The *Pseudomonas cellulosa* glycoside hydrolase family 51 arabinofuranosidase exhibits wide substrate specificity

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To investigate the mechanism by which *Pseudomonas cellulosa* releases arabinose from polysaccharides and oligosaccharides, a gene library of *P. cellulosa* genomic DNA was screened for 4-methylumbelliferyl- α -L-arabinofuranosidase (MUAase) activity. A single MUAase gene (*abf51A*) was isolated, which encoded a non-modular glycoside hydrolase family (GH) 51 arabinofuranosidase (Abf51A) of 57000 Da. The substrate specificity of the Abf51A showed that it preferentially removed α 1,2- and α 1,3-linked arabinofuranose side chains from either arabinan or arabinoxylan, and hydrolysed α 1,5-linked arabino-oligosaccharides, although at a much lower rate. The activity of Abf51A against arabinoxylan was similar to a GH62 arabinofuranosidase encoded by a *P. cellulosa* gene. Glu-194 and Glu-321 of Abf51A are conserved in GH51 enzymes, and it has been

suggested that these amino acids comprise the key catalytic acid/base and nucleophile residues, respectively. To evaluate this hypothesis the biochemical properties of E194A and E321A mutants of Abf51A were evaluated. The data were consistent with the view that Glu-194 and Glu-321 comprise the key catalytic residues of Abf51A. These data, in conjunction with the results presented in the accompanying paper [Beylot, Emami, McKie, Gilbert and Pell (2001) Biochem. J. 358, 599–605], indicate that *P. cellulosa* expresses a membrane-bound GH51 arabinofuranosidase that plays a pivotal role in releasing arabinose from a range of polysaccharides and oligosaccharides.

Key words: arabinose, catalytic mutant.

INTRODUCTION

The plant cell wall is a composite structure consisting mainly of a complex mixture of structural polysaccharides. L-Arabinose is a common component of these polysaccharides, and is present at high concentrations in arabinans, arabinoxylans and arabinogalactans [1]. The arabinan and arabinoxylan backbone comprise α 1,5-linked arabinofuranose units and β 1,4-linked xylopyranose moieties, respectively. Both polysaccharides are decorated at C-2 and/or C-3 with arabinofuranose molecules [1]. The release of arabinose side chains by arabinofuranosidases from both hemicellulose and arabinan is a critical step in the microbial degradation of these polysaccharides [2]. At least four types of arabinofuranosidase remove arabinose side chains from hemicellulose and pectic polymers. Arabinofuranosidases can exhibit wide substrate specificity and remove the furanose sugars from arabinans and arabinoxylans substituted at C-2 or C-3 [3], display restricted specificity and attack only the side chains of arabinan, exemplified by α -L-arabinofuranosidase I from *Streptomyces chartreusis* [4], or hydrolyse exclusively the glycosidic bonds between arabinofuranose units and the xylan backbone of arabinoxylans [5]. All three arabinofuranosidases act only on polysaccharides substituted at C-2 or C-3, although an arabinofuranosidase that preferentially removes arabinose molecules from xylose moieties which are substituted by the furanose sugar at both C-2 and C-3 has been described [6]. The arabinan backbone that is free of side-chain constituents is hydrolysed by endo- α 1,5-arabinanases, which can act as typical endo-enzymes, generating a mixture of arabino-oligosaccharides [2]. The α 1,5-

linked arabino-oligosaccharides generated can be hydrolysed by the general-acting arabinofuranosidases, although exo-acting enzymes that specifically remove arabinose moieties from these oligosaccharides have been described [4].

Arabinofuranosidases have been grouped into glycoside hydrolase families (GHs) based on primary-structure similarities and hydrophobic cluster analysis [P. M. Coutinho and B. Henrissat (2000) Carbohydrate-Active Enzymes Server at <http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>]. These enzymes are located in GH43, GH51, GH54 and GH62. In general, the enzymes in GH43 and GH62 hydrolyse unsubstituted α 1,5-arabinan/ α 1,5-arabino-oligosaccharides [4,7] and arabinoxylans, respectively [8], whereas the GH51 and GH54 arabinofuranosidases are believed to exhibit more relaxed substrate specificity [9].

The bacterium *Pseudomonas cellulosa* degrades all the major plant structural polysaccharides, and expresses an extensive repertoire of glycoside hydrolases that cleave the backbones of these polysaccharides. However, the mechanism by which the bacterium removes the arabinose side chains from hemicellulose and arabinan is unclear. A *P. cellulosa* gene (*abf62A*; formerly *xynC*) that encodes a GH62 arabinofuranosidase (Abf62A, formerly XylC) has been described [8]. Abf62A attacks arabinoxylan but its specificity for the different linkages in arabinoxylan is unknown. Given that GH62 enzymes are generally specific for arabinoxylan, it is unclear whether Abf62A exhibits unusually wide substrate specificity and removes arabinose side chains from arabinan as well as hemicellulose, or whether the bacterium synthesizes additional arabinofuranosidases that attack the pectic

Abbreviations used: GH, glycoside hydrolase family; MUA, 4-methylumbelliferyl- α -L-arabinofuranoside; MUAase, 4-methylumbelliferyl- α -L-arabinofuranosidase; LB, Luria–Bertani broth; IPTG, isopropyl β -D-thiogalactoside; HPAEC, high-performance anion-exchange chromatography; ORF, open reading frame.

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The nucleotide sequence data reported for *abf51A* and flanking sequences will appear in the GenBank® Nucleotide Sequence Databases under the accession number AY043167.

polysaccharide. This report and the accompanying paper [10] use biochemical and genetic approaches, respectively, to dissect the mechanism by which *P. cellulosa* removes arabinose side chains from hemicellulose and arabinan. In this paper we show that *P. cellulosa* contains a second arabinofuranosidase gene (*abf51A*) that encodes a GH51 enzyme (Abf51A). The biochemical properties of Abf51A indicate that it is capable of removing arabinose side chains from a range of polysaccharides, and questions the functional significance of Abf62A in *P. cellulosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The *Escherichia coli* strains used in this study were, BL21(DE3):pLysS (Novagen) and XL1-Blue (Stratagene). The bacteriophage employed was λ ZAPII (Stratagene). The plasmid vectors used were as follows: pBluescript SK⁻ (Stratagene), pCR-Blunt (Invitrogen), pET19b and pET21a (Novogen). All *E. coli* strains containing recombinant plasmids were cultured in Luria-Bertani broth (LB) supplemented with 50 μ g/ml ampicillin or kanamycin, as appropriate, at 37 °C unless otherwise stated. *E. coli* cells used to propagate bacteriophage were grown on LB supplemented with 10 mM MgSO₄ and 0.2 % maltose, and were plated out on NZYM top agar (0.7 %). To screen the library of *P. cellulosa* DNA constructed in λ ZAPII, approx. 40 000 recombinant phage were plated out on NZYM top agar at a density of 3 plaques/cm². After incubation at 37 °C for 24 h, the plates were overlaid with 4 ml of 0.7 % agarose containing 4 mM 4-methylumbelliferyl- α -L-arabinofuranoside (MUA), and incubated at room temperature for up to 30 min. The plates were inspected regularly under UV light for fluorescent haloes surrounding recombinant plaques, which is indicative of MUA hydrolysis. To generate the *P. cellulosa* proteins encoded by pET-based expression vectors, recombinant strains of *E. coli* BL21(DE3) harbouring pLysS were cultured to the mid-exponential phase (A_{550} , 0.5) in LB containing ampicillin at 30 °C, after which time isopropyl β -D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and the cultures were incubated for a further 4 h at 30 °C.

Recombinant DNA techniques

Standard recombinant DNA techniques such as Southern and Northern hybridization, plaque hybridization, bacterial transformation, agarose gel electrophoresis, plasmid DNA preparation, restriction digestion and ligation were as described previously [11]. The nucleotide sequence of DNA was determined using an ABI Prism[®] Ready Reaction DyeDeoxy[™] Terminator Cycle Sequencing Kit. The sequencing reactions were electrophoresed, and the fluorescent signals emitted by the DNA molecules were detected using an Applied Biosystems 377A Sequencing System. The complete sequence of the DNA (both strands) was determined using a series of custom-made primers to initiate DNA synthesis. The compiled DNA sequence was analysed using the DNAsis computer program. To generate mutants of the arabinofuranosidase gene the QuikChange kit supplied by Stratagene was used. The mutagenic primers employed to generate the two mutations were as follows (the nucleotides in bold comprise the introduced mutations): E194A, 5'-GCAATTGGTAATGCGGCGTGGGGCTGC 3' and 5'-GCTGCCACGCGCATTACCAATTGC-3'; E321A, 5'-TCT-ATGTGGACGCGTGGGGCACCTGG-3' and 5'-CCAGGTGCCACGCGTCCACATAGA-3'.

Construction of recombinant plasmids

DNA sequences encoding full-length and mature forms of the *Pseudomonas* arabinofuranosidase were amplified by PCR using the following primer pairs. Full-length gene, 5'-GCGCATATGCGCCGTTTGAACCCCTC-3' and 5'-GCGCTCGAGTTCACCCGCAACCACACC-3'; gene encoding the mature arabinofuranosidase (nucleotides 64–1551), 5'-CGCCATATGAATACCCACATCACCATAGAT-3' and 5'-GCGGGATCCGCGCCCTATTCCACCCGCAAC-3'.

PCRs were performed using 2 units of the thermostable DNA polymerase Vent[®] (New England Biolabs). The reactions contained 2 mM MgSO₄, 1 $\times$ Thermopol Reaction Buffer (New England Biolabs), 0.5 μ M primers and 60 ng of target DNA in a final volume of 100 μ l. The reactions were subject to 25 cycles at the following temperatures: 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The resultant DNA was cloned into pCR-Blunt and sequenced to ensure that no mutations had occurred during amplification. To clone DNA sequences into pET21a, amplified DNA (full-length *abf51A*) and the pET vector were digested with *Nde*I and *Xho*I and ligated together. To clone DNA sequences into pET19b, amplified DNA (truncated *abf51A* encoding mature Abf51A) and the pET vector were digested with *Nde*I/*Bam*HI and ligated together. The different forms of Abf51A encoded by recombinant plasmids derived from pET21a and pET19b contained C-terminal and N-terminal His tags, respectively.

Purification of proteins

Cell-free extracts of *E. coli* strains, in which the expression of recombinant proteins had been induced with IPTG, were prepared as described previously [12]. The proteins, which contain His tags, were purified by metal-ion affinity chromatography, using a Talon[™] matrix, as described previously [12]. The purity of enzymes subject to affinity chromatography was evaluated by SDS/PAGE [13].

Assays

Protein was measured by the dye-binding method of Sedmak and Grossberg using BSA as the standard [14]. CD spectroscopy was performed as described previously [15]. Arabinofuranosidase activity was measured using several different substrates. To assay against 4-nitrophenyl-glycosides, the substrate, at a final concentration of 1 mM in 50 mM sodium phosphate buffer, pH 7.0, was incubated with the enzyme at 37 °C and the increase in A_{400} was measured against time. The amount of product generated was calculated using a molar extinction coefficient for 4-nitrophenol at pH 7.0 of 10 500 M⁻¹·cm⁻¹ [15]. To measure the activity of the arabinofuranosidase against polysaccharides and oligosaccharides, the release of arabinose was determined using high-performance anion-exchange chromatography (HPAEC). Briefly, the enzyme (1–500 μ g) was incubated with appropriate substrates for up to 16 h. At regular intervals aliquots were removed, boiled for 10 min to inactivate the enzyme, and then subjected to HPAEC using a Carbowac 100 column; chromatographed sugars were detected using a pulsed amperometric detector as described previously [16]. The amount of arabinose produced was quantified by comparing the arabinose peak generated by the enzyme with the peak area produced by standard amounts of the furanose sugar. The substrates used in these assays were as follows: sugar beet arabinan, wheat arabinoxylan, linear arabinan and arabino-oligosaccharides comprising arabinobiose to arabinohexaose (all supplied by Megazyme, County Wicklow, Ireland). The other substrates used were

supplied by the laboratory of A.G.J.V. (2-*O*-arabinofuranosyl-xylotetraose, 3-*O*-arabinofuranosyl-xylotetraose and 2,3-*O*-arabinofuranosyl-xylotetraose) and by G. Williamson [Institute of Food Research, Norwich, U.K.; 2-*O*-(*trans*-feruloyl)- α -L-arabinofuranosyl-(1 $\rightarrow$ 5)-L-arabinofuranose]. To measure the activity of the enzyme at different pH values, Abf51A was incubated with arabinobiose in the following buffers: 50 mM sodium acetate, pH 4–6, 50 mM sodium phosphate, pH 6–7.5, 50 mM Tris/HCl, pH 7.5–9, and 50 mM Caps [3-(cyclohexylamino)propane-1-sulphonic acid], pH 9–10, and the hydrolysis of the disaccharide was determined by HPAEC. The susceptibility of the arabinofuranosidase to thermal and proteinase inactivation was evaluated as described previously [17].

RESULTS

Cloning of *abf51A*

Previous studies have identified two *P. cellulosa* enzymes that hydrolyse arabinose-containing polysaccharides; a GH62 arabinofuranosidase [8] and an endo/processive arabinanase [7] which cleaves linear arabinans almost exclusively to arabinotriose. To investigate whether *P. cellulosa* contains genes encoding other arabinofuranosidases, a genomic library of the bacterium constructed in λ ZAPII was screened with MUA, which is hydrolysed by arabinofuranosidases to release the highly fluorescent molecule 4-methylumbelliferone. Approx. 20 4-methylumbelliferyl- α -L-arabinofuranosidase (MUAase)-positive phage were isolated from a total of 40000 recombinants. The pseudomonad inserts in three of the MUAase phage were excised into the plasmid pBluescript SK⁻ to generate the recombinant plasmids pMB1, pMB2 and pMB3. A combined restriction map of the three recombinant plasmids is presented in Figure 1, and the arabinofuranosidase gene present in the plasmids and its encoded enzyme were designated *abf51A* and Abf51A, respectively. Plaque hybridization showed that all the phage contained DNA that cross-hybridized with the pseudomonad insert in pMB1, suggesting that all the recombinants contain *abf51A* (results not shown). In the accompanying paper [10], Southern-blot hybridization was used to show that a single copy of *abf51A* was present in the pseudomonad's chromosome.

Sequence of *abf51A* and Abf51A

To investigate the structure of *abf51A* and its encoded product, the sequences of the *Pseudomonas* inserts in pMB1, pMB2 and pMB3 were determined in both strands. The data revealed three complete open reading frames (ORFs) of 1551 bp, 1269 bp and 909 bp, respectively, and one ORF of > 1359 bp that extended beyond the 5' end of the pseudomonad insert in pMB1. The sequence of the 1551 bp ORF is presented in Figure 2. Comparison of the polypeptides encoded by the ORFs with proteins in the databases searched by Fasta3 (at the EMBL European Bioinformatics Institute, <http://www.ebi.ac.uk/>) showed that the 1551 bp, 1269 bp and 909 bp ORFs encoded enzymes that exhibit sequence identity with GH51 arabinofuranosidases, the GH26 mannanase Man26A (identical sequence to *man26A* [18], implying it is this gene) and cysteine synthases, respectively. The incomplete ORF showed homology to uronate isomerases. The GH51 enzymes that exhibited > 46% identity with the 1551 bp ORF were arabinofuranosidases I and II from *Cytophaga xylanolytica*, arabinosidase 2 from *Bacteroides ovatus*, arabinofuranosidase B from *Clostridium stercoarum* and arabinofuranosidase 2 from *Bacillus subtilis* (Figure 3). To confirm that the 1551 bp ORF encoded a functional arabinofuranosidase, derivatives of pMB1, pMB2 and pMB3 were constructed and their capacity to direct the synthesis of a functional MUAase was evaluated. The data (Figure 1) showed that plasmids in which the 1551 bp ORF was intact encoded a functional arabinofuranosidase. The presence of a single arabinofuranosidase gene in plasmids pMB1–3 confirmed that all the 20 MUAase-positive phage contain *abf51A*, which comprises the 1551 bp ORF.

Inspection of *abf51A* revealed a typical prokaryotic ribosome-binding sequence (AGGAGA) located 7 bp upstream of the putative translation initiation codon. The predicted molecular mass of Abf51A is 57000 Da and the codon usage of *abf51A* is typical of other *P. cellulosa* plant-cell-wall hydrolase genes [7,8,18,19]. The N-terminus of Abf51A appears to constitute a prokaryotic signal peptide [7,8]. It contains three basic residues at the N-terminus, followed by 15 small hydrophobic amino acids capable of forming an α -helical structure. Based on the signal-peptide-prediction program signalP version 1.1 (<http://www.cbs.dtu.dk/services/SignalP/>), the cleavage site is likely

Figure 1 Restriction map of recombinant plasmids containing *abf51A*

The positions of the cleavage sites for the following restriction enzymes are as follows: *EcoRI* (R), *EcoRV* (E), *HindIII* (H), *KpnI* (K), *PstI* (P), *SmaI* (S) and *SstI* (Ss). The solid arrows show the extent and orientation of the genes encoding Abf51A (*abf51A*), Man26A (*man26A*), truncated uronate isomerase (*uriA*) and cysteine synthase (*cysS*). The plasmids pMB1, pMB2 and pMB3 were excised from recombinant phage, pMB4 and pMB5 were derived from PCRs of pMB3 and pMB6 was generated by cloning the appropriate restriction fragment from pMB2 into pBluescript SK⁻.

A D E F T A F N R D I E Q V Y O R P T L
GCCGATGAATTCACAGCGTTCAATCGCGACATCGAGCAGGTCTATCAGCGTCCGACCTG -195

I V K *
ATAGTTAAATAGTCTTTCTCTTGACTGATTCGCCGCCCGCCAGGTTGCCGGGGCGGCGA -135

ATTTCCTGCTTAACTGATACCCGGGATAACCGTAAAAATAGGTTAAATTGTAGGACAA -75

TAATATCTTATGGCCTGTATTCTCCACGGTTTCACGTTTGATAAAAAATCCCGGATAACA -15

M R R L K P L I A A L G L S F
ACAAAGGAGAATCCCATGCGCGTTTGAACCCCTCATCGCGCACTTGGCTTGAGTTT +45

⁺¹
S I G S F A N T H I T I D T T K S G P V
AGCATCGGCAGCTTCGCCAATACCCACATCACCATAGATACCAACCAATCCGGTCCGCTG 105

I N K N I Y G Q F A E H L G R G I Y E G
ATTAAACAAGACATCTATGCGCAATTTGCGGAGCATCTGGGACGAGGTATTTATGAAGGA 165

L W V G P E S G I P N T R G W R N D V V
CTGTGGGTGGTCCGGATCCGCATCCCAACCCCGCGGTTGGCGCAATGACGTGGTG 225

G A L K D I N V P L V R W P G G C F A D
GGTCGCTCAAGGATATTAAAGTGGCCCTGGTGGTGGGCCGGTGGCTGTTTGGCGGAT 285

E Y H W R D G I G P R D Q R P V K V N T
GAATACCACTGGCGTATGGCATAGGCCCGCGCAGCAGCGCCGGTGAAGGTCAATACC 345

N W G G V E E D N A V G T H E F F D L V
AACTGGGGGGCGTGAAGAGGATAATGCGTGGGGACCCAGGTTTTCGACCTGGTG 405

E I L G A E A Y V N G N L G T G T P Q E
GAGATTCTGGGGCTGAAGCCTATGTGAATGGCAACCTGGGCACCGGAACGCCCGAGGA 465

M A E W L E Y M T A E G K S T L A E L R
ATGGCCGAATGGTGAATATATGACGCGGAGGGTAATCAACCTTGGCGGAATTCGCG 525

R K N G R D K P F Q V Y F A I G N E A
CGTAATAATGGCGCGATAAACCTTCACGATTCAGTATTTGCAATTGGTAATGAGCGG 585

W G C G G N M T P E Y Y T N L Y N H Y A
TGGGGCTCGGTGGCAATATGACCGCCGAGTACTACCAACCTGTACAACCACTACGCG 645

T F L K A P A H N A P K L I A S G G H T
ACTTCTCGTGAAGGCCCTGCCACAAATGCACCAATTTGATTGCCAGCGCGCGCATACC 705

E D T S W A A H L T A N V K P N W S L K
GAAGACACCGCTGGGCTGCCACCTGACCGCAATGTCAAACCACTGGAGTTTGAAA 765

M D A V S F H Y Y T L P T G K W D K K G
ATGGATCCGCTTAGTTTCCACTACTACACCTGCCACGGGTAATGGGATAAGAAGGT 825

A A I G F P E A E W M S T L V N T L R M
GCGGCCATCGGCTTCCCGAGGCGGAGTGTCTACGCTGGTGAATACCCCTGCCATG 885

D D F I V N N K K V M D K N D P E K K V
GATGACTTTATCGTCAACCAAAAAAGTCATGGATAAAAACGATCCCGAGAAAAAGTG 945

G F Y V D E W G T W D V E A G E N P G
GGCTTCTATGTGGACGATGGGGCACCTGTGACGCTGGAAGCGGGCGAGAATCCCGGT 1005

F L Y Q Q N S L R D A V V A A L N F N I
TTCCTTTACCAAGAACAGTTTGGCGGATGGGTTAGCGGCACCTTAATCAATATT 1065

F H K H A D R V H M T N I A Q M V N V L
TTCACAAGCATGACAGTCCGGTGACATGACCAACATTGCGCAGATGGTCAATGTGTTG 1125

Q A M I L T D K E K M I L T P T Y Y A Y
CAGGCCATGATTTTACCGATAAGGAAAAATGATCTGACGCCACCTACTATGCGTAC 1185

K M Y V P P F Q D A T S L P V S L K K V S
AAGATGATGTACCTTCCAGGATGCAACATCGCTGCTGAGCTTGAAAAAGTGTC 1245

Q Y R L G K S S V P A I S A S A A R G K
CAATACGCTTTGGGAAATCCTCGTGCCGGCATCAGCGCTTACGCCGCGCGGTAAG 1305

D G K V Y L A L V N A N P N Q A E T V A
GATGGCAAGGTACTCGGCCCTGGTCAATGCCAATCCCAACAGGCCGAGACGGTAGGG 1365

L A L P G V T A S G V S G Q L L T A T A
CTTGCAATACCTGGTGTAACTGCCAGCGGGGTGAGTGCCAGTTGCTGACAGCGACAGG 1425

M D A H N T F A N P N A I K P V S Y S A
ATGGATGCGCATACACCTTTGCCAATCCCAATGCGATCAAGCCGGTGAATTTCTGCG 1485

K A V N G K L S L E L P A K S V V V V A
AAAGCCGTGAATGGCAATTTGCGTGGAGCTGCCTGCTAAATCGGTGCTGTGGTGGG 1545

V E *
GTGGAATAGGCGCGCTTTTTCAGATGGAACCGGAGTCCGGTCTGTTGGTGTAAATACCGAC 1605

GACCGGGTTTGGTTAAGTGGAGTGACAATAATTAACCAAAATCTCAATCGACAAATA 1665

GCGCTCGCGGTATCGTAATGAAATACGACGCGGCTACCGGGTTTCATATCAGCTTG 1725

Figure 2 The nucleotide sequence of *abf51A* and the deduced primary structure of Abf51A

The 3' end of *man26A* and the C-terminal sequence of Man26A is shown in italics, the 1551 bp ORF and the encoded protein are *abf51A* and Abf51A, respectively. The experimentally determined N-terminal sequence of Abf51A is in bold. The potential stem-loop structure between *man26A* and *abf51A* is shown as opposing arrows.

to be between amino acids Ala-15 and Asn-16. Downstream of the signal peptide is a 502-amino-acid module that exhibits extensive sequence identity with GH51 arabinofuranosidases, and is thus presumed to comprise the catalytic domain. Unlike the majority of *P. cellulosa* plant-cell-wall hydrolases, Abf51A did not appear to be a modular protein. The enzyme did not contain *P. cellulosa* hydroxyamino-acid-rich sequences that link discrete modules, and the protein showed no homology to non-catalytic domains, such as the family 2a and 10 carbohydrate-binding modules found in numerous *Pseudomonas* cellulases and xylanases [8,19]. It would appear, therefore, that Abf51A is a non-modular GH51 arabinofuranosidase.

Purification of Abf51A

To investigate the biochemical properties of Abf51A, the regions of *abf51A* encoding full-length and mature (lacking the signal peptide) forms of the protein were amplified by PCR and cloned into pET-based expression vectors to generate pMB4 and pMB5, respectively. *E. coli* BL21(DE3):pLysS harbouring pMB5 expressed high levels of soluble Abf51A, as evidenced by the appearance of a 55000 Da polypeptide, whereas cells containing pMB4 synthesized low levels of soluble Abf51A (results not shown). Full-length Abf51A was purified by nickel-ion chromatography (contains a C-terminal His tag) and its N-terminal sequence of NTHITIDTT indicated that the signal-peptide cleavage site of the enzyme, at least in *E. coli*, was between Ala-15 and Asn-16. Mature Abf51A, which contains an N-terminal His-tag (introduced during the construction of pMB5) was purified from *E. coli* by nickel-ion chromatography to apparent homogeneity, as judged by SDS/PAGE (Figure 4).

Biochemical properties of Abf51A

The capacity of purified Abf51A to hydrolyse a range of different substrates was evaluated. The data, presented in Table 1, showed that the arabinofuranosidase hydrolysed aryl- α -arabinofuranosides, sugar beet arabinan, linear arabinan and wheat arabinoxylan. But the enzyme did not cleave aryl-arabinopyranose (either α - or β -linked), α - or β -linked aryl-xylosides and α -glucosides, mannan, galactomannan, hydroxyethyl cellulose, acid-swollen cellulose or β 1,4-galactan (results not shown). HPAEC analysis of the reaction products generated by Abf51A revealed that the enzyme released only arabinose from sugar beet arabinan, linear arabinan and wheat arabinoxylan (results not shown), demonstrating that it cleaved terminal arabinofuranose units exclusively (Table 1). Although Abf51A attacked linear arabinan the k_{cat}/K_m value could not be determined; the low solubility of the polysaccharide and the high K_m of Abf51A for the substrate prevented accurate determination of the individual kinetic constants. To investigate the substrate specificity of Abf51A in more detail, its capacity to hydrolyse oligosaccharides was evaluated. The data, presented in Table 2, show that the enzyme exhibits the greatest activity against xylotetraose containing a single arabinose side-chain-linked α 1,3. It was two times less active against xylotetraose with an α 1,2-linked arabinose side chain and was approx. 1000 times less active against xylotetraose in which the middle xylose moiety was substituted at both the 2' and 3' positions with arabinofuranose units. Abf51A exhibited low activity against arabinotriose-linked α 1,5 and displayed no detectable activity against arabinobiose in which the non-reducing end of the oligosaccharide contained a ferulic acid residue linked to C-2. The capacity of the arabinofuranosidase to cleave a range of α 1,5-linked arabino-

	80
ABF51A	MRLKPLIAALGLSFSIGSFANTHITIDTTKSGPVINKNIYGFQAEHLGRGIYEGWLWGPESGIPNTRGWRNDVVGALKD
068279	GALVLAFMLTMTCTPKPPQNDVVKMSVDVTQSGPEINRHIFGQAEHLGTGIYGGIWLWGPESGIPNTRGIRNDVVKALKA
068278	MKRISPTLALLPLS-----AQTKITLQADQAKERINPEIYGHFAEHLGRLIYGGILVEPGSQIDNIRGFRDVIQALRD
Q59219	MKAKLLVTAFLAASVLSAQKSATITVHADQGKEIIPKEIYGFQAEHLGSCIYGGIWLWGENSDIPNIGKGYRTDVFNALKD
008457	-----MAKYVINTLNKRSKINREIYGHFSEHLGRCIYGGFYVGENSPINPTKIGIRNDVVNALKK
P94552	-----MSEHQAVIQTDAIKGTINKNIYGHFAEHLGRGIYEGIWLWGTDSIPNINIGIRKDVLEALKQ
	160
ABF51A	INVPLVRWPGGCFADYHWRDGIQPRDQRPVKVNTNWGGVEEDNAVGTHEFFDLVEILGAEAYVNGNLGTGTPQEMAEWL
068279	IKVPNVWRWPGGCFADQYHWRDGIQAPERKSRINVSWGGSPPEANTFGTHEYDFDISQIGSEAFISANVSGSTVQESADWL
068278	MKVPIILRWPGGCFADTYNWRDGIQPKQNRPSIVNIHWGGVTEDNSFGTHEFFDFCELIGAEPIVNLNVGSGTVREAAQWV
Q59219	LSVPVLRWPGGCFADYHWMDDGIQPKENRPMVNNWGGTIEDNSFGTHEFFNLCEMLGCEPYVSGNVGSGTVEELAKWV
008457	IRIPVLRWPGGCFADYHWMDDGIQPKQRRKMINTHWGGVVE-ASFGTDEFMELCRQLECEPYINGNLGSGTVKEMQDWI
P94552	LHIPVLRWPGGCFADYHWANGVG---DRKTMNLTHWGGTIESNEFGTHEFFMMLCELLECEPYICGNVSGSTVQEMSEWI
	240
ABF51A	EYMTAEGKSTIAELRRKNGRDQKPFQVQYFAIGNEAWGCGGNMTPEYTYNLYNHYATFLKAPAHNAPKLIASGGHTEDTSW
068279	EYLTASG-STLALERARNGHDPDPYDVAFWGVGNEVWCGGPFPTQEXITELKKFATFTNNYNANVTQFVAVGPDSEDFESF
068278	EYITSNPSPMTNLKENGDRDQKWDIYFCIGNENWCGCGHMTPEYADLYRNYATYCGGVKYK----IAGPNVDDYRW
Q59219	EYMTSDGDSPMANLRRKNGRDQKAWKLYLGVGNEVWCGGSMRPEYADLYRRYSTYCRNYDGNRLFKIASGASDYDYKW
008457	EYLTFDGISPMSELRAKNGRKEPYRLKYFCIGNENWCGGGMREPEFYADMYRRYQTYVRNYGNKNIYKIACGNPSDDYNW
P94552	EYMTFEEGTPMSDWRKQNGREEPWKLKYGFGVNEVWCGGGMHPEYADLYRRFYTYVRNYSNGNDIYKIAGGANVDDFNW
	*
	320
ABF51A	AAHLTANVKPNWSLKMDAVSFHYTTLPTGKWDKKGAAIGFPEAEWMSTLVNLTMRDDFIVNNKVMMDKNDPEKKVGFYVD
068279	GAIMEAWAKKTWTWDIQGLSLHMYT--RGGWPAVIPATGFNESGYAAVIKETLEMDKFISDNRAIMDKFDPKHKVSIIMVD
068278	TEVLMNKTQHHKFL-MQGLSLHYTTFP-GRWEDKGSSIVFDEQSWHQTLRHLRMQELISKHGEIMDKYDPKKQIGLIVD
Q59219	TDVLMNRV----GHRMDGLSLHYTTP-TGWSGSGKSATQFNKDDYWTMGKCLEVEDVLKKHCTIMDKYDQDKKIALLLD
008457	TETV---VKIAGRF-MDAISLHYTTPV-GTWSKGSSTKFTEEEWYITLFTKLMDELITKHTAIRRYDPDNRIGLVVD
P94552	TDVL---MKKAAGL-MDGLSLHYTTPGDFWKGKGSATEFTEDEWFITMKKAKYIDELIQKHGTIMDRYDPEQRVGLIID
	400
ABF51A	EWGTWYDVEAGENPGFLYQONSLRDVAALNFNI FHKHADRVHMTNIAQMNVNLQAMILTDKEKMILTPTTYAYKMYVP
068279	EWGTWYAPTEGTNPGFLYQONSLRDVAALNFNI FIRAERVKGANIAQMNVNLQAMILTEGEKMVLTPPTTYHTFRMYVP
068278	EWGTWYDVMPGTNPGFLYQONSLRDVAALNFNI FNNNADRVKMANIAQMNVNLQAVILTQGDDEMILTPTTYVFKMYNV
Q59219	EWGTWWDDEPGTIKGHLYQONSLRDVAALNFNI FVSLSDVFKYTDRLKMANIAQIVNVNLQSMILTQDKEMVLTPPTTYVFKMYKV
008457	EWGTWYDVEPGTNPGFLYQONSLRDVAALNFNI FNKHSKDVVMANLAQTINVNLQAVILTEGEKMILTPTTYHVFDYKE
P94552	EWGTWFDPEPGTNPGFLYQONTIRDALVAASHFHFHQHCRRVQMANIAQTVNVNLQAMILTEGERMLLTPTTYHVFNMFKV
	*
	480
ABF51A	FQDATSLPVSLKKVSQYRLGKSSVPAISASAARGKDGKYLALVNANPNQAETVALALPGVTASGVSGQLLTATAMDAHN
068279	FQDATRLPINFNK-GFYKEGTIELPRVDAIAAKGRDGKIYVAITNIDPKNNASLDLSFDEQTIKDVKGETLYASDAIDAVN
068278	HHGATLIPMNIQ-TQDYVMGDQKIPMVNASASI-KDKTVSVTLCLNLHASQSTKVEIDVTGFEGKTATGQIITGEKITDYN
Q59219	HQDATYLPIDLTCEKMSVRDNTVPMVSATASKNKDGVIIHISLSNVDADEAQEITINLGDTKAKKAI GEILTASKLTDYN
008457	HQGAELVELLLKTEKKSQTKYTVPNLHESASVNADGKLHITLCLNLSLESYRVETEII GKKAENVTRILTGK-MNDHN
P94552	HQDA-SLLATETMSADYEWNGETFPQISISASKQAEGDINITICNIDQNKAEA-EIELRGLKAADHSGVILTAEKMNHN
	517
ABF51A	TFANPNAIKPVSYSAKAVNGKLSLELPAKSVVVVAVE
068279	TFDNPNNVAPTSINASLTGSNVSVTVQPQSVTVLEIV
068278	DFGKPEMVGLKAFNAKPKGNKLTVEIPAKSVVLVQMK
Q59219	SFEKPNIVKPAFPKEVKINGTMKVLPKASIVTLELQ
008457	TFDAPETVKPQTFDKISVNG-LVIEIPPVSVVSVITVD
P94552	TFDDPHHVKPESFRQYTLNKLKVLPPMSVVLTLR

Figure 3 Comparison of Abf51A with family 51 enzymes

Abf51A was compared with sequences in protein databases using the Fasta3 search engine at the European Bioinformatics Institute. The accession numbers of those proteins which exhibit greater than 46% sequence identity are shown. Residues which are identical in all the sequences are in bold, and the putative catalytic amino acids are denoted by asterisks. 068279 and 068278, arabinofuranosidases II and I from *Cytophaga xylanolytica*; Q59219, arabinosidase 2 from *Bacteroides ovatus*; 008457, arabinofuranosidase from *Clostridium stercorarium*; P94552, arabinofuranosidase 2 from *Bacillus subtilis* 168.

oligosaccharides was assessed. The data (Table 3) showed that the enzyme hydrolysed arabino-oligosaccharides comprising three, four, five and six arabinose units at similar rates, but was approx. eight times less active against arabinobiose compared with the other arabino-oligosaccharides. To assess whether the pattern of arabino-oligosaccharide hydrolysis by Abf51A is typical of GH51 enzymes, the activity of arabinofuranosidase A from *Aspergillus niger* (GH51 enzyme [20]) against these substrates was assessed. The data showed that its activity was not significantly higher against arabinotriose and arabinohexaose, compared with arabinobiose (Table 3), suggesting that the pattern

of hydrolysis of α 1,5-linked arabino-oligosaccharides by GH51 enzymes is variable.

Biophysical properties of Abf51A

The catalytic activity of Abf51A at different pH values was assessed. The enzyme exhibited optimal activity at approx. pH 5.5, which is much lower than other *P. cellulosa* plant-cell-wall hydrolases. The glycoside hydrolase was rapidly inactivated at temperatures above 55 °C, and was completely resistant to

Figure 4 SDS/PAGE of wild-type and mutant forms of Abf51A

Wild-type Abf51A (lane 1) and the mutants E194A (lane 2) and E321A (lane 3) were purified by immobilized metal-ion chromatography and subjected to SDS/PAGE, together with Sigma high-molecular-mass standards (lane 4), using a 10% polyacrylamide gel. The size of Abf51A is shown in kDa.

proteinase inactivation over a 3 h incubation under conditions in which typical enzymes are completely inactivated within 20 min (results not shown).

Biochemical properties of Abf62A

Previous studies [8] showed that *P. cellulosa* contains a gene that encodes an arabinofuranosidase, designated Abf62A (formerly XylC), which hydrolyses arabinoxylans and belongs to GH62. To compare the properties of Abf62A and Abf51A, purified forms of the two enzymes were incubated with arabinose-containing polysaccharides. The data, presented in Table 1, showed that while Abf62A hydrolysed arabinoxylans, it exhibited no activity against arabinans, demonstrating that the enzyme was specific for arabinoxylans. Surprisingly, Abf62A exhibited very similar k_{cat} and K_m values to Abf51A. Thus although Abf62A is an arabinofuranosidase that is specifically designed to hydrolyse arabinoxylans, it does not hydrolyse this substrate more efficiently than a non-specific arabinofuranosidase that does not discriminate between the sugars linked to the arabinofuranose moieties. To investigate in more detail the properties of Abf62A, the capacity of the enzyme to hydrolyse arabinoxylo-oligosaccharides was assessed. The data, presented in Table 2,

Table 2 Activity of Abf51A and Abf62A against oligosaccharides

The two enzymes (20 μg) were incubated with 1 μmol of substrate and the rate of oligosaccharide hydrolysis was determined by HPAEC. The activity is expressed relative to the hydrolysis of A-2-X3 by Abf51A (3.7 mol of product/mol of enzyme $^{-1} \cdot \text{s}^{-1}$), which was defined as 1. Substrate abbreviations: A-2-X4, 2-*O*-arabinofuranosyl-xylotetraose; A-3-X4, 3-*O*-arabinofuranosyl-xylotetraose; A-2,3-X4, 2,3-*O*-arabinofuranosyl-xylotetraose; FAA2, 2-*O*-(*trans*-feruloyl)- α -L-arabinofuranosyl-(1 $\rightarrow$ 5)-L-arabinofuranose. NA, no activity detected.

Enzyme	Substrate	Relative activity
Abf51A	A-2-X4	1
Abf62A	A-2-X4	4.5×10^{-1}
Abf51A	A-3-X4	2.2×10^{-1}
Abf62A	A-3-X4	3.4×10^{-3}
Abf51A	A-2,3-X4	2.5×10^{-4}
Abf62A	A-2,3-X4	NA
Abf51A	FAA2	NA
Abf62A	FAA2	NA
Abf51A	Arabinotriose	3.3×10^{-3}
Abf62A	Arabinotriose	NA

Table 3 Activity of Abf51A against oligosaccharides

The enzymes were incubated with an equimolar amount of the five different arabinoo-oligosaccharides and the rate of hydrolysis was determined by HPLC. The rate of arabinobiose cleavage was defined as 1 for both enzymes.

Enzyme	Substrate	Relative activity
Abf51A	Arabinobiose	1
Abf51A	Arabinotriose	6.5
<i>Aspergillus niger</i> GH51	Arabinotriose	1.4
Abf51A	Arabinotetraose	7.2
Abf51A	Arabinopentaose	6.9
Abf51A	Arabinohexaose	8.3
<i>Aspergillus niger</i> GH51	Arabinohexaose	1.2

show that the enzyme cleaved arabinose units linked 3' to xylo-oligosaccharides at a similar rate to Abf51A, but was much less active against α 1,2-glycosidic bonds. In addition, Abf62A had no activity against substrates in which the xylose moieties were substituted at the 2' and 3' positions with arabinofuranose units.

Key catalytic residues of Abf51A

Glycoside hydrolases mediate glycosidic bond cleavage via acid/base-assisted catalysis [21]. These enzymes contain two

Table 1 Catalytic activity of *P. cellulosa* Abf51A and Abf62A

PNPA, 4-nitrophenyl- α -arabinofuranoside; NA, no activity detected; ND, although enzyme activity was present it was too low to measure the individual kinetic constants.

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)
Abf51A	Sugar beet arabinan	4.3 ± 0.29	0.33 ± 0.005	13
Abf62A	Sugar beet arabinan	NA	NA	NA
Abf51A	Linear arabinan	ND	ND	0.0006
Abf62A	Linear arabinan	NA	NA	NA
Abf51A	MUA	25.1 ± 0.67	0.26 ± 0.019	96.2
Abf62A	MUA	ND	ND	0.03
Abf51A	Wheat arabinoxylan	5.4 ± 0.19	0.27 ± 0.06	20
Abf62A	Wheat arabinoxylan	4.6 ± 0.36	0.36 ± 0.03	12.8
Abf51A	PNPA	30 ± 0.56	0.18 ± 0.01	167
Abf62A	PNPA	NA	NA	0.05
Abf51A	Arabinobiose	5.1 ± 0.57	1.58 ± 0.158	3.2
Abf62A	Arabinobiose	NA	NA	NA

Table 4 Catalytic activity of Abf51A mutants

The data for the wild-type enzyme are from Table 1, and are included for ease of comparison with results for the mutants. NA, no activity detected; ND, although enzyme activity was present it was too low to measure the individual kinetic constants.

Enzyme	Substrate	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)
Wild type	PNPA	30 ± 0.56	0.18 ± 0.01	167
E194A	PNPA	0.29 ± 0.009	0.042 ± 0.004	6.9
E321A	PNPA	NA	NA	NA
Wild type	Arabinobiose	5.1 ± 0.57	1.58 ± 0.16	3.2
E194A	Arabinobiose	0.003 ± 0.001	1.7 ± 0.2	0.002
E321A	Arabinobiose	NA	NA	NA
Wild type	Sugar beet arabinan	4.3 ± 0.29	0.33 ± 0.015	13
E194A	Sugar beet arabinan	ND	ND	0.003
E321A	Sugar beet arabinan	NA	NA	NA

amino acids, either glutamate or aspartate, that play a direct role in catalysis. Inspection of the GH51 enzymes revealed two glutamate residues (corresponding to Glu-194 and Glu-321 in Abf51A) which were invariant. Zverlov et al. [22] suggested that two carboxylic amino acids comprise the acid/base and nucleophile catalysts, respectively. To test this hypothesis, we constructed two mutants of Abf51A, E194A and E321A, purified the mutant enzymes (Figure 4) and evaluated their catalytic properties. Table 4 shows that E321A was completely inactive against all substrates evaluated. In contrast, E194A retained some activity against aryl- α -arabinofuranosides, although there was a substantial drop in k_{cat} and K_{m} . To assess the influence of these mutations on the secondary structure of Abf51A, the mutant and wild-type forms of the enzyme were subjected to CD spectroscopy. The data (not shown) revealed that the mutations had not caused a significant perturbation in the secondary structure of the arabinofuranosidase. These data are consistent with Glu-194 and Glu-321 comprising the catalytic nucleophile and acid/base residues of Abf51A, respectively.

DISCUSSION

This report shows that *P. cellulosa* expresses a GH51 arabinofuranosidase (Abf51A) that will cleave arabinofuranose units linked α 1,2, α 1,3 or α 1,5 to arabinose or xylose polymers. Previous studies [2] have shown that GH51 enzymes preferentially remove the arabinose side chains from sugar beet arabinan rather than removing the sugar at the non-reducing end of the backbone polymer. It was thought that this mode of action simply reflected the abundance of side-chain sugars compared with the non-reducing end of the arabinan main chain. While this is clearly so, data reported here show that Abf51A is approx. 300-fold less active against α 1,5-linked arabino-oligosaccharides than arabinofuranose moieties joined α 1,2 or α 1,3. These data are consistent with a study which showed that a GH51 enzyme from *S. chartreus* exhibited a strong preference for methyl 2-*O*- α -L-arabinofuranosyl- α -L-arabinofuranoside and methyl 3-*O*- α -L-arabinofuranosyl- α -L-arabinofuranoside, compared with methyl 5-*O*- α -L-arabinofuranosyl- α -L-arabinofuranoside [4]. Thus it would appear that GH51 enzymes exhibit a strong preference for α 1,2- and α 1,3-linked arabinofuranose units rather than α 1,5-glycosidic bonds. However, within GH51 there are clear differences in substrate specificity. For example, the *S. chartreus* GH51 enzyme does not hydrolyse arabinoxylo-oligosaccharides and exhibits very low activity against arabinoxylan compared with arabinan, which is in sharp contrast to Abf51A. In addition, Abf51A is clearly more active on α 1,5 arabino-oligosaccharides comprising three or more sugars than

the disaccharide, while the GH51 enzyme from *A. niger* exhibits very similar activities against arabinobiose and arabino-oligosaccharides up to arabinohexaose. These differences in activity could reflect the nature of the substrate available to the enzymes. Thus the endo- α 1,5-arabinanase synthesized by *P. cellulosa* is a processive enzyme that generates primarily arabinotriose from linear arabinan, whereas the corresponding enzyme from *A. niger* produces a mixture of arabino-oligosaccharides including large amounts of arabinobiose [7]. Thus the GH51 enzymes from *A. niger* and *P. cellulosa* appear to have evolved substrate specificities that are consistent with the substrates released by their respective endo-acting enzymes.

In a previous study Zverlov et al. [22] suggested that GH51 is a member of the glycoside hydrolase clan GH-A, and that the equivalent residues to Glu-194 and Glu-321 of Abf51A comprise the catalytic acid/base and nucleophile residues, respectively. The complete lack of catalytic activity of E321A is entirely consistent with Glu-321 being the catalytic nucleophile of the enzyme. The reduction in the catalytic activity of E194A (compared with wild-type protein) against arabinobiose, which was not associated with a change in K_{m} , indicates that the mutation affects the acid/base catalytic power of the enzyme. To conclusively prove the identity of catalytic carboxylic residues in glycoside hydrolases, nucleophile and acid/base mutants of these enzymes have been reactivated by the presence of high concentrations of ions, such as azide, that can act as nucleophiles [23,24]. The covalent attachment of azide to the cleaved substrate provides definitive proof that the mutant enzymes lack one of the catalytic amino acids. Attempts to reactivate the E194A and E321A mutants of Abf51A failed, probably because the ions could not penetrate the pocket active site (typical of glycoside hydrolases that remove terminal sugars from polysaccharides) of the enzyme when occupied by the substrate. Thus although the data presented in this paper are consistent with Glu-194 and Glu-321 comprising the key catalytic residues, this view must be treated with some caution.

Conclusions

The observation that *P. cellulosa* Abf51A releases arabinose side chains from arabinoxylan as efficiently as Abf62A implies functional redundancy between the two enzymes. The accompanying paper [10], however, shows that Abf62A is not expressed by *P. cellulosa*, and deletion of the *abf51A* gene compromises the capacity of the bacterium to grow on sugar beet and linear arabinan, and reduces the growth rate and final biomass when the pseudomonad is cultured on media containing arabinoxylan as sole carbon source. In conclusion, the data published in this

report and in the accompanying paper [10] show that Abf51A plays a major role in the capacity of *P. cellulosa* to remove arabinose side chains from plant structural polysaccharides, and thus the enzyme is pivotal to the degradation of the plant cell wall by this bacterium.

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