PERGAMON

Phytochemistry 59 (2002) 689–696

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

A β-glucosidase/xylosidase from the phytopathogenic oomycete, *Phytophthora infestans*

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Received 27 November 2001; received in revised form 17 January 2002

Abstract

An 85-kDa β-glucosidase/xylosidase (BGX1) was purified from the axenically grown phytopathogenic oomycete, *Phytophthora infestans*. The *bgx1* gene encodes a predicted 61-kDa protein product which, upon removal of a 21 amino acid leader peptide, accumulates in the apoplastic space. Extensive *N*-mannosylation accounts for part of the observed molecular mass difference. BGX1 belongs to family 30 of the glycoside hydrolases and is the first such oomycete enzyme deposited in public databases. The *bgx1* gene was found in various *Phytophthora* species, but is apparently absent in species of the related genus, *Pythium*. Despite significant sequence similarity to human and murine lysosomal glucosylceramidases, BGX1 demonstrated neither glucocerebroside nor galactocerebroside-hydrolyzing activity. The native enzyme exhibited glucohydrolytic activity towards 4-methylumbelliferyl (4-MU) β-D-glucopyranoside and, to lesser extent, towards 4-MU-D-xylopyranoside, but not towards 4-MU-β-D-glucopyranoside. BGX1 did not hydrolyze carboxymethyl cellulose, cellotetraose, chitosan or xylan, suggesting high substrate specificity and/or specific cofactor requirements for enzymatic activity.

Keywords: Chromista; Glycoside hydrolase; β-Glucosidase/xylosidase; Mannosylation; Oomycete; *Phytophthora infestans*

1. Introduction

Various species of the phytopathogenic oomycete genera *Phytophthora*, *Peronospora* and *Pythium* cause devastating diseases of crop plants (Govers et al., 1997; Kamoun et al., 1999). For example, *Phytophthora infestans* is the causal agent of potato late blight and is considered one of the most costly biotic constraints to global food production (Fry and Goodwin, 1997). The molecular analysis of oomycete pathogenicity is believed to reveal targets for phytotherapeutic intervention and, hence, may provide the basis for efficient disease control on crop plants (Fry, 1996; Knogge, 1998; Kamoun et al., 1999).

Hydrolytic enzymes secreted by phytopathogenic fungi are believed to facilitate penetration of the plant cuticle and cell wall and subsequent colonization of host plants. Such enzymes include cutinases, cellulases, glycosidases, pectinases, and proteases (Kolattukudy et al., 1995; Knogge, 1996, 1998). However, individual hydrolytic enzymes have been shown to be dispensible for fungal pathogenicity on host plants (Stahl and Schäfer, 1992; Apel-Birkhold and Walton, 1996; Murphy and Walton, 1996; Görlach et al., 1998; Scott-Craig et al., 1998; Wegener et al., 1999), suggesting that a 'cocktail' of hydrolytic enzyme activities with overlapping substrate specificities may contribute to the establishment of proper infection structures (Knogge, 1996, 1998).

Like numerous phytopathogenic fungi, *P. infestans* forms specialized penetration organs, called appressoria, at the tip of growing hyphae (Erwin and Ribeiro, 1996; Govers et al., 1997). Appressorium formation is followed by invagination of the plant plasma membrane and subsequent formation of an infection peg and

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0031-9422/02/$ - see front matter
PII: S0031-9422(02)00045-6

haustoria. In comparison to phytopathogenic fungi, however, much less is known about the role in pathogenicity of hydrolytic enzymes produced by phytopathogenic oomycetes, being most closely related to brown algae (Govers et al., 1997).

In contrast to transformation-mediated gene disruption and gene replacement strategies developed for fungi, functional analysis of oomycete enzymes and their encoding genes has been significantly hampered by the lack of efficient transformation technologies. However, recently, transformation of the diploid oomycete, *P. infestans*, with antisense, sense, and promoter-less constructs of the coding sequence of the elicitin gene, *inf1*, resulted in inheritable transcriptional silencing of both the transgene and the endogenous gene (van West et al., 1999). Thus, a systematic survey of secreted oomycete hydrolytic enzyme activities and their possible implication in pathogenicity has become feasible. To investigate the role of such enzymes in the biology of the oomycete, we have isolated and characterized a mixed function β-glucosidase/xylosidase (BGX1) from in vitro grown *P. infestans*.

2. Results

The aryl β-glycoside, 4-methylumbelliferyl-β-D-glucopyranoside (MUG), was used as substrate to monitor purification of glucohydrolytic enzymes (Daniels and Glew, 1984) from the culture filtrate of the in vitro grown oomycete, *P. infestans*. A combination of DE52-cellulose anion exchange chromatography and phenyl superose hydrophobic interaction chromatography yielded a homogenous preparation of an 85-kDa protein (BGX1) (Fig. 1, lane 1). Protein microsequencing of the N-terminus and of internal peptides (Fig. 2) enabled isolation of the encoding gene by PCR using *P. infestans* genomic DNA as template. A 1-kb product was used to screen a cDNA library prepared from mycelial RNA. Nine cDNAs differing in length were isolated which, on sequencing, were found to be identical. All cDNA clones contained 3′ ends identical to that of the PCR product used for screening. The largest of these clones (2.0 kb, GenBank accession No. AF352032, *bgx1*) contained an open reading frame (ORF) of 572 codons, a 180 bp 3′-untranslated region and a 6 bp poly(dA) stretch (Fig. 2). This clone hybridized to a 2.2-kb mRNA when used as a probe in RNA blot analyses (Fig. 3 A). Hybridization of a 1.4-kb PstI-BamHI fragment (Fig. 2) to *P. infestans* genomic DNA showed that BGX1 appears to be encoded by a single gene (Fig. 3 B). Digestion of genomic DNA with the restriction endonuclease, SacI, produced two hybridizing bands, which is consistent with a SacI cleavage site within the 2.0-kb clone (Fig. 2). DNA blot analyses with genomic DNA from various oomycete species revealed that this gene is a feature of the genus *Phytophthora*, but is apparently not present or structurally different in the taxonomically related genus, *Pythium* (Fig. 3 C). Absence of the gene in *P. undulata*, previously named *Pythium undulatum* (Dick, 1989), may be explained by the close evolutionary relationship of this oomycete with *Pythium* species.

The *N*-terminus of BGX1 corresponded to codon 22 of the ORF (Fig. 2), which is consistent with a 21 amino acid leader peptide upstream of the mature *N*-terminus. This sequence exhibited the hallmarks of a eukaryotic signal peptide, such as a hydrophobic core region and two small uncharged amino acids at positions-3 and -1 reminiscent of a signal peptidase cleavage site (von Heijne and Abrahmsen, 1989). The protein encoded by the 2.0-kb cDNA had a predicted pI of 4.2, which is in agreement with the value of 3.5 as determined by isoelectric focusing (not shown). The deduced molecular mass of 60,680 Da was substantially smaller than 85,000 Da as calculated on the basis of the electrophoretic mobility of BGX1. Five putative *N*-glycosylation sites were found in the translated protein sequence (Fig. 2), suggesting that glycosylation may account for the discrepancy in molecular masses. An enzyme immunoassay employed for the detection of sugars in glycoconjugates revealed glycosylation of BGX1 (Fig. 1, lane 2). Plant lectins recognizing different carbohydrate moieties of glycoproteins were utilized to elucidate the nature of

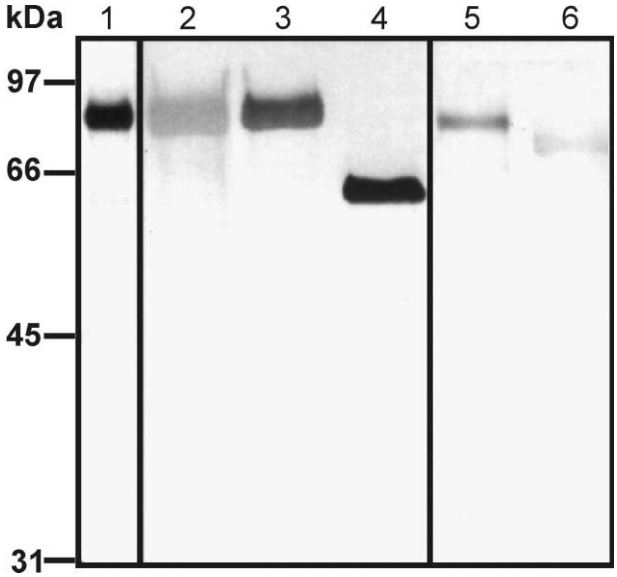

Fig. 1. Isolation and molecular characterization of BGX1. BGX1 was purified to apparent homogeneity and analyzed as described in Experimental. Purified BGX1 (1 μg) was separated by SDS–PAGE and stained with silver (lane 1), or transferred to nitrocellulose (lanes 2, 3 and 5, 6) for enzyme-linked immunodetection of the carbohydrate moiety (lane 2) and immunochemical detection of mannosylation with *Galanthus nivalis* agglutinin (lanes 3, 5). Carboxypeptidase Y (0.5 μg) was used as positive control for protein mannosylation (lane 4). *N*-Glycosidase F-digested BGX1 (lane 6). Size of formula weight markers are indicated on the left.

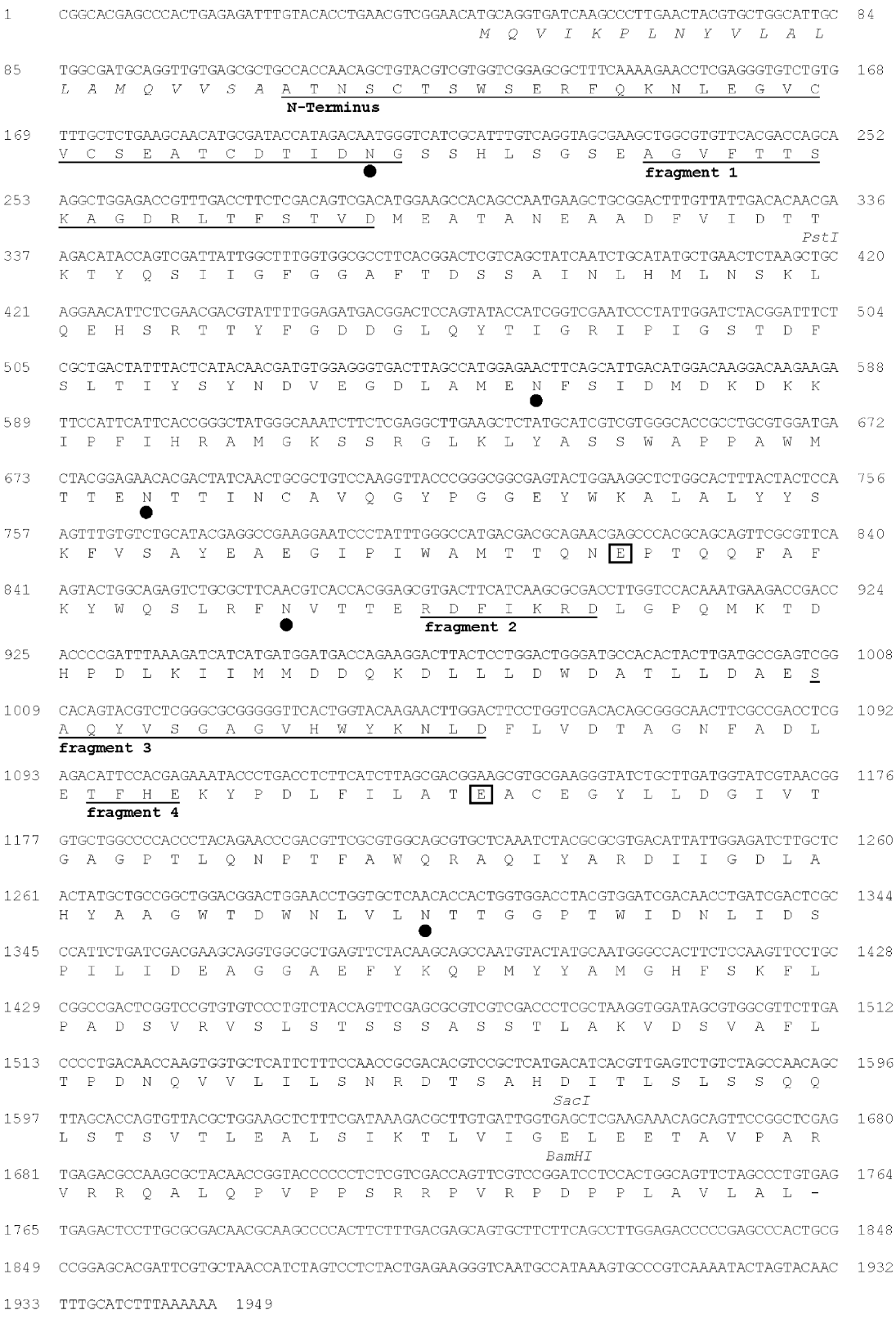

Fig. 2. Nucleotide sequence of the BGX1-encoding cDNA and deduced amino acid sequence. Locations of relevant restriction sites are indicated. Closed circles denote potential *N*-glycosylation sites. The *N*-terminal leader peptide is given in italics. Underlined protein sequences of the mature *N*-terminus and of BGX1 fragments (1–4) generated by endoproteinase Glu-C treatment were determined by Edman degradation. Boxed glutamic acid residues (E258, E365) refer to the catalytic proton donor and catalytic nucleophile as inferred from alignment of BGX1 with related enzymes of glycoside hydrolase clan, CH-A (Henrissat, 2001). The nucleotide and amino acid sequences have been assigned GenBank accession number AF352032.

carbohydrate side chain. Among the six lectins tested, only *Galanthus nivalis* agglutinin strongly recognized the 85-kDa protein (Fig. 1, lane 3), which suggested mannosylation of BGX1 similar to that reported for various yeast glycoproteins (Shibuya et al., 1988). *N*-Glycosidase F treatment reduced the molecular size of BGX1 by approximately 12 kDa, indicating sizable asparagine-linked *N*-glycosylation of the protein (Fig. 1, lanes 5, 6). *O*-Glycosidase treatment did not alter the molecular mass of the protein (not shown). Gel filtration analysis of the protein revealed a molecular mass of 154 kDa, suggesting a homodimeric subunit structure of native BGX1 (not shown).

A nomenclature system, which classifies all known glycoside hydrolases in 82 families according to their primary sequence homologies as well as their 3-D structures (Henrissat and Davies, 1997; Czjzek et al., 2000; Henrissat, 2001), grouped BGX1 into glycoside hydrolase family 30. Glycoside hydrolases with significant homology to BGX1 were from species being as diverse as *Caenorhabditis elegans*, human, mouse, or *Caulobacter crescentus*. Highest homology (37% identity over 470 aa) was found to a putative glycoside hydrolase from *C. elegans* (AF125971). Similar degrees of homology were found to acid β-glucosidases (glucosylceramidase, EC 3.2.1.45) from mouse (36% identity over 473 aa, M24119), human (34% identity over 529 aa, AF023268), and a glycoside hydrolase from *C. crescentus* (27% identity over 425 aa, AE005849). Since the fold of proteins is better conserved than their sequences, several glycoside hydrolase families can be grouped in 11 clans (Henrissat, 2001). According to this, BGX1 belongs to clan GH-A, of which members are characterized by a $(\beta/\alpha)_8$ fold structure and two glutamic residues considered to form the active site of these enzymes (catalytic proton donor and catalytic nucleophile). From the alignment of BGX1 with enzymes from families for which the catalytic function of these residues has been experimentally shown, residues E258 and E365 (Fig. 2) were inferred to constitute the active site of BGX1.

Sequence homology to mouse and human glucosylceramidase prompted us to investigate such a substrate specificity of purified BGX1. However, neither one of five glucocerebroside preparations [*A. fumigatus* GlcCer (Cer = d18:2 [4*E*,8*E*]-9-methyl-4,8-sphingadienine + 2-hydroxy fatty acids); *A. fumigatus* GalCer (Cer = d18:2 [4*E*,8*E*]-9-methyl-4,8-sphingadienine + 2-hydroxy fatty acids); Gauchers spleen GlcCer (Cer = d18:1 sphingosine + non-hydroxy fatty acids); bovine brain GalCer (Cer = d18:1 sphingosine + 2-hydroxy fatty acids); bovine brain GalCer (Cer = d18:1 sphingosine + non-hydroxy fatty acids) tested in a liposomal assay system with or without phosphatidylinositol (Sarmientos et al., 1986) was hydrolyzed by BGX1. In addition, conduritol B epoxide, which completely inactivates glucosylceramidases at micromolar concentrations (Daniels and Glew, 1984), reduced MUG-hydrolyzing activity of the *P. infestans* enzyme by 50% only at a concentration of 1 mM (not shown). Inhibitory effects at millimolar conduritol B epoxide concentrations were reported for acid β-glucosidases

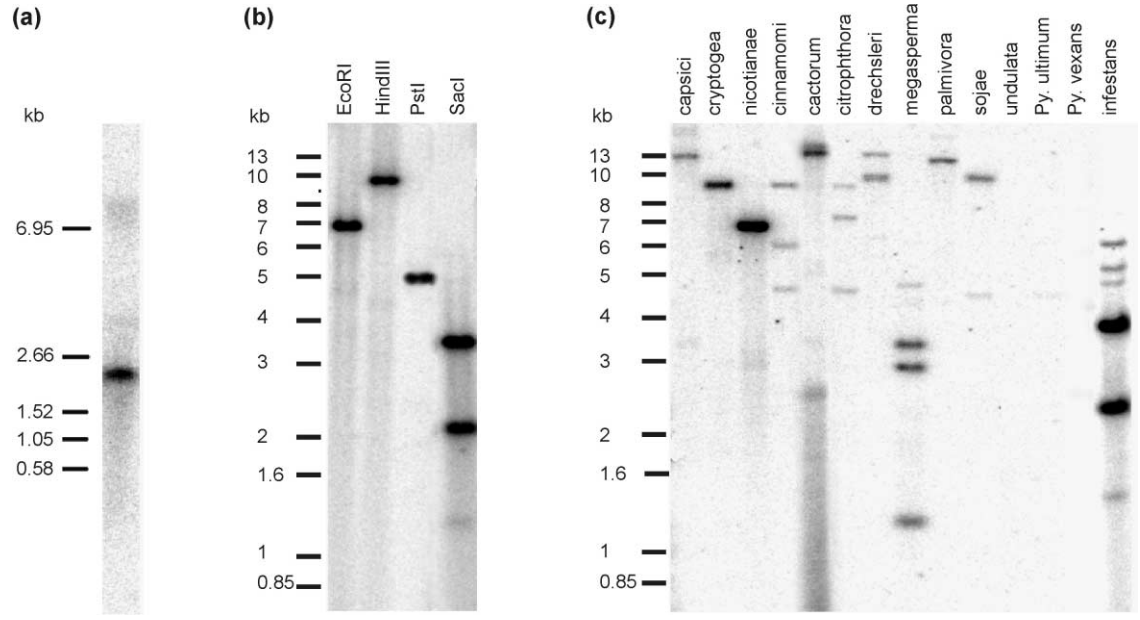

Fig. 3. RNA and DNA blot analysis of the BGX1-encoding gene, *bgx1*. (A) Poly (A^+) RNA (2 μg) prepared from *Phytopthora infestans* mycelium was separated on agarose, transferred onto a nylon membrane, and hybridized with a [^{32}P]-α-dATP-labeled 1.4 kb PstI-BamHI fragment of *bgx1* (Fig. 2). (B, C) Genomic DNA (2 μg) prepared from *P. infestans* (B) or the *Phytophthora* and *Pythium* (Py.) species given in (C), respectively, was digested as indicated (B) or with SacI (C), separated on agarose, and transferred onto nylon membranes. Hybridization was performed as in (A).

Table 1
Substrate specificity of purified BGX 1

Substrate	Specific activity (nkat/g)
4-MU-β-D-glucopyranoside	2800.0
4-MU-α-D-glucopyranoside	2.8
4-MU-β-D-xylopyranoside	195.0
4-MU-β-D-galactopyranoside	14.2
4-MU-β-D-mannopyranoside	2.1
4-MU-β-D-lactopyranoside	1.8
4-MU-*N*-acetyl-β-D-glucosaminide	2.4
4-MU-β-D-glucuronide	0.0

different from glucosylceramidases (Daniels and Glew, 1984; Legler and Bieberich, 1988; Li et al., 2001).

A series of 4-methylumbelliferyl glycosides was employed to study the substrate specificity of BGX1. MUG was cleaved most efficiently, but 4-MU-β-D-xylopyranoside was also hydrolyzed (Table 1). In contrast, hydrolysis of the related mannopyranoside, galactopyranoside, glucosaminide, or glucuronide was not observed. Stereoselectivity was detected as the enzyme did not hydrolyze the α-anomer, 4-MU-α-D-glucopyranoside. MUG was used as substrate for the kinetic characterization of enzyme activity. β-Glucosidase activity was maximal between pH 4.0 and 6.0, and the temperature optimum of enzyme activity was found to be at 50 °C (not shown). The K_M values determined were 0.32 mM (Hanes) or 0.76 mM (Lineweaver–Burk), respectively. Maximum reaction velocity and specific enzyme activities were 2.8 pkat or 2.8 μkat/g (Hanes) and 2.9 pkat or 2.9 μkat/g (Lineweaver-Burk), respectively (not shown). The purified enzyme did not catalyze the transfer of UDP-glucose to *p*-nitrophenol, suggesting that it does not act as a glucosyl transferase.

To search for potential natural substrates of BGX1 we tested the following compounds: arabinogalactan, arabinoxylan, cellobiose, cellotetraose, chitosan, citrus pectin, carboxymethylcellulose, galactomannan, laminarin, mannan, polygalacturonic acid, starch, xylan and *Tamarindus indica* xyloglucan. However, none of these potential oomycete or plant cell wall-derived components was hydrolyzed (not shown).

3. Discussion

O-Glycoside hydrolases (EC 3.2.1.-) selectively hydrolyze the glycosidic bond in oligosaccharides, polysaccharides and their conjugates (Henrissat and Davies, 1997; Henrissat, 2001). Among the more than 1000 glycoside hydrolases from bacteria, fungi, plants, non-vertebrates and vertebrates deposited in public databases, no such activity of oomycete origin has been reported. Originally considered fungi, oomycetes differ from all other taxa in the kingdom fungi, and are now categorized in a separate monophyletic clade, named chromista (Govers et al., 1997). Here we show that *P. infestans* produces and secretes significant amounts of β-glucosidase activity (EC 3.2.1.21). BGX1 cleaves β-glycosidic, but not α-glycosidic bonds in synthetic aryl glycosides. β-glucosidases, which constitute a major group among glycoside hydrolases (Henrissat and Davies, 1997; Henrissat, 2001), catalyze the hydrolysis of terminal, non-reducing β-D-glucose residues often with a specificity for several β-D-glycosides. Similarly, BGX1 hydrolyzed 4-MU-β-D-glucopyranoside and 4-MU-β-D-xylopyranoside. β-glucosidases possessing both activities are known in other organisms as well, but the ratios of the two activities vary greatly (McCleary and Harrington, 1988; Yasui and Matsuo, 1988; Vroemen et al., 1995). The well-characterized specificity towards artificial substrates contrasts often largely with the limited knowledge on the natural substrates of β-glucosidases. On the other hand, the wealth of potential natural substrates makes sensible predictions rather difficult. Our failure to identify a native substrate of BGX1 among 15 oomycete or plant-derived compounds may reflect (i) the high substrate specificity of the enzyme, or (ii) specific cofactor requirements for enzymatic activity and thus inadequate assay conditions. Precedence for that was provided by a β-glucosidase/xylosidase from the plant pathogenic bacterium, *Erwinia chrysanthemi*, which also did not hydrolyze carboxymethyl cellulose or xylan (Vroemen et al., 1995). It is further conceivable that modification of carbohydrate chains through removal of lateral sugar residues or aglycone release may be the intrinsic function of such enzymes. Thus, in order to identify the native substrate of BGX1 a very broad spectrum of potential substrates needs to be tested.

Alignment of the BGX1 primary sequence with more than 1000 glycoside hydrolase sequences compiled in a specific database (Henrissat, 2001) revealed homology to human and murine glucosylceramidases (glucocerebrosidase; EC 3.2.1.45) (Sorge et al., 1985; O'Neill et al., 1989), a particular class of β-glucosidases which hydrolyze glucosylceramides (Sandhoff and Kolter, 1996). Both glucosylceramides and ceramides, the substrates and products of glucosylceramidase action, have been found in oomycete plant pathogens (Lhomme et al., 1990; Moreau et al., 1998). However, several lines of evidence argue against glucosylceramide-hydrolyzing activity of BGX1. (i) Glucosylceramidases are involved in lysosomal degradation of plasma membrane-derived glycosphingolipids after endocytotic internalization (Sandhoff and Kolter, 1996). Thus, these enzymes are located intracellularly, while BGX1 was primarily secreted to the extracellular medium. (ii) Several glucosylceramide preparations were tested as substrates in a liposomal assay system (Sarmientos et al., 1986), but neither experimental conditions provided evidence for

ceramide-releasing activity of BGX1. It should be noted that human glucosylceramidase activity requires specific, heat-stable sphingolipid activator proteins as cofactors in vivo (Sandhoff and Kolter, 1996; Linke et al., 2001). Hence, it cannot be ruled out that yet unknown oomycete activator proteins are required for glucocerebroside hydrolysis by BGX1. However, neither untreated nor heat-treated *Phytophthora*-derived cell extracts restored glucosylceramidase activity when added to the reaction mix (not shown). (iii) As β-glucosidase activity, glucosylceramidase activity can be assayed by using the non-physiological substrate MUG under specified conditions (Daniels and Glew, 1982). MUG-hydrolyzing activity of BGX1, however, did not require detergents or lipids for catalysis. (iv) Glucosylceramidases are inhibited completely and irreversibly by the active-site reagent, conduritol B epoxide, at micromolar concentrations (Daniels and Glew, 1984). BGX1 activity was affected only at concentrations of the inhibitor similar to those reported to affect β-glucosidase activity (Daniels and Glew, 1984; Legler and Bieberich, 1988; Li et al., 2001).

Identification of the intrinsic function of the *P. infestans*-derived enzyme, its role during the life cycle of oomycetes and/or its possible implication in oomycete pathogenicity will be most appropriately addressed by inactivation of the encoding gene. Isolation of *bgx1* and establishment of efficient transformation technologies for diploid oomycetes (van West et al., 1999) now provide the tools for this experiment.

4. Experimental

4.1. Purification of BGX1

P. infestans race 4 (mating type A1, isolate 88069 from tomato, Agricultural University Wageningen, The Netherlands) was grown on synthetic medium (Henniger, 1959) for 4–6 weeks at 16 °C in the dark. Preparation of secreted proteins from the *P. infestans* culture filtrate was performed as described (Rohwer et al., 1987). Proteins were applied to a DE52-cellulose column (Whatman International Ltd., Maidstone, UK) equilibrated in 50 mM Tris–HCl, pH 8.0, and bound protein was eluted with a gradient of 0–0.5 M KCl in the same buffer. Fractions containing BGX1 activity were pooled, adjusted to 1.5 M $(NH_4)_2SO_4$ and subjected to hydrophobic interaction fast protein liquid chromatography (FPLC) on phenyl superose (Amersham Pharmacia, Freiburg, Germany) equilibrated with 1.5 M $(NH_4)_2SO_4$ in 50 mM Tris–HCl, pH 8. BGX1 was eluted with a linear gradient of 0–100% 50 mM Tris–HCl, pH 8. Gel filtration FPLC analysis of BGX1 was performed on Superose 12 (Amersham Pharmacia) in 50 mM Tris–HCl, pH 8, 150 mM NaCl.

4.2. Protein biochemistry

SDS-polyacrylamide gelelectrophoretic separation of proteins and silver staining was performed as described (Blum et al., 1987). Transfer of proteins onto nitrocellulose was carried out in a semi-dry blotting device (Biotech Fisher, Schwerte, Germany) in 39 mM glycine, 48 mM Tris–HCl, pH 8.3, 0.037% SDS, 20% methanol for 2 h at 1 mA/cm^2. Analysis of the carbohydrate moiety of BGX1 was performed with digoxygenin glycan detection and digoxygenin differentiation kits according to the supplier's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

BGX1 (2 μg) was deglycosylated by either 4 U *N*-glycopeptidase F (Roche Diagnostics) in 200 μl of 100 mM sodium phosphate, pH 7.0, 25 mM EDTA, 0.5% Nonidet P-40, 1% β-mercaptoethanol, or *O*-glycosidase treatment (5 mU, Roche Molecular Biochemicals) in 200 μl 20 mM sodium phosphate, pH 7.2, for 16 h at 37 °C. BGX1 was boiled for 5 min in 20 μl 1% SDS prior to enzymatic deglycosylation.

To generate internal peptide fragments, BGX1 (20 μg) was treated with 4 μg endoproteinase Glu-C (Roche Molecular Biochemicals, 25 °C, 24 h) in 100 μl 25 mM NH_4HCO_3. Peptides were fractionated by reverse-phase HPLC as described in (Nürnberger et al., 1994). *N*-Terminal sequencing was performed by Edman degradation on a G1050A protein sequencer according to the manufacturer's protocol (Hewlett-Packard, Palo Alto, California).

4.3. DNA and RNA techniques

Genomic DNA from mycelia of various oomycete species was isolated according to Pieterse et al. 1996. Restriction endonuclease digestion of genomic DNA, gelelectrophoretic DNA separation on 1% agarose, DNA transfer onto Hybond-N membranes (Amersham Pharmacia), and hybridization with a [^{32}P]-α-dATP-labeled 1.4 kb PstI-BamHI fragment of *bgx1* was performed as described in (Sambrook and Russell, 2001). *P. infestans* poly(A)$^+$-RNA was purified from 100 μg total RNA (Dunsmuir et al., 1989) using oligo(dT) agarose (Amersham Pharmacia). Agarose gel electrophoresis, RNA transfer onto Hybond-*N* membrane, and hybridization with the 1.4 kb PstI-BamHI *bgx1* fragment was carried out as in (Sambrook and Russell, 2001). The last wash was performed with 0.1×SSC at 68 °C.

4.4. Isolation of bgx1

Total *P. infestans* RNA (50 μg), 1 μM 5′-TCTAGAACTAGTGGATCAAGCT$_{17}$-3′ primer and 10 U RNase inhibitor were dissolved in a total volume of 12.5 μl water, denatured for 10 min at 70 °C, and cooled on

ice. After addition of 4 μl 5×Superscript first strand buffer (Gibco BRL, Paisley, UK), 2 μl 0.1 M DTT, and 1 μl 10 mM dNTP-Mix the mix was heated to 37 °C, and, upon addition of 1 μl Superscript II reverse transcriptase (Gibco BRL), incubated at 37 °C for 1 h. PCR amplification was performed in 50 μl aliquots of PCR buffer (Appligene Oncor, Heidelberg, Germany) containing 2 μl of the reverse transcriptase reaction, 100 pmol oligonucleotide primer 5′-TCTAGAACTAGTGGATCAAGC-3′, 100 pmol degenerate oligonucleotide primer 5′-GCN CAA/G TAC/T GTN A/TG/CN GGN GCN GGN GT-3′ encoding amino acids (one-letter code) AQY VSG AGV derived from *bgx1* fragment 3 (Fig. 2), 200 nmol dNTP, and 2 U Taq-polymerase. The following PCR conditions were used: 4 min, 95 °C; 30 cycles (1 min, 95 °C; 1 min, 50 °C; 1 min, 74 °C); 1 cycle (1 min, 95 °C; 1 min, 50 °C; 10 min, 74 °C). The 1-kb PCR fragment was subsequently cloned into linearized pCRII (Invitrogen, Leek, The Netherlands). To isolate *bgx1* full-length cDNAs, a total of 500,000 plaques of a *P. infestans* λ-ZAPII cDNA library (Pieterse et al., 1994) was screened with the digoxigenin-dUTP-labeled 1-kb PCR fragment obtained by PCR with sequence-specific oligonucleotide primers. Positive plaques were isolated, subjected to in vivo excision of pBluescript SK- (Stratagene, Heidelberg, Germany), and the cloned inserts characterized by DNA sequencing (Sambrook and Russell, 2001).

4.5. Enzymatic assays

4-Methylumbelliferyl glucopyranoside-hydrolyzing activity was assayed as described (Daniels and Glew, 1984) using 1.1 mM 4-methylumbelliferyl glucopyranoside (or compounds listed in Table 1) in 220 mM sodium acetate, pH 5.5. Enzyme reactions (250 fkat BGX1, 1 h, 37 °C) were terminated by addition of 100 μl 0.4 M sodium carbonate, and fluorescence of 4-methylumbelliferone was quantified (460 nm emission wavelength) in a Cytofluor II fluorescence microplate reader (Millipore, Dassel, Germany). β-Glucosidase activity on plant and fungal cell wall polysaccharides was performed by determining the generation of reducing groups with the PABAH assay (Lever, 1972). Reaction mixtures contained 47 μl polysaccharide solution and 3 μl BGX1 solution (250 fkat BGX1 in 0.1% w/v sodium acetate, pH 5.5, 0.02% w/v sodium azide) or buffer as control. Reactions were incubated for 1 h at 37 °C, followed by spectrophotometric quantification (405 nm) of the absorbance of the reaction products.

Acknowledgements

We are grateful to Dr. Gundo Wilkening and Professor Konrad Sandhoff (University Bonn, Germany) for performing glucosylceramidase activity assays with purified BGX. Many thanks go to Dr. Sabine Rosahl for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Nu 70/2–2), Kleinwanzlebener Saatzucht AG Einbeck, and Fonds der Chemischen Industrie.

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