Study of the mode of action of a polygalacturonase from the phytopathogen *Burkholderia cepacia*

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We have recently isolated and heterologously expressed BcPeh28A, an endopolygalacturonase from the phytopathogenic Gram-negative bacterium *Burkholderia cepacia*. Endopolygalacturonases belong to glycoside hydrolase family 28 and are responsible for the hydrolysis of the non-esterified regions of pectins. The mode of action of BcPeh28A on different substrates has been investigated and its enzymatic mechanism elucidated. The hydrolysis of polygalacturonate indicates that BcPeh28A is a non-processive enzyme that releases oligomers with chain lengths ranging from two to eight. By inspection of product progression curves, a kinetic model has been generated and extensively tested. It has been used to derive the kinetic parameters that describe

the time course of the formation of six predominant products. Moreover, an investigation of the enzymatic activity on shorter substrates that differ in their overall length and methylation patterns sheds light on the architecture of the BcPeh28A active site. Specifically the tolerance of individual sites towards methylated saccharide units was rationalized on the basis of the hydrolysis of hexagalacturonides with different methylation patterns.

Key words: *Burkholderia cepacia*, capillary electrophoresis (CE), glycoside hydrolase, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), kinetic model, pectin degradation.

INTRODUCTION

Primary cell walls are important structures of plant cells that are involved in a variety of physiological processes [1]. These structures are composed mainly of polysaccharides such as cellulose, hemicellulose and pectin. Cellulose microfibrils, composed of 30–36 chains of β -1,4-linked glucose, together with hemicelluloses, form a cohesive network that is embedded in a soluble matrix of polysaccharides, glycoproteins and low-molecular-mass compounds. Pectic substances are the most abundant components within this matrix. On the basis of backbone structures, pectic substances can be divided into three classes: HGA (homogalacturonan), RG-I (rhamnogalacturonan I) and RG-II (rhamnogalacturonan II). HGA is a linear homopolymer composed of 1,4-linked GalA (α -D-galacturonic acid) residues, where several GalA residues can be methyl-esterified or acetylated depending on the plant source. RG-I has a backbone of alternating rhamnose and GalA residues, where several rhamnose residues can be substituted at C-4 with neutral and acidic oligosaccharide side chains. RG-II is composed of at least seven 1,4-linked α -D-GalA residues, to which branched side chains are attached [2].

The modification of such a complex substrate is required in the course of plant physiological processes, such as cell wall turnover and remodelling [3], but it also occurs as a consequence of a pathogen attack [4,5]. Cell-wall-degrading enzymes isolated from pathogenic organisms include exopolygalacturonases and endoPGs (endopolygalacturonases), pectin and pectate lyases, rhamnogalacturonases and rhamnogalacturonan lyases. More-

over, other accessory enzymes are needed to degrade the branched side groups, such as acetyl and methyl esterases [6]. Interestingly, many pathogenic organisms produce multiple isoenzymes, suggesting that each isoform harbours a specific catalytic, and thus physiological, function [7].

Among the pectin-degrading enzymes, a large number of endoPGs have been isolated from pathogenic fungi such as *Aspergillus niger* [8] and *Botrytis cinerea* [9], and their specific hydrolytic activities have been investigated. EndoPGs are secretory enzymes responsible for the hydrolysis of the α -1,4 glycosidic bond in HGA. On the basis of their amino acid sequence similarities, these enzymes have been grouped into the glycoside hydrolase family 28, which also includes exopolygalacturonases, rhamnogalacturonases and xylogalacturonan hydrolases. The catalytic action of endoPGs proceeds according to an acid–base mechanism: a conserved aspartate residue (Asp²⁰¹ in *A. niger* endoPGII; see Figure 1) acts as proton donor, while a water molecule, structurally conserved in two of the known endoPGs crystal structures [10,11], is the nucleophile. The activation of this water molecule is ascribed to two aspartic acid residues (Asp¹⁸⁰ and Asp²⁰² in *A. niger* endoPGII; see Figure 1) that act as general bases. These three aspartate residues are strictly conserved among the endoPGs [12].

In order to define the mode of action of endoPGs, the most commonly used experimental approach consists of the detection of the products that are released upon hydrolysis of a substrate chain [13–18]. On the basis of the hydrolysis product progression profile, endoPGs have been classified as single-attack or

Abbreviations used: 4-ABN, 4-aminobenzonitrile; BcPeh28A, an endopolygalacturonase from the phytopathogenic Gram-negative bacterium *Burkholderia cepacia*; CE, capillary electrophoresis; DP, degree of polymerization; endoPG, endopolygalacturonase; ESI-TOF-MS, electrospray ionization MS with a time-of-flight analyser; GalA, α -D-galacturonic acid; HGA, homogalacturonan; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; ID %, percentage identity; MEKC, micellar electrokinetic capillary chromatography; OGA, oligogalacturonides; PGA, polyGalA; RG-I, rhamnogalacturonan I; RG-II, rhamnogalacturonan II; rmsd, root-mean-square deviation.

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Figure 1 Three-dimensional structure of *A. niger* endoPGII

The β -helix encompasses ten complete rungs (numbered 1–10). The amino acid residues associated with subsites –3 to +3 are reported in the scheme [28]. Asp¹⁸⁰, Asp²⁰¹ and Asp²⁰² are involved in catalysis. The residues at subsites –1 and +1 shown in the table are strictly conserved among fungal endoPGs [12]. The Figure was prepared with PyMOL (DeLanoScientific; <http://www.pymol.org>). Amino acids mentioned in the table forming the bottom panel are given in one-letter code.

Table 1 Fungal endoPGs whose mode of action has been elucidated

The enzymes have been grouped into two subgroups according to their mode of action on PGA. For each of the entries the source, the Swiss-Prot ^(a) or EMBL/GenBank[®] ^(b) database accession numbers (acc. no.), the PDB code and the relevant bibliographic reference(s) [Ref(s)] have been included.

Multiple-attack enzymes (processive behaviour)					Single-attack enzymes (non-processive behaviour)				
Name	Source	Database acc. no.	PDB code	Ref(s)	Name	Source	Database acc. no.	PDB code	Ref(s)
endoPGI	<i>A. niger</i>	P26213 ^a	1NHC	[14,19]	endoPGII	<i>A. niger</i>	P26214 ^a	1CZF	[11,14]
PGA	<i>A. niger</i>	Y18804 ^b	–	[16]	PGB	<i>A. niger</i>	Y18805 ^b	–	[16]
PGC	<i>A. niger</i>	Q12554 ^a	–	[14]	PGE	<i>A. niger</i>	Y14386 ^b	–	[13]
PGD	<i>A. niger</i>	Q9P4W2 ^a	–	[15]	Fm_pg	<i>F* moniliforme</i>	Q07181 ^a	1HG8	[17,20]
BcPG3	<i>B. cinerea</i>	AY665554 ^b	–	[18]	BcPG1	<i>B. cinerea</i>	AY665552 ^b	–	[18]
BcPG6	<i>B. cinerea</i>	AY665557 ^b	–	[18]	BcPG2	<i>B. cinerea</i>	AY665553 ^b	–	[18]
					BcPG5	<i>B. cinerea</i>	AY665556 ^b	–	[18]

^a *Fusarium*.

multiple-attack enzymes. Accordingly, in the case of single-attack enzymes, substrate molecules are cleaved only once after the formation of the ES (enzyme–substrate) complex, resulting in the release of oligomers of different chain lengths. The multiple-attack enzymes, also termed ‘processive’ enzymes, instead cleave the same substrate molecule several times, and small oligomers, or even monomers, are released during the initial period of the reaction. The available information on the mode of action of several fungal endoPGs is reported in Table 1. In particular, a comparative study of the modes of action of *A. niger* endoPGI and

endoPGII has been carried out, combining the information taken from the amino acid sequence alignment, and from biochemical and structural studies. These two enzymes, despite their high degree of amino acid sequence identity (71.4 %) and similarity (74.2 %), exhibit a different mode of action on polygalacturonate. The two structures are very similar, with a relative rmsd (root-mean-square deviation) of 0.8 Å (1 Å = 0.1 nm) on 335 corresponding C α atoms [11,19]. Both enzymes adopt a right-handed β -helix fold that has been observed in all the known structures of endoPGs [11,12,20–22]. The structure of *A. niger*

endoPGII, which includes ten 'rungs', each consisting of three β -strands separated by turn regions, is depicted in Figure 1. Site-directed mutagenesis studies on *A. niger* endoPGI and endoPGII demonstrated that the switch between a processive and a non-processive mode of action can be ascribed to the presence of an arginine or a serine residue respectively in a conserved segment belonging to rung 2 of the β -helix [23].

Little information is available on the mode of action of bacterial endoPGs, and our current understanding has been gathered from data on enzymes isolated from *Agrobacterium vitis*, *Ralstonia solanacearum* and *Erwinia carotovora* that have been previously classified as endo-acting enzymes. By analysing their product progression profile, the former enzyme has been classified as a dimer-releasing enzyme and the latter ones as trimer-releasing enzymes [24]. The three dimensional crystal structure of *E. carotovora* endoPG has been elucidated [11], but no structure–function relationship studies have been carried out so far.

Other studies aimed at characterizing the active site of endoPGs have been carried out by analysing the products released following the hydrolysis of pure OGAs (oligogalacturonides) that differ in their DP (degree of polymerization) and in their methyl-esterification patterns [13–18,25].

The active site of depolymerizing enzymes includes several saccharide-unit-binding sites, referred to as subsites, that have been labelled, by convention, from $-n$ to $+n$, where $-n$ represents the non-reducing end and $+n$ the reducing end of the sugar moieties respectively [26]. The cleavage occurs between the subsites -1 and $+1$. The latter subsites have been most extensively studied, since they include strictly conserved amino acid residues that are involved in catalysis (Figure 1) [27]. The other subsites are mainly involved in substrate recognition and have been less well characterized, since no significant conservation in the amino acid residues is detectable at these positions. Structure-based site-directed mutagenesis studies of the *A. niger* endoPGII unveiled a total of seven subsites, from -4 to $+3$, allowing for several amino acid residues to be assigned to each of them (Figure 1) [23,28].

To the best of our knowledge, no characterization of the structural determinants responsible for the catalytic activity of bacterial endoPGs has been reported.

We have recently heterologously expressed and purified to homogeneity BcPeh28A, an endoPG secreted by the plant-pathogenic Gram-negative bacterium *Burkholderia cepacia*, strain A.T.C.C. 25416, referred to also as 'pectic hydrolase enzyme A' [29]. BcPeh28A does not exhibit a significant amino acid sequence identity with respect to fungal and other bacterial endoPGs: *A. niger* endoPGI (ID % (percentage identity) 21.5), *A. niger* endoPGII (ID % 21.3) and *E. carotovora* (ID % 23.8), *R. solanacearum* (ID % 22.8) and *A. vitis* (ID % 22.9) endoPGs.

With the aim of characterizing the active site of BcPeh28A, the evident lack of sequence similarity notwithstanding, we now, for the first time, report on the mode of action of a bacterial endoPG on polygalacturonate and on shorter substrate molecules that differ in their overall length and in their methylation patterns.

EXPERIMENTAL

Production and purification of BcPeh28A

BcPeh28A was heterologously expressed and purified as reported elsewhere [29].

Amino acid sequence alignments

The pairwise alignments were performed, with default parameters, by algorithms available within the EMBOSS bio-

informatics suite accessible at the following URL: <http://www.ebi.ac.uk/emboss/align/>.

Substrates

PGA (polyGalA)

PGA sodium salt from citrus fruit was purchased from Sigma–Aldrich. According to the manufacturer, the raw material was de-esterified with NaOH and the final level of esters was approx. 5%. The product was purified from high-grade pectin with a molecular-mass distribution of 50–150 kDa.

Oligogalacturonides

A sample containing a mixture of OGAs with DPs ranging from 1 to 8 was kindly provided by Dr Giovanni Salvi, Dipartimento di Biologia Vegetale, Università 'La Sapienza', Rome, Italy. It was used as the standard in the analysis by CE (capillary electrophoresis) and HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection). The mixture was obtained as described in [30].

Decagalacturonide

A fully unmethylated decagalacturonide, purified from a pectin digest as described in [31], was kindly given by Professor Knud J. Jensen, Department of Natural Sciences, University of Copenhagen, Copenhagen, Denmark.

Hexagalacturonides

The hexagalacturonides (1–4) (Figure 7 below), which differ in their methylation patterns, were synthesized as reported in [32].

Time-course experiment

BcPeh28A (0.5 $\mu\text{g/ml}$) was incubated at 20 °C in 2.0 ml of 50 mM sodium acetate buffer, pH 3.5, in the presence of 0.25 % PGA as substrate. Aliquots of 50 μl were taken at regular intervals, and the reaction was stopped by adding an equal volume of derivatization solution (described below). The samples were then boiled at 95 °C for 20 min. CE, as described below, was used for identifying and assessing the molar number distribution of the OGAs present in the mixture, following the enzymatic reaction.

Kinetic model

The detailed kinetic characterization of BcPeh28A, using PGA as substrate, was done in several steps. At first, direct information on the reaction path was gathered by inspecting the product progression curves. Then a family of independent and likely reaction schemes was constructed, and, for each of them, the corresponding sets of differential equations were derived. Each reaction scheme was then tested by simultaneous fitting to the progress curves. A non-linear regression analysis was performed by a computer program that fitted the parameters of a set of stiff differential equations to multiple experimental curves [33]. In a system of differential equations that describes the time course of six products, more than 12 parameters should be evaluated. In most, if not all cases, it is necessary to make rational assumptions in order to reduce the number of unknowns, due to the limitations in the experimental data. Hence, the bimolecular rate constants for enzyme–substrate binding were assumed to be close to the diffusion rate limit either for the PGA or for the oligomers ($k_1 = k_3 = k_5 = k_7 = 5 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$); the catalytic constants for OGAs with DPs of 4–6 were assumed to be equal ($k_4 = k_6 = k_8$); the dissociation constant of the E–PGA (enzyme–PGA) complex,

Table 2 BcPeh28A kinetic parameters for different substrates

For each derived kinetic constant, the associated S.D. is also reported.

Substrate	Binding constant ($M^{-1} \cdot s^{-1}$)	Dissociation constant (s^{-1})	Catalytic constant (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
PGA	$k_1 = 5 \times 10^8$	$k_{-1} = 0$	$k_2 = 155 \pm 39$	3×10^{-7}	5×10^8
DP 4	$k_3 = 5 \times 10^8$	$k_{-3} = (1.67 \pm 0.41) \times 10^5$	$k_4 = 31 \pm 4.9$	3.3×10^{-4}	9.3×10^4
DP 5	$k_5 = 5 \times 10^8$	$k_{-5} = 8526 \pm 3869$	$k_6 = 31 \pm 4.9$	1.7×10^{-5}	1.8×10^6
DP 6	$k_7 = 5 \times 10^8$	$k_{-7} = 5326 \pm 2496$	$k_8 = 31 \pm 4.9$	1.1×10^{-5}	2.9×10^6

k_{-1} , always converging to very small values, was set equal to zero; the initial enzyme concentration was set to 2.0×10^{-8} M (see under 'Time-course experiment' above). Table 2 reports the imposed and derived kinetic constants in detail. The basic criteria for the goodness-of-fit were obeyed throughout the analysis [34].

Mode of action on OGAs

Hydrolysis of decagalacturonide

BcPeh28A ($0.075 \mu\text{g/ml}$) was incubated at 37°C with decagalacturonide ($50 \mu\text{M}$) in 50 mM sodium acetate buffer, pH 3.5. Aliquots ($100 \mu\text{l}$) of the reaction mixture were taken at regular time intervals and the reaction was stopped by the addition of an equal volume of 2.0 mM Tris/HCl and 50 mM NaOH, which resulted in a final pH of 8.3–8.5. In order to avoid the spontaneous breakdown of the obtained OGAs, reported to occur at high pH [13], the samples were immediately analysed by HPAEC-PAD, as described below. The identification of the hydrolysis products was done in accordance to the retention time of a standard containing a mixture of OGAs with DPs in the range 1–8.

Hydrolysis of hexagalacturonides

BcPeh28A ($1.0 \mu\text{g/ml}$) was incubated at 37°C with $25 \mu\text{M}$ hexagalacturonides (1–4) in 50 mM sodium acetate buffer, pH 3.5, for different incubation times. The enzymatic digest was analysed by ESI-TOF-MS (electrospray ionization MS with a time-of-flight analyser), as described below.

Analytical techniques

Capillary electrophoresis

CE experiments were carried out using a Hewlett–Packard HP3D CE system, equipped with a diode-array UV detector. Fused capillaries were from Agilent Technologies (total length: 104 cm; effective length: 95.5 cm; internal diameter $50 \mu\text{m}$; extended light path). Before sample injection, a 4 min conditioning of the capillary with the running buffer was necessary, preceded by a 2 min wash with 0.1 M NaOH [pressure equal to 950 mbar; 1 bar = 10^5 Pa]. Signals were acquired by continuously monitoring UV absorbance at 195 and 285 nm. All analyses were done at 25°C . The separation potential was equal to 27 kV. Samples were injected at 50 mbar for 6 s. The monomer, dimer and trimer of GalA (Sigma–Aldrich), as well as a sample containing a mixture of OGAs with DPs of 1–8, were used as standards. To permit sensitive UV detection, the saccharides were derivatized by reaction with 4-ABN (4-aminobenzonitrile) in the presence of NaCNBH₃ (sodium cyanoborohydride) [35]. The derivatization solution was 0.16 M NaCNBH₃ and 0.5 M 4-ABN in methanol/acetic acid (95:5, v/v). Only one molecule of the 4-ABN chromophore is attached to each oligomer, leading to a UV response that is independent of the length of the OGAs. This represents an advantage over chromatographic techniques

such as HPAEC-PAD, where the electrochemical response must be determined for each oligomer [36]. The derivatization solution was diluted 1:1 with an aqueous solution of the uronic acid(s), and the derivatization mixture was heated for 20 min at 95°C . Prior to injection into the CE system, samples were spun down for 1 min at 13 000 g. The analysis was performed by MEKC (micellar electrokinetic capillary chromatography) as described in [36]. The buffer used was 600 mM boric acid, containing 75 mM SDS (pH 8.0). The peak areas divided by migration time (A/t) were used for quantitative purposes [37]. To obtain a calibration curve, electropherograms for different amounts of GalA were recorded, and their corresponding A/t values were plotted as a function of nmol of GalA. Calibration was linear over the range 1.0–14.0 nmol.

HPAEC-PAD

The analysis of oligomers released upon hydrolysis of decagalacturonide by BcPeh28A was carried out with a HPAEC-PAD system (Dionex Corporation) equipped with a GP50 gradient pump, an ED50 electrochemical detector and an LC25 chromatography oven. The separation was performed using a CarboPac PA-200 Analytical anion-exchange column (3 mm $\times$ 250 mm) with Guard column (3 mm $\times$ 50 mm). The volume of the injection loop was $25 \mu\text{l}$, and the column temperature was 35°C . The flow-through cell of the pulsed amperometric detector consisted of an gold working electrode (1.0 mm diameter) and a pH–silver/silver chloride combination reference electrode; the titanium body of the cell served as the counter-electrode. The sequence of potentials (E) applied to the electrode was set as recommended by the manufacturer for carbohydrate detection, as follows:

$t = 0.00$ s, $E = 0.10$ V

$t = 0.20$ s, $E = 0.10$ V (integration begins)

$t = 0.40$ s, $E = 0.10$ V (integration ends)

$t = 0.40$ s, $E = -2.00$ V

$t = 0.40$ s, $E = -2.00$ V

$t = 0.43$ s, $E = 0.60$ V

$t = 0.40$ s, $E = -0.10$ V

$t = 0.50$ s, $E = -0.10$ V.

The software Chromeleon, version 6.60 (Dionex Corporation) was used for the acquisition and processing of the chromatograms. The column was equilibrated with 0.1 M NaOH/0.2 M sodium acetate. The samples were eluted by applying a linear gradient of 0.2–0.8 M sodium acetate in 0.1 M NaOH at 0.5 ml/min for 60 min. The identification of the hydrolysis products was done by correlating with the retention times of a standard containing a mixture of OGAs with DPs of 1–8.

Figure 2 Electropherograms of a sample containing a mixture of OGAs with DPs 1–8 (A) and of the reaction mixture after 30 min of incubation at 37 °C on 0.25% PGA (B)

Details are given in the Experimental section.

ESI-TOF-MS

ESI-TOF-MS was performed on a microTOF Focus system (Bruker Daltonics) equipped with a nitrogen generator N2LCMS1 (Claind). The samples were diluted 5-fold with methanol/water/formic acid (49:49:2, by vol.) and injected with a syringe pump in the mass spectrometer. The injection flow rate was 180 μ l/h; the capillary voltage was 4500 V and the end-plate offset was 500 V (positive mode); the dry temperature was 180 °C, the dry gas flow was 4 l/min and the nebulizer pressure was 0.4 bar.

RESULTS AND DISCUSSION

Time-course experiment

The mode of action of BcPeh28A on a polymeric substrate was investigated in a time-course experiment that monitored the products released upon PGA hydrolysis by collecting electropherograms. The suitability of MEKC with UV detection for the separation of uronic acids [36] was tested on a standard sample containing a mixture of OGAs with DPs of 1–8, as shown in Figure 2(A).

The product progression profile (Figure 3A) indicates that, during the first few minutes of the reaction, OGAs with DPs ranging from 3 to 8 were produced. This confirms that BcPeh28A is an endo-acting enzyme. The electropherogram of the reaction mixture shown in Figure 2(B) shows the molar product distribution present in the enzymatic digest. The occurrence of OGAs with different chain lengths clearly demonstrates that BcPeh28A can be classified as single-attack enzyme. The minimum length of the released OGAs varies among pectin-degrading enzymes and provides further information on the active-site architecture of the enzyme. For the PGA hydrolysis catalysed by BcPeh28A, the end products of the reaction are monomers, dimers and trimers, with a clear excess of trimers (Figure 3B). To verify whether BcPeh28A is capable of further degrading the trimers, a commercial sample of tri-

Figure 3 Progression profile curves for released products on incubation of PGA (0.25 g/l) with BcPeh28A at 20 °C over 10 min (A) and over 1.5 h (B)

Δ , DP 1; $\blacklozenge$, DP 2; $\blacktriangle$, DP 3; $\blacksquare$, DP 4; $\square$, DP 5; $+$, DP 6; $\times$, DP 7; $*$, DP 8. Details are given in the Experimental section.

GalA was incubated with the enzyme. No cleaved products were observed (results not shown). Hence the substrate-binding cleft of BcPeh28A is likely to span at least four subsites. This finding allows us to classify BcPeh28A as a trimer-releasing enzyme, in keeping with other bacterial endoPGs [24].

In order to gain a more detailed insights into the catalytic site of BcPeh28A, its amino acid sequence was pairwise and structure-based aligned with that of the well-studied non-processive enzyme *A. niger* endoPGII. It appears (Figures 1 and 4) that the catalytic amino acids forming subsites -1 and $+1$, belonging to rungs 5, 6, 7 and 8, are very well conserved, whereas the amino acid residues associated with the distal subsites are more variable (Met¹⁵⁰/Glu¹⁹², Asn¹⁸⁶/Gly²³⁴, Glu²⁵²/Asp³¹⁰, Asp²⁸²/Gln³⁴², Gln²⁸⁸/Thr³⁴⁷, Tyr³²⁶/Glu⁴¹⁷). It is noteworthy that a serine residue, Ser¹⁰⁴, is present in putative rung 2 of BcPeh28A, thus supporting the previously established criteria according to which a single amino acid residue can determine the processive or non-processive behaviour of endoPGs [23].

Kinetic model

A kinetic model that describes the BcPeh28A mode of action on PGA has been constructed (see 'Kinetic model' in the

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RUNG 1  Anig_PGII  61  LDLTGLTSG-----TKVIFEGTTTFQYEE----WAGPL  89
        BcPeh28A  61  PGLVDLASNNGISTAVITSVNLANIVLKLEKGF1TLKGSPA 101
                .*. : . . . : * : . . . :
RUNG 2  Anig_PGII  90  -ISMSGEHITVTGASGHLINCDGAR-----WWDGKGTSGKKKPKF 128
        BcPeh28A 102  QPS-SGAMLTGSNLSNLTITGTGAIDGDGQDYWPAAVGQNNTARPKL 147
                * * : * : . * . * * * * . * . . . : * :
RUNG 3  Anig_PGII 129  FYAHG-----LDSSSITG--LNIKNTPL1MA 151
        BcPeh28A 148  IAITGSNLQIGSNFTDAGKSQSIVAFSPSSNATGSALIIRNSPKEQ 193
                : * * * * . * . * * * * :
RUNG 4  Anig_PGII 152  FSVQAN--DITFTDVTINNADGDTQGG----HNT1DA 181
        BcPeh28A 194  LVIESGSKNVTIDGVWIYANPNRNASGDDLAPNTDA 229
                : : . : * : . * * . . . * * * *
RUNG 5  Anig_PGII 182  F1DVGNSVGVNIIKPWVHNQ1DDC 203
        BcPeh28A 230  IDIIGTQTATIKNCLLDTGDDD 251
                : * : . . * : . . * *
RUNG 6  Anig_PGII 204  LAVNSGE-----NIWFTGGTCIG1HG 224
        BcPeh28A 252  IAIKSNAGGAATSSVNVSHCVVGGGHG 278
                : * : * . . : . : * * * *
RUNG 7  Anig_PGII 225  LSI1GSVGD1RSNNVVKN---VTIEHSTVSN1E 252
        BcPeh28A 279  ISIGGQEAAGTTLAKPGVSQVTVD1TMQFSGTD 310
                : * * . . . . : * * * : . * : :
RUNG 8  Anig_PGII 253  NAV1RI1KTISGAT--GSVSEITYSNIVMSGIS1D 282
        BcPeh28A 311  YGYRIKTDQTAKDSGATTGVTYRNTCMRNVQQ 342
                . * * * * . * . * : : * * * * . . :
RUNG 9  Anig_PGII 283  YGVVI1Q1QDYEDGKP-----TGKPTNGVTIQDVKLESVTGSVDSGA 322
        BcPeh28A 343  P-FLFTYAYASGTG[G356-L387]MGVPKSGDTGISITNSQISGGKA1FSV 413
                . : * . * . * * * * . . . . : .
RUNG 10 Anig_PGII 323  TEI1YLLCGSGS-CSDWTWDDVKVT 345
        BcPeh28A 414  TDGELQLGSHSSATTSTGSNGQVV 437
                * : * * * * : * . : : *

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Figure 4 Structure-based amino acid sequence alignment of *A. niger* endoPGII (PDB code 1CZF) and BcPeh28A

The amino acid residues of *A. niger* endoPGII shown in Figure 1 are in boldface and those that are conserved in BcPeh28A are also underlined. Ser⁹¹ (*A. niger* endoPGII) and Ser¹⁰⁴ (BcPeh28A) are underlined. They belong to rung 2 and are responsible for the non-processive enzymatic behaviour. The asterisk (*), colon (:) and dot (.) symbols indicate identical residues, conserved substitutions and semi-conserved substitutions respectively.

Experimental section). The reaction scheme has been rationalized, as illustrated in Figure 5(A). By inspecting the product progress curves (Figure 3), it is possible to assume that OGAs with DPs of 3–6 are produced rapidly after mixing PGA with the enzyme, whereas monomers and dimers emerge only later as second-generation products. Additionally, as previously mentioned, monomers, dimers and trimers accumulate over time, whereas OGAs with DPs of 4–6 appear at the beginning of the reaction and are cleaved later to yield shorter ones. Thus trimer formation is the result of a rapid release from either PGA or DP 4–6 reprocessing. The percentage of uncleaved PGA at the end of the reaction has been evaluated to be 99%, confirming that the time-course experiment was performed in a large excess of substrate. The excellent agreement between the experimental progression curves shown in Figure 3(B) and those obtained as the result of the kinetic model based on a numerical integration method, is shown in Figure 5(B).

A number of conclusions can be drawn from the kinetic constants derived from the model (see Table 2). It has been

assumed that the dissociation rate constant of the E-PGA complex, k_{-1} , can be approximated to zero. As a consequence, the specificity constant (k_{cat}/K_m) value for PGA approaches the diffusion-controlled rate limits, i.e. the specificity constant for BcPeh28A for PGA approaches its maximum. This means that the catalytic velocity on PGA is only restricted by the rate at which BcPeh28A encounters the polymer in solution. Hence, every encounter between the enzyme and the substrate is productive. Moreover, the k_{cat} value for PGA is higher if compared with the k_{cat} values for oligomers, suggesting that BcPeh28A degrades long polymeric chains faster than the short OGAs.

With regard to the hydrolysis of OGAs, it has been assumed that, once BcPeh28A binds to an oligomer with a DP of 4–6, catalysis takes place at the same rate, independently of the length of the substrate bound ($k_4 = k_6 = k_8$). This implies that the rate-determining step of the enzymatic reaction on OGAs is the formation of a stable and productive ES complex. At this point, it is noteworthy that the only kinetic parameters that enable differentiation of oligomers from one another are the dissociation constants,

Figure 5 Reaction scheme (A), progression curves (B) and a graphical view of binding modes (C)

(A) Reaction scheme constructed on the basis of the product progression curves reported in Figure 3(B). (B) Progression curves of OGAs with DPs of 1–6 derived from the experimental data (continuous lines) and reconstructed according to the kinetic model (broken lines). (C) A graphical view of the productive binding modes between the enzyme and the substrates (oligomers with a DP of 4, 5 and 6 respectively). The reducing end of OGAs is indicated by the symbol $\emptyset$, whereas an arrow indicates the cleavage sites. The percentage of occurrence of each binding mode and the associated S.D. values are also reported.

whose values decrease with increasing chain length of the substrates ($k_{-3} > k_{-5} > k_{-7}$). In order to explain this trend, it is convenient to introduce the concept of binding modes. They can be referred to as the multiple putative modes of interaction that may occur between the enzyme subsites and the saccharide units of the substrate chain. From a catalytic point of view, only the binding modes that involve binding at the subsites -1 and $+1$ can lead to a product release, thereby permitting discrimination between productive and non-productive binding modes.

Longer substrate molecules, such as those with a DP of 6, that can interact with a large number of enzyme subsites, exhibit a higher overall binding affinity when compared with those of shorter OGAs such as those with a DP of 4 or 5. Binding of shorter OGAs is unfavourable, which is confirmed by their higher

Figure 6 HPAEC-PAD analysis of the hydrolysis of the decagalacturonide before (A) and after (B) incubation for 5 min at 37 °C with BcPeh28A

Details are given in the Experimental section.

dissociation constants. Such a trend of dissociation constants consequently determines the decreasing trend of K_m values with the increase in the substrate chain length. It should be emphasized that, since the binding of oligomers such as those with a DP of 4 or 5 can occur in more than one way (Figure 5C), one would expect different affinities for each binding mode. The kinetic model indirectly allowed the identification of the favoured binding modes by introducing a partition coefficient for the distribution of the end products. The resulting distribution led us to conclude that the subsites at positions -3 , -2 , -1 and $+1$ display the highest binding affinities, being those that are most likely to be involved in the formation of the favoured productive ES complexes (Figure 5C). Similar conclusions have been reported by studying the active-site architectures of endo-(1 $\rightarrow$ 5)- α -L-arabinanases from *A. niger* and *A. aculeatus* [38] and of the endo- β -(1 $\rightarrow$ 4)-xylanase from *Penicillium simplicissimum* [39]. Crystallographic studies on the *P. simplicissimum* xylanase and biochemical data on the hydrolysis of xylo-oligosaccharides with DPs of 2–6 revealed that 4 is the minimum substrate length for cleavage. This suggests that the two catalytic residues divide the binding cleft into a 'substrate-recognition area', from the active site towards the non-reducing end of a bound xylan chain, with strong and specific binding for the first three xylose sugar units, and a 'product-release area' with considerably weaker and less specific binding.

Mode of action on OGAs

Hydrolysis of decagalacturonide

To investigate whether BcPeh28A displays a preferential cleavage mode on shorter substrate chains, its mode of action on a pure decagalacturonide was studied. The hydrolysis products were analysed by the highly sensitive HPAEC-PAD technique, since the available amount of the initial substrate was under the sensitivity limit for OGA detection by MEKC-UV. The purity of the decagalacturonide, prior to the enzymatic incubation, was checked by HPAEC-PAD. Small amounts of octa- and nona-galacturonides were detected (Figure 6A). Following the incubation with the enzyme (Figure 6B), the decagalacturonide sample was hydrolysed, and the formation of OGAs with DPs of 3–7 was detected. The reaction was stopped as previously described, by adding 2.0 mM Tris/HCl and 50 mM NaOH. This buffer produced a large peak that masked the signal of monomers and dimers, making their detection impossible. HPAEC-PAD analysis of the decagalacturonide digestion products indicated that BcPeh28A is an endo-acting enzyme that displays a non-processive behaviour also on shorter substrates. As previously

The reducing end is indicated by the symbol Ø.

Figure 8 Mass spectra of the products released after the enzymatic hydrolysis by BcPeh28A of compound 1 after 1 h of incubation at 37 °C (A and B) and of compound 3 after 20 min of incubation at 37 °C (C and D)

The molecular masses indicated by the arrows were assigned as follows: peak 1: m/z 1139.25 $[M + Na]^+$, hexagalacturonide 1; peaks 1a and 3a: m/z 407.08 $[M + Na]^+$, monomethylated dimer; peak 1b: m/z 773.16 $[M + Na]^+$, dimethylated tetramer; peak 3b: m/z 583.11 $[M + Na]^+$, monomethylated trimer. Details are given in the Experimental section.

3a: m/z 407.08, $[M + Na]^+$ and monomethylated trimer (peak 3b: m/z 583.11, $[M + Na]^+$) were identified as end products. The identification of these two hydrolysis products strongly suggests that two productive ES complexes can occur (Figure 9, panels 2 and 3). In either case, BcPeh28A will accommodate three consecutive unmethylated saccharide units at subsites -2 , -1 and $+1$.

The ESI-TOF-MS analyses showed that compound 1 was not fully hydrolysed by BcPeh28A after 1 h of incubation at 37 °C (Figure 8B), in contrast with compound 3, which, under the same conditions, was completely degraded after 20 min. This finding suggests that compound 3 is a substrate preferred over compound 1. Their different susceptibilities to the enzymatic hydrolysis can be ascribed to the two binding modes in contrast with the one productive binding mode that probably exist for compounds 3 and 1 respectively (Figure 9). However, it should be emphasized that these findings are of a qualitative nature. The ionization efficiency of methylated OGAs is influenced by their chain length and methylation pattern. Namely, the degree of methylation of hexagalacturonides 1 and 3 may affect changes in their volatility and hydrophobicity [43]. Therefore ESI-TOF-MS was reliable only for a qualitative assessment of the mixtures analysed.

Overall, the observed action patterns of BcPeh28A on compounds 1 and 3 and its inability to hydrolyse compounds 2

Figure 9 BcPeh28A binding modes of compounds 1 (panel 1) and 3 (panels 2 and 3)

The reducing end is indicated by the symbol Ø, whereas an arrow indicates the cleavage sites.

and 4 indicate that, in order to form a productive ES complex, the substrates should at least contain three consecutive unmethylated saccharide units. In addition, among the six subsites identified, those referred to as -2 , -1 and $+1$ can be supposed to be

responsible for crucial ES contacts. Indeed, methylated saccharide units cannot be accommodated at these subsites, thus excluding the formation of the productive ES complex observed for compounds 2 and 4. The subsites -4 , -3 , $+2$, $+3$ and $+4$, if all of them exist, are likely to be less critical for the formation of a productive ES complex, since they can accommodate a methylated saccharide unit (Figure 9). This finding is in agreement with results obtained from the digestion of pectins with a different extents of methyl-esterification by *A. niger* and *E. carotovora* endoPGs [44].

In perspective, further studies are needed to fully elucidate the structural determinants that are responsible for the binding affinities of each subsite by using pure unmethylated and *ad hoc* methyl-esterified OGAs with DPs of 5–8, in concert with a site-directed mutagenesis approach aimed at determining the exact topology of the amino acid residues at each subsite.

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