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Purification and characterization of extracellular β-xylosidase and α-arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum* [1]

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Abstract

The filamentous fungus *Cochliobolus carbonum* produces extracellular β-xylosidase (Xyp) and α-arabinofuranosidase (Arf) when grown on maize cell walls. Low concentrations of sucrose in a medium containing other carbon sources stimulate production of both enzymes, while high sucrose concentrations partially repress enzyme production. Xyp and Arf were purified 49-fold and 162-fold with 24% and 15% yields, respectively, by cation-exchange, hydrophobic-interaction, and gel-filtration HPLC. Xyp has a molecular mass of 42 kDa, a low level of α-arabinosidase, but no arabinanase activity, a pH optimum of 5.5–6.5, and a temperature optimum of 37 °C. Two peptides derived from Xyp show strong similarity to a bacterial bifunctional β-D-xylopyranosidase/α-L-arabinofuranosidase. Arf has a molecular mass of 63 kDa, a low level of β-xylosidase and arabinanase activity, a pH optimum of 3.5–4.0, and a temperature optimum of 50 °C. Xyp but not Arf is glycosylated. Xyp liberates xylose from oat spelt xylan, while Arf releases both arabinose and larger oligosaccharides, suggesting that while Xyp behaves as a β-xylosidase on natural substrates, Arf acts as both an α-arabinosidase and an arabinanase.

Keywords: β-Xylosidase, extracellular; α-Arabinofuranosidase, extracellular; Fungi; *Cochliobolus carbonum*

1. Introduction

Degradation of the plant cell wall is a common phenomenon during infection of plants by pathogens and may be necessary in penetration, the release of nutrients, and the generation of signal molecules [1]. Cell-wall degrading enzymes (CWDE) are produced by all classes of cellular plant pathogens as well as saphophytic bacteria, fungi, and nematodes [1]. Al-

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[1] Abbreviations: HPLC, high-pressure liquid chromatography; HI-HPLC, hydrophobic interaction HPLC; Xyp, *C. carbonum* β-D-xylopyranosidase; XP, β-D-xylopyranosidase; Arf, *C. carbonum* α-L-arabinofuranosidase; AF, α-L-arabinofuranosidase; NP, *p*-nitrophenyl.

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though some CWDE have been shown, either directly or indirectly, to influence the interactions between plants and pathogens, the role of most CWDEs is not known.

Xylans are important components of the hemicellulosic fraction of the cell walls of most plants. The primary cell walls of monocotyledons contain up to 40% β-(1 → 4)-xylan, and in maize, arabinoxylan (β-(1 → 4)-xylan substituted with short chains of α-linked arabinose) is the major water-insoluble, non-cellulosic component [2,3]. Enzymes that can degrade arabinoxylans [including endo-β-(1 → 4)-xylanase, arabinanase, β-xylosidase (here abbreviated XP), and α-arabinosidase (AF)], have been described from many microorganisms, including plant pathogenic bacteria and fungi (e.g., Maino et al. [4]; Wong et al. [5]). Xylanase genes have been isolated from *Cochliobolus carbonum* [6], *Magnaporthe grisea* [7], and *Erwinia chrysanthemi* [8]. Arabinanases are produced by many pathogenic and saprophytic filamentous fungi (e.g., Fuchs et al. [9]). Arabinanases and xylanases are the first enzymes produced when several pathogens of monocotyledonous plants are grown on isolated monocotyledonous cell walls [10]. Extracellular α-arabinosidase has been purified from the plant pathogenic fungus *Sclerotinia sclerotiorum* [11]. Genetically uncharacterized mutants of *S. fructigena* and *S. trifoliorum* that are deficient in α-arabinosidase activity have reduced virulence [12,13]. *Cochliobolus heterostrophus* (*Helminthosporium maydis*) makes high levels of β-xylosidase when grown on isolated maize cell walls but not when grown on bean walls [14]. β-xylosidase and α-arabinosidase genes have been isolated from rumenal bacteria (e.g., Whitehead and Hespell [15]), but apparently not from any plant pathogenic fungi.

As part of a concerted effort to determine the role of CWDEs in Northern leaf spot disease of maize caused by *C. carbonum*, we are constructing specific mutants by transformation-mediated targeted gene disruption of a variety of CWDE genes alone and in combination. Our general strategy has been to use amino acid sequences from purified and characterized CWDEs to isolate the corresponding genes using either PCR [16] or labelled oligonucleotides [6]. Heterologous probes have also been used successfully, but lack of knowledge of the biochemical nature of the gene product can make subsequent analysis of engineered mutants difficult [17].

Since substituted xylans are major components of the walls of maize, and since AF and XP in cooperation with endoxylanases could be important for the complete degradation of those xylans to monomers, we have undertaken an analysis of the AF and XP activities made by *C. carbonum* in culture. Purification of these enzymes provides an avenue to the isolation and disruption of the encoding genes in order to test their possible involvement in pathogenicity.

2. Experimental

Fungal cultures.—Growth conditions for isolate SB111 (ATCC 90305) or 367-1 (derived in this lab from SB111) of *Cochliobolus carbonum* Nelson (anamorph *Helminthosporium carbonum* or *Bipolaris zeicola*), and preparation of maize cell walls were as described [17,18].

Enzyme purification.—*C. carbonum* was grown in still culture in 1 L flasks containing 125 mL of modified Fries' medium [19] containing one-tenth (2 g/L) the normal concentration of sucrose, plus 8 g/L maize cell walls. The culture liquid was filtered though Whatman #1 paper, and the filtrate (2 L) was reduced to 200 mL by rotary evaporation at 30 °C. The volume of the filtrate was further reduced ten-fold by ultrafiltration though a 10 kD MWCO ultrafiltration membrane, and the retained material was dialyzed against 2 L of 25 mM sodium acetate, pH 5 (buffer A) for 6 h with two buffer changes. The dialysate was filtered though a 0.22-μm syringe filter and fractionated in several runs by polysulfoethyl aspartamide cation-exchange HPLC (The Nest Group, Southboro, MA) with a 30-min gradient at 1 mL/min of 0–0.4 M KCl in 25 mM sodium acetate, pH 5.0 [18]. The fraction(s) from each cation-exchange HPLC separation containing the greatest amount of β-D-xylopyranosidase (XP) or α-L-arabinofuranosidase (AF) activity were pooled, mixed with an equal volume of 3.4 M $(NH_4)_2SO_4$ in 50 mM sodium acetate, pH 5.0, clarified by centrifugation, and fractionated by hydrophobic-interaction (HI) HPLC (TSK-Phenyl-5PW, 75 × 7.5 mm) with a 30 min gradient at 1 mL/min of 1.7–0 M $(NH_4)_2SO_4$ in 25 mM sodium acetate, pH 5.0 [19]. The single fraction (1 mL) from HI-HPLC containing the greatest amount of AF activity was concentrated to 0.2 mL with a Centricon 10 ultrafiltration device and applied to a Superdex 200 HR 10/30 FPLC column (Pharmacia-LKB). Proteins were eluted with 150 mM NaCl in 50 mM potassium phosphate, pH 7.0, at a flow rate of 0.5 mL/min.

Enzyme assays.—β-D-Glucopyranosidase, α-D-mannopyranosidase, α-L-arabinofuranosidase, β-D-

xylopyranosidase, and α-L-arabinopyranosidase activities were determined by production of *p*-nitrophenol from the corresponding *p*-NP conjugates (Sigma). Enzyme (100 μL), 200 μL of 50 mM sodium acetate, pH 5, and 100 μL 10 mM *p*-NP substrate were incubated for 20 min at 25 °C. Sodium carbonate (600 μL of 1 M) was added, and the absorbance was measured at 400 nm. One unit (U) of enzyme activity is defined as the amount producing 1 μg of *p*-nitrophenol min^{-1} mL^{-1} at 25 °C, and specific activities were defined similarly as the amount producing 1 μg of *p*-nitrophenol min^{-1} mg $protein^{-1}$ at 25 °C. For determination of pH dependence, Prideaux–Ward universal buffer [20] (10 mM boric acid, 10 mM phenylacetic acid, 30 mM phosphoric acid) titrated to the proper pH with NaOH was substituted for sodium acetate buffer in the reaction mixture.

Reducing sugar assays were performed using *p*-hydroxybenzoic acid hydrazide [18,21]. Reactions (total volume 500 μL) contained 0.1% polysaccharide substrate and 0.05 U of enzyme in 50 mM sodium acetate buffer, pH 5.0. Aliquots of 30 μL of the reaction mixture were removed at each time point and mixed with 1.5 mL of *p*-hydroxybenzoic acid hydrazide working solution (1 vol of 5% *p*-hydroxybenzoic acid hydrazide in 0.5 M HCl mixed with 4 vol 0.5 M NaOH). Assay mixtures were heated to 100 °C for 10 min, allowed to cool, and the absorbance at 410 nm was determined. The substrates used were oat spelt xylan (Sigma), birchwood xylan (Fluka), larchwood xylan (Aldrich), xylobiose (Sigma), and wheat arabinoxylan, sugar-beet arabinans, and debranched arabinan (all from Megazyme, Sydney, Australia).

Thin-layer chromatography.—The enzymatic digestion reactions consisted of 0.1% polysaccharide substrate and 0.05 U of enzyme in 100 μL 50 mM sodium acetate, pH 5, and proceeded at 42 °C for 2 or 6 h. The products were lyophilized, dissolved in 20 μL water, centrifuged to remove insoluble material, and separated by thin-layer chromatography on Kieselgel 60 TLC plates (0.25 mm) (E. Merck, Germany) with a solvent system of 1:3:1 nitroethane–ethanol–water [15]. The plates were sprayed with 6.5 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride in methanol containing 3% sulfuric acid, and the reaction products were visualized after heating at 100 °C for 10 min [22].

SDS-PAGE and glycoprotein detection.—SDS-PAGE was performed as described [19] on 12% (w/v) acrylamide separating gels. Gels were fixed in 10% acetic acid plus 40% methanol and stained with colloidal Coomassie G-250 as described by Neuhoff et al. [23].

Proteins were transferred to PVDF membranes (Applied Biosystems) by the procedure of Matsudaira et al. [24]. Glycoproteins were detected with periodic acid–Schiff base [25]. Ovalbumin and bovine serum albumin served as positive and negative glycoprotein controls, respectively.

3. Results

Cochliobolus carbonum secretes both a β-D-xylopyranosidase (Xyp) and an α-L-arabinofuranosidase (Arf) when grown on Fries' medium containing a variety of complex carbohydrate carbon sources. The greatest amount of XP activity was in filtrates from cultures grown on oat spelt xylan or maize cell walls, and AF activity was highest in filtrates of cultures grown on maize cell walls (data not shown). Low concentrations of sucrose (1–5 g/L) in medium containing other carbon sources stimulated both XP and AF activities but concentrations higher than 5 g/L repressed enzyme production (Fig. 1). The XP and AF activities are less sensitive to carbon repression than endo-polygalacturonase, endo-β-(1 → 4)-xylanase, and exo-β-(1 → 3)-glucanase, which are completely repressed at 20 g/mL sucrose [18,19,26]. Both XP and AF activity in culture filtrates increased steadily as a function of culture age up to nine days when grown on medium supplemented with corn cell walls (Fig. 2); after nine days little further increase in either activity was observed.

The concentrated, clarified, and desalted culture filtrate was passed over a DEAE-cellulose column to

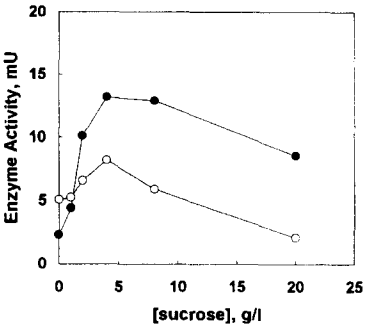

Fig. 1. Effect of sucrose concentration on β-xylosidase (●) and α-arabinosidase (○) activity in culture filtrates of *C. carbonum* grown on modified Fries' medium containing 0.8% maize cell walls.

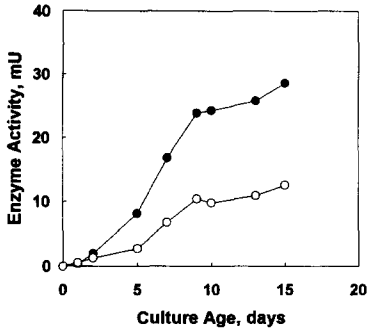

Fig. 2. Effect of culture age on β-xylosidase (●) and α-arabinosidase (○) activity in culture filtrates of *C. carbonum* grown on modified Fries' medium (0.2% sucrose) containing 0.8% maize cell walls.

remove strongly anionic species and pigments. No detectable XP or AF activity remained bound to the DEAE-cellulose. Following fractionation by cation-exchange HPLC, the XP and AF activities each eluted as single, slightly overlapping peaks (Fig. 3). The fraction containing the greatest amount of XP or AF activity (approximately 60–70% of the total activity eluted from the column) was separated by HI-HPLC. XP activity was associated with a major

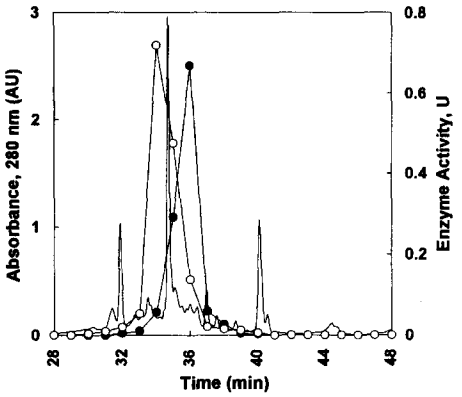

Fig. 3. Cation-exchange HPLC fractionation of culture filtrate of *C. carbonum*. OD_{280} (———); β-xylosidase activity (●); α-arabinosidase activity (○).

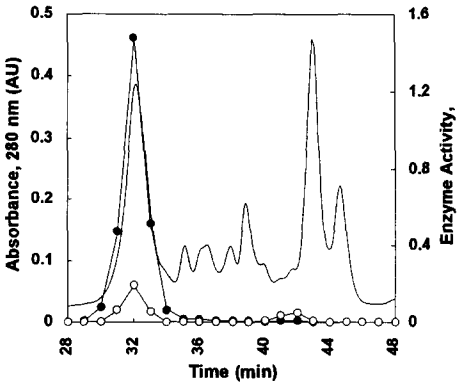

Fig. 4. HI-HPLC fractionation of Xyp in the cation-exchange fractions (Fig. 3) eluting between 33 and 34 min. OD_{280} (———); β-xylosidase activity (●); α-arabinosidase activity (○).

peak of UV absorbance (Fig. 4), and the Xyp protein appeared as a single 42-kDa band on SDS-PAGE (Fig. 6, lane 2). Xyp has an apparent mass of 35 kDa by gel filtration. Associated with purified Xyp was a small amount of AF activity, approximately equivalent to 12% of its XP activity (Fig. 4). Xyp was purified 49-fold with a 24% yield (Table 1).

The AF activity from cation-exchange HPLC coeluted with a peak of UV absorbance from HI-HPLC (Fig. 5). Following an additional purification step by gel filtration, the AF activity was associated with a single peak of UV absorption and the Arf protein ran as a single 62 kDa band on SDS-PAGE (Fig. 6, lane 3). The purified Arf was also associated with a small amount (2% of its Arf activity) of XP activity (data not shown). Arf has an apparent mass of 33 kDa by gel filtration, considerably smaller than its M_r estimated by SDS-PAGE (Fig. 5, lane 3). Likewise, endo-(1 → 4)-xylanase behaves by gel filtration as a protein considerably smaller than the size estimated by either SDS-PAGE or predicted from the gene sequence [6,18]. Arf was purified 162-fold with a yield of 15% (Table 2).

Table 1
Purification of Xyp from culture filtrates of *Cochliobolus carbonum*

	Total protein (mg)	Total β-xylosidase (U)	Specific activity (U mg protein^{-1})	Yield (%)	Purification (fold)
1. Culture filtrate [a]	107	15.7	0.15	100	1.0
2. U.F. Retentate	69	12.9	0.20	82	1.3
3. Dialysate	40	12.2	0.31	77	2.1
4. DEAE-Cellulose eluant	35	11.6	0.33	74	2.2
5. Cation-exchange HPLC	1.6	5.6	3.5	36	23
6. HI-HPLC	0.5	3.7	7.4	24	49

[a] Original volume 1800 mL.

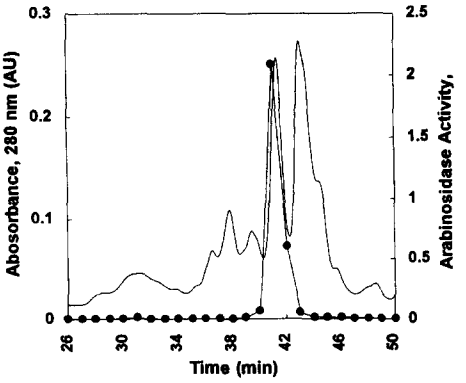

Fig. 5. HI-HPLC fractionation of Arf in the cation-exchange fractions (Fig. 3) eluting between 35 and 37 min. OD_{280} (———); α-arabinosidase activity (●).

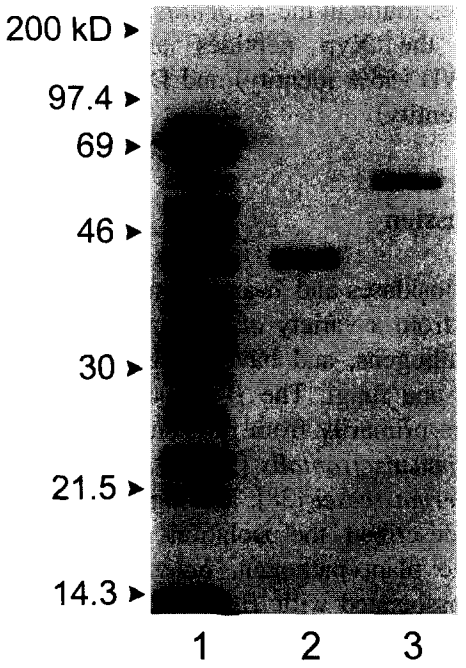

Fig. 6. SDS-PAGE analysis of purified Xyp and Arf. Lane 1: proteins (20 μg) from unfractionated concentrated culture filtrate. Lane 2: purified Xyp (3 μg). Lane 3: purified Arf (3 μg). Positions of M_r markers are indicated at the left (sizes in kDa).

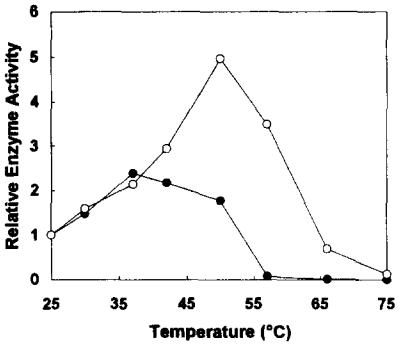

Fig. 7. Temperature dependence of purified Xyp (●) and Arf (○) activities, relative to activity at 25 °C. All reactions were run for 20 min with 5 mU of enzyme.

Xyp exhibited maximum activity between 37 and 42 °C (Fig. 7) and over the pH range of 5.5–6.5 (Fig. 8). Arf activity was five-fold higher at its optimum temperature (50 °C) than at 25 °C (Fig. 7) and showed highest activity in the pH range of 3.5–4.0 (Fig. 8).

Both enzymes hydrolyzed xylobiose to xylose and released reducing sugars from a variety of commercial xylans and from wheat arabinoxylan (Table 3). Arf also released significant quantities of reducing sugars from sugar-beet arabinan (Table 3). TLC anal-

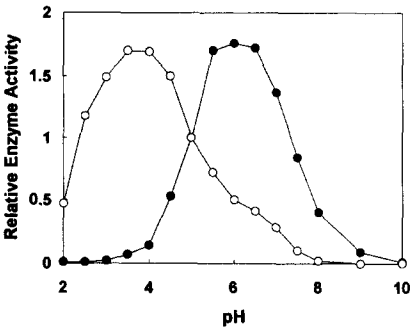

Fig. 8. pH dependence of Xyp (●) and Arf (○) activities, relative to activity at pH 5. All reactions were run for 20 min with 5 mU of enzyme.

Table 2
Purification of Arf from culture filtrates of *Cochliobolus carbonum*

	Total protein (mg)	Total α-arabinosidase (U)	Specific activity (U mg protein^{-1})	Yield (%)	Purification (fold)
1. Culture filtrate [a]	107	14.3	0.13	100	1.0
2. U.F. Retentate	69	11.9	0.17	83	1.3
3. Dialysate	40	11.1	0.28	78	2.2
4. DEAE-Cellulose eluant	35	9.4	0.26	66	2.0
5. Cation-exchange HPLC	1.0	6.6	6.6	46	51
6. HI-HPLC	0.4	4.1	10.3	29	79
7. Gel-filtration HPLC	0.1	2.1	21	15	162

[a] Original volume 1800 mL.

Table 3
Hydrolysis of various substrates by Xyp and Arf

Mixtures containing:	Reducing groups (μmol/12h) released in reaction	
Polysaccaride substrate	Xyp	Arf
Xylobiose	6.60	6.20
Oat-spelt xylan	0.72	1.13
Larch wood xylan	0.59	0.54
Birch wood xylan	1.26	1.13
Wheat arabinoxylan	1.42	2.54
Sugar-beet arabinan	0.05	4.47

ysis revealed that the products of a 2 h reaction at 42 °C containing oat spelt xylan and Arf were oligosaccharides (Fig. 9, lane 4 and 5), while after a 6 h digestion only arabinose was detected (data not shown). Xylose was the primary product detected in reactions containing Xyp and oat spelt xylan at 2 h (Fig. 9, lane 3), and the sole product after a 6 h incubation (data not shown). Arf exhibited Xyp activity equal to only 2% of its Arf activity, and Xyp had Arf activity equal to 12% of its Xyp activity when assayed under standard conditions using *p*-NP-β-D-xylopyranoside and *p*-NP-α-L-arabinofuranoside (data not shown). Neither enzyme released NP from *p*-NP-conjugated β-D-glucopyranoside, α-D-mannopyranoside, or α-L-arabinopyranoside (data not shown).

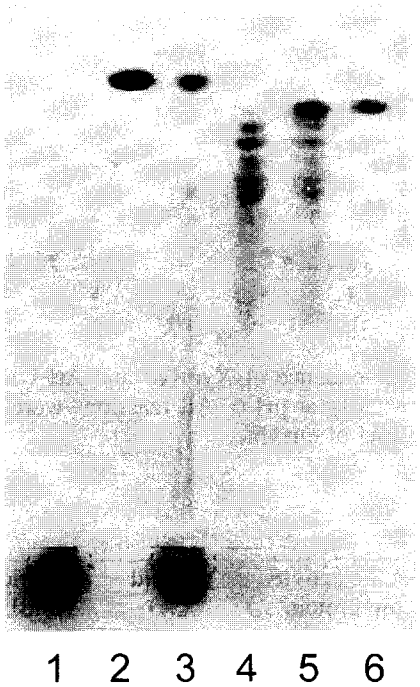

Fig. 9. Silica gel TLC of products released from oat spelt xylan by purified Xyp and Arf. Lane 1, control reaction (no enzyme); lane 2, 20 μg/mL xylose standard; lane 3, Xyp reaction products; lane 4, Arf reaction products; lane 5, Arf reaction products +20 μg/mL arabinose standard; lane 6, 20 μg/mL arabinose.

Xyp is a glycoprotein based on staining with periodic acid–Shiff base, while Arf is not glycosylated (data not shown).

Neither Xyp nor Arf yielded N-terminal sequence despite repeated attempts. Amino acid sequences from Xyp were obtained from ten peptides generated by AspN or trypsin digestion. Sequences of two peptides, LLDLVDIPWATQQLWA and DKPLGKFLPELNPI, were similar to a bifunctional β-xylopyranosidase–α-arabinofuranosidase from *Bacteriodes ovatus* [15] (Genbank accession #U04957). The sequences found in the *B. ovatus* enzyme corresponding to the Xyp peptides are VLSTEDIPWAGRQLWD (56% identity) and DKPYGPFIPEANPM (64% identity).

4. Discussion

β-Xylosidases and α-arabinosidases have been described from a variety of microorganisms, including plant pathogens, and have been purified from both bacteria and fungi. The β-xylosidases purified from fungi are primarily from rumenal organisms such as *Neocallimastix frontalis* [27] or saprophytes such as *Trichoderma reesei* [28], although Waksman [29] recently described the isolation of a β-glucosidase from the plant pathogen, *Sclerotinia sclerotiorum*, that is associated with β-xylosidase activity. To the best of our knowledge, Xyp is the first β-xylosidase enzyme purified from a fungal plant pathogen. While several genes encoding β-xylosidases have been isolated and characterized from nonpathogenic microorganisms [30–32], the only β-xylosidase gene isolated from a plant pathogen is the *bgxA* gene from the bacterium *Erwinia chrysanthemi* [8]. α-Arabinosidases have been isolated from bacterial [33] and fungal [11] plant pathogens, and genes encoding similar activities have been cloned from nonpathogenic microorganisms (e.g., Gasparic et al. [34]; Sakka et al. [35]).

The M_r of Xyp as determined by SDS-PAGE (42 kD) is less than the M_rs of the β-D-xylosidases previously described from other fungi, which range from 53 kDa to 102 kDa [27,28,36], or from rumenal bacteria, which range from 56 kDa to 75 kDa [30–32]. The pH optimum of Xyp was also less acidic (pH 5.5–6.5) than the optima for other fungal XP's, which

range from pH 3.3 to 4.5 [37–39]. The M_r (62 kD) and pH optimum (pH 4.0–4.5) of Arf are similar to those of the AF from *S. sclerotiorum* [11]. Other fungal AFs also have pH optima between pH 3 and pH 6 but vary in M_r from 53 kDa to 500 kDa, and in some cases are composed of several subunits [40]. Neither the purified, native Arf nor Xyp of *C. carbonum* had an M_r which would suggest a multiple-subunit structure; indeed, both enzymes were eluted from a gel-filtration column with M_rs less than their estimated M_rs by SDS-PAGE.

It was surprising that Arf is able completely to hydrolyze xylobiose to xylose (Table 3) and that the major products released after 2 h from oat spelt xylan by Arf are oligosaccharides (Fig. 9, lane 4). These results suggest that although Arf does not behave as a bifunctional AF/XP enzyme using *p*-NP substrates, it has AF and arabinanase activities against natural substrates and would be characterized as an XP by its ability to digest xylobiose. The AF of *S. sclerotiorum*, while sharing several physical properties with the *C. carbonum* Arf, was unable to degrade xylan, although it did release a relatively small amount of reducing sugar from galactan [11]. Xyp completely hydrolyzes xylobiose to xylose and releases xylose from several xylans and from wheat arabinoxylan (Table 3), confirming that the Xyp enzyme is a β-D-xylopyranosidase. Although Xyp has a small amount of activity against *p*-NP-α-L-arabinofuranoside, the enzyme does not release arabinose from oat spelt xylan (Fig. 9, lane 3), nor does it release reducing sugars from sugar-beet arabinan (Table 3). Unlike the β-glucosidase from *S. sclerotiorum* [29] which is associated with a β-xylosidase activity, Xyp has no detectable activity against *p*NP β-D-glucoside.

The Xyp enzyme, like the major xylanase of *C. carbonum*, has a blocked N-terminus. Peptides generated from Xyp by tryptic or AspN digestion show similarity to a bifunctional XP/AF from *B. ovatus*, supporting our conclusion that Xyp is a β-D-xylopyranosidase. We are currently in the process of cloning the genes for Xyp and Arf, which will permit the isolation of the genes encoding both activities and a subsequent test of their involvement in pathogenesis.

Acknowledgements

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