1.6	682 ± 41
30	4.5 ± 1.6	1030 ± 82	4.7 ± 1.5	950 ± 105
40	7.9 ± 2.0	2023 ± 595	9.2 ± 2.5	1966 ± 652

[a] Arrhenius activation energies = 9.1 ± 1.4 kcal. [b] Arrhenius activation energies = 10.5 ± 2.1 kcal.

subsequent experiments. The induction of ethylene was dependent on the amount of EIX protein in the incubation medium with maximal induction for six leaf discs requiring about 200 ng of EIX (Fig. 1B). For a given amount of protein, Cellulysin EIX was approximately twice as effective as *T. viride* EIX at inducing ethylene biosynthesis in these tobacco tissues, reflecting the specific bioactivities reported previously for these enzymes (7).

Kinetic Parameters of EIX Endoxylanase Activity

The xylanase activity for both EIXs was optimal at pH 5.0, and neither buffer composition nor concentration affected this activity. Temperature optima in a 10-min assay were 53 and 50°C for the Cellulysin and *T. viride* EIXs, respectively; thus, 50°C was selected as the standard assay temperature for subsequent experiments. Each EIX enzyme was assayed for xylanase activity at several substrate and protein concentrations in order to generate Lineweaver-Burk plots from which to calculate the K_m and V_{max} values shown in Table I. These kinetic constants were determined at several different temperatures, allowing calculation of Arrhenius activation energies of 9,100 and 10,500 cal for the Cellulysin and *T. viride* EIX xylanase enzymes, respectively.

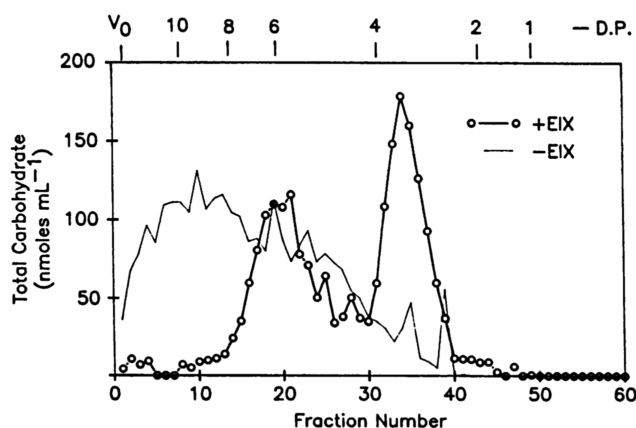

Figure 2. Gel filtration chromatography of oligosaccharides released from birchwood xylan by EIX digestion. Oligosaccharides released from birchwood xylan (2 mg/mL) incubated for 3 d with (+EIX) or without (−EIX) *T. viride* EIX were fractionated by gel filtration chromatography on Bio-Gel P2. The column was calibrated with blue dextran (V_O), low mol wt malto-oligosaccharides (degrees of polymerization [D.P.] = 10–2), and D-xylose (D.P. = 1). Total carbohydrate in eluted fractions was quantified by phenol-sulfuric acid assay (19).

Composition Analysis of the Reaction Products of EIX Endoxylanase Activity

Treatment of birchwood xylan with either Cellulysin or *T. viride* EIX converted the relatively high mol wt polysaccharide substrate into two classes of oligosaccharides with apparent degrees of polymerization of approximately 6 and 3 to 4 (Fig. 2). Glycosyl composition analysis of the birchwood xylan substrate indicated that xylose was the only neutral sugar present in this polysaccharide. Glycosyl-linkage analysis revealed a structure containing intermittent branch points consisting of one 2,4-linked xylosyl and one terminal xylosyl/18 4-linked xylosyl residues (Table II). Glycosyl-linkage composition ascertained by methylation analysis of the material contained in the two peak fractions generated by EIX digestion indicated that the late eluting peak contained unbranched

Table II. *Composition and Linkage Analysis of Substrate and of Bio-Gel P2 Size-Fractionated Oligosaccharides Released from Birchwood Xylan during Incubation with or without EIX*

Treatment	Fraction No.	Glycosyl Linkage	Composition
			mol %
Without EIX	Substrate	*t*-Xylose	7.7
		4-Xylose	87.4
		2,4-Xylose	4.9
	7	*t*-Xylose	7.0
		4-Xylose	85.9
		2,4-Xylose	7.1
	19	*t*-Xylose	17.4
		4-Xylose	82.5
	35	ND[a]	
With EIX	7	ND	
	19	*t*-Xylose	35.1
		4-Xylose	47.0
		2,4-Xylose	17.9
	35	*t*-Xylose	22.5
		4-Xylose	77.5

[a] Not detected.

1,4-linked xylotriose and xylotetraose fragments. The xylan fragments in the early peak appeared to consist primarily of hexamers with single xylosyl branches attached at the carbon 2 of residues on the 1,4-linked xylosyl backbone.

Digestion of oat-spelt xylan with EIX also led to conversion of high mol wt polysaccharide into oligosaccharides. The structural heterogeneity of these fragments reflected the more complex composition and structure of the arabinoxylan substrate (Table III). However, it was apparent from the composition analysis that the released material was primarily a 1,4-linked xylose backbone with branches containing xylosyl and arabinosyl residues.

EIX also released branched and unbranched fragments of xylan from ground corn cobs (Table IV), but small oligosaccharides were not detected in EIX digests of isolated tobacco mesophyll cell walls (Table V). Little or no detectable xylobiose or free xylose was released in any of the xylan or cell wall digests. Thus, the EIX xylanase activity is strictly *endo*. Neither birchwood nor oat spelt xylan digests (10–100 μg total carbohydrate digest/mL bioassay media) contained heat-stable material capable of inducing ethylene biosynthesis in tobacco leaf discs.

Table III. *Composition and Linkage Analysis of Substrate and of Bio-Gel P2 Size-Fractionated Oligosaccharides Released from Oat Spelt Xylan during Incubation with or without EIX*

Treatment	Fraction No.	Glycosyl Linkage	Composition
			mol %
Without EIX	Substrate	*t*-Arabinose	4.5
		t-Xylose	2.1
		2-Arabinose	0.5
		4-Xylose	69.7
		3,4-Xylose	8.4
		2,4-Xylose	2.9
		Free arabinose	1.4
		4-Glucose	0.7
		Free xylose	9.8
	7	*t*-Arabinose	4.4
		t-Xylose	9.1
		4-Xylose	62.4
		3,4-Xylose	14.0
		2,4-Xylose	10.2
	19	*t*-Xylose	19.5
		4-Xylose	69.3
		3,4-Xylose	10.3
		2,4-Xylose	0.9
	35	ND[a]	
With EIX	7	ND	
	19	*t*-Arabinose	3.2
		t-Xylose	32.6
		2-Arabinose	4.2
		4-Xylose	30.9
		3,4-Xylose	24.7
		2,4-Xylose	4.3
	35	ND	

[a] Not detected.

Table IV. *Composition and Linkage Analysis of Substrate and of Bio-Gel P2 Size-Fractionated Oligosaccharides Released from Ground Corn Cobs during Incubation with or without EIX*

Treatment	Fraction No.	Glycosyl Linkage	Composition
			mol %
Without EIX	Substrate	*t*-Arabinose	2.2
		t-Xylose	1.7
		5-Arabinose	0.5
		4-Xylose	51.7
		t-Glucose	11.2
		t-Galactose	0.7
		3,4-Xylose	6.3
		2,4-Xylose	2.4
		4-Glucose	16.8
		Free xylose	4.4
		4,6-Glucose	2.2
	7	ND[a]	
	19	ND	
	35	ND	
With EIX	7	ND	
	19	*t*-Arabinose	3.5
		t-Xylose	16.2
		4-Xylose	20.7
		3,4-Xylose	59.5
	35	*t*-Xylose	33.3
		3,4-Xylose	66.7

[a] Not detected.

DISCUSSION

EIX treatment of Xanthi tobacco leaves induced levels of ethylene biosynthesis approaching those associated with the ripening climacteric in banana and avocado (20), but the time course was much shorter. The accumulation of ethylene from EIX-treated tobacco leaf discs appeared to mirror the rates of production reported for intact, detached tobacco leaves treated with EIX (1). However, induction of ethylene biosynthesis by EIX is about 10 times more efficient for a given ratio of EIX protein to leaf tissue in the detached leaf system. This greater efficiency of the detached leaf assay probably represents a more even distribution of EIX to the mesophyll tissue in the interior of the leaf. The drop-off in ethylene accumulation and production rates in both the leaf disc and detached leaf systems coincided with cellular lysis in the mesophyll tissue, an event known to halt the terminal step of ethylene biosynthesis (29). The basis of the higher specific activity for ethylene biosynthesis induction by the EIX isolated from Cellulysin is not yet understood.

The xylanase activity kinetics of the EIXs isolated from

Table V. *Composition and Linkage Analysis of Substrate and of Bio-Gel P2 Size-Fractionated Oligosaccharides Released from Tobacco Cell Walls during Incubation with or without EIX*

Treatment	Fraction No.	Glycosyl Linkage	Composition
			mol %
Without EIX	Substrate	*t*-Arabinose	3.9
		t-Xylose	3.6
		5-Arabinose	3.5
		2-Rhamnose	7.8
		4-Xylose	2.3
		t-Glucose	2.3
		t-Galactose	4.0
		2,4-Rhamnose	4.7
		4-Galactose	2.1
		4-Mannose	2.1
		4-Glucose	55.1
		4,6-Glucose	8.8
	7	ND[a]	
	19	ND	
	35	ND	
With EIX	7	ND	
	19	ND	
	35	ND	

[a] Not detected.

Cellulysin and *T. viride* cultures are very similar to those displayed by xylanases purified from a variety of other sources (2, 21, 27, 28). Many of these other enzymes are also incapable of releasing free xylose or of debranching xylan (22, 28). A related *Trichoderma* xylanase was shown to effectively release oligosaccharides from corn cell walls, but very little material was released from bean cell walls (2). That EIX released little or no carbohydrate material from tobacco leaf cell walls is not surprising, because most of the 5 to 6% xylose identified in this substrate (Table V) is likely attached to xyloglucan, a cell wall polysaccharide constituting 1 to 5% of the primary cell wall (12) that is not a substrate for EIX (JFD Dean, unpublished observation). However, unbranched β-1,4-D-xylan has been identified in tobacco stalks (10), suggesting that a possible substrate for EIX xylanase activity does exist in the plant *in vivo*. Most of this tobacco stalk xylan is probably contained in secondary walls of vascular tissue.

Although several laboratories have demonstrated stimulation of plant defense responses with oligosaccharides (3, 6, 24), our inability to identify heat-stable xylanase products capable of eliciting ethylene biosynthesis during this and previous work (13) has led us to consider alternative induction mechanisms. Experiments documenting the induction of ethylene biosynthesis, tissue necrosis (1), or synthesis of pathogenesis-related proteins (17), only in those tobacco tissues actually containing EIX, likewise have suggested that products of EIX xylanase activity may not have a direct role in eliciting these defense responses. Given that plant defenses can be stimulated by direct interaction with polypeptides of fungal origin (9, 25), perhaps a specific receptor for the EIX protein existing on Xanthi tobacco cells is responsible for the induction of ethylene biosynthesis. This does not completely discount the possibility that xylan fragments produced by EIX may be responsible for some of the other pathologic symptoms seen in EIX-treated tissues, particularly given the observation that heat-stable cell wall fragments released by another fungal xylanase can kill plant cells (6). Further work is needed to determine whether the EIX xylanase and ethylene biosynthesis-inducing activities can be resolved from one another.

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