**pgaA and pgaB encode two constitutively expressed endopolygalacturonases of Aspergillus niger**

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**INTRODUCTION**

Aspergillus niger is a saprophytic micro-organism producing a vast number of extracellular enzymes involved in degradation of plant cell-wall material. Pectin is one of the major heteropolysaccharides found in the middle lamella and the primary plant cell wall, and has an important function in the structural organization of other cell-wall components, such as cellulose and hemicellulose [1].

A. niger enzymes degrading the homogalacturanon part of the pectin molecule include several pectin lyases [2–4], a single pectate lyase (J. A. E. Benen, H. C. M. Kester, L. Parënicová and J. Visser, unpublished work), pectin methylesterase [5] and a family of endopolygalacturonases (EC 3.2.1.15) (PGs) [6–9].

At present, more than 30 different fungal pga sequences have been deposited in the Databases, the majority of which have been determined for genes from phytopathogenic micro-organisms. As in A. niger [8], PG-encoding genes were discovered in the phytopathogenic fungi Sclerotinia sclerotiorum [10] and Botrytis cinerea [11]. However, little is known about the role of the different PGs in these micro-organisms. Only recently, studies on pga gene expression in the phytopathogenic fungi Colletotrichum lindemuthianum [12] and A. flavus [13], and the analysis of secreted PG isoforms during the growth of S. sclerotiorum on polygalacturonate [14] showed differential expression of these genes and secretion of the proteins. These results suggest that, although fungal PGs often show a high degree of sequence identity, their physiological function is different.

In order to understand the nature of the individual A. niger PG-encoding genes and their corresponding enzymes, we decided to analyse all of these, in detail, at the molecular and biochemical level. The A. niger pga gene family consists of seven different genes [8]. To date, the genes pgaI [7], pgaII [6], pgaC [8] and pgaE [9] as well as the corresponding enzymes [9,15] have been characterized. In the present study, two novel pga genes of A. niger, pgaA and pgaB, and the two enzymes they encode are described.

**EXPERIMENTAL**

**Strains**

For induction studies, the wild-type laboratory strain A. niger N400 (=CBS 120.49) was used. The A. niger strain NW188 (cspA1, pyrA6, leuA1, prlF28, goxC17), which is derived from A. niger N400, was used for transformation of expression plasmids. The A. niger multicopy transformants, 617.42-1 and 617.49-15 of pIM3763 and pIM3773 (for a description see below), were used for overproduction and purification of PGA and PGB respectively. For cloning and propagation of plasmids and phages, Escherichia coli DH5α [16] and E. coli LE392 [17], were used.

**Phages and plasmids**

Isolation of recombinant pBluescriptII phages was described by Bussink et al. [8]. The phagemid pBluescriptSK- [18] and plasmids pUC18 [19], pGEM-7 (Promega, Madison, WI, U.S.A.) and pEMBL19 [20] were used for subcloning of chromosomal DNA fragments or the construction of expression plasmids. The pGEM-T Easy kit (Promega) was used for cloning of PCR fragments.

Plasmid pGW635 carrying the pyrA gene, which encodes orotidine-5’-phosphate decarboxylase from A. niger [21], served as a selection marker for restoration of uridine protrophy.

Abbreviations used: CREA, carbon catabolite repressing protein A; (GalpA)n, oligogalacturonate with n degrees of polymerization; PG, endopolygalacturonase; BCF, bond-cleavage frequency; HPAEC-PAD, high performance anion-exchange chromatography with pulsed amperometric detection.

1 To whom correspondence should be addressed (e-mail office@algemeen.mgim.wau.nl). The nucleotide sequence data for pgaA and pgaB have been deposited with the EMBL, GenBank and DDBJ Databases under accession numbers Y18804 and Y18805 respectively.
The expression plasmids containing the pkiA promoter–gene fusion were constructed in the following way. The 0.7 kb BamHI–NsiI fragment of the pkiA promoter was isolated from pUC18HN [9]. In the next step, plIM3760 served as a template to introduce the NsiI restriction site at the translational start of pgaA by PCR using Taq polymerase (Pharmacia Biotech., Uppsala, Sweden). For the PCR synthesis of the NsiI–KpnI pgaA fragment (see Figure 1), two primers were designed: primer pgaA-NsiI, a nineteen-residue long oligonucleotide, 5'CGCAATCATGCTATCTGCC 3', localized at position 1907–1925 bp, and primer pgaA-KpnI, a twenty-residue long oligonucleotide, 5'AGATCCGCGCGAAGTAG 3', annealing at position 2640–2621 bp of the pgaA gene. The PCR conditions were: denaturation for 5 min at 95 °C, 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 1 min extension at 72 °C. The final extension time was increased to 5 min. The resulting 734 bp pgaA PCR product was checked by sequencing for PCR errors. The pkiA promoter BamHI–NsiI fragment and the pgaA NsiI–KpnI fragments were ligated into pGEM-7. In the final ligation, the 2.5 kb KpnI–EcoRI fragment (see Figure 1), containing the remainder of the pgaA gene and its downstream sequence, was cloned into pGEM-7 harbouring the pkiA–pgaA fragment, resulting in plIM3763. For construction of plIM3773, the pkiA–pgaB expression plasmid, the presence of the NsiI site localized at the translational start of the pgaB gene was exploited. The 1.6 kb XhoI fragment (see Figure 1) containing the pgaB promoter sequence and the 5' end of the pgaB coding sequence was subcloned into pBluescriptSK+. In the next step, the pgaB promoter part of this subclone was replaced by the 0.7 kb BamHI–NsiI fragment of the pkiA promoter from pUC18HN. The final construct was obtained after ligation of the 1.3 kb XhoI fragment, consisting of the pgaB gene and an additional 170 bp of sequence downstream of the 3' end of pgaB.

**DNA and RNA manipulations**

The phage and plasmid DNA isolation and other molecular techniques were essentially carried out as described by Sambrook et al. [17]. The restriction enzymes were used as described by the supplier (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, U.S.A.). The nucleotide sequences were determined using either the Cy5™ AutoCycle Sequencing Kit (Pharmacia Biotech.) or the Cy5™-dATP Labeling Mix (Pharmacia Biotech.) with universal and reverse primers or with gene-specific primers respectively. The reactions were analysed with an ALFexpress™ DNA sequencer. Computer analysis was done using the program GeneRunner (Hastings Software, Inc., Hastings, NY, U.S.A.).

The total RNA was isolated using TRIzol™ Reagent according to the supplier (Gibco BRL). The RNA concentration was estimated spectrophotometrically at 260 nm. RNA (20 μg) was loaded on to and separated in 1.2% (w/v) agarose gel under glyoxal denaturation conditions [17]. After electrophoresis, the gels were capillary blotted overnight on to Hybond-N+ membranes (Amersham International, Little Chalfont, Bucks., U.K.).

Prehybridization and hybridization of Southern blots were carried out at 56 °C or 68 °C for heterologous or homologous screening respectively [17]. Northern blots were hybridized at 42 °C in hybridization buffer containing 50% (v/v) of formamide. For pgaA the 335 bp XhoI–XbaI fragment, localized in the 3' part of the gene, and the 3' non-translated region was used as a probe, and for pgaB the NsiI–KpnI 200 bp fragment from the 5' end of the gene served this purpose (see Figure 1).

Transformation of *A. niger* was done as described previously [3], using 1 μg of plgW635 and 20 μg of co-transforming plasmid DNA.

**cDNA synthesis**

The *A. niger* transformants, 617.42-1 and 617.49-15, were grown for 18 h on minimal medium containing 3% (w/v) fructose. Total RNA isolated from the mycelium was used for the partial cDNA synthesis by reverse transcription-PCR. The reactions were performed according to Gilliland et al. [23] with minor modifications; 1 μg of RNA was used in the synthesis of the first strand cDNA, using the Moloney murine leukemia virus reverse transcriptase under the conditions described by the supplier (Gibco BRL). Gene-specific primers were used. In the case of pgaA, primer pgaA-KpnI was used in the first reaction (see above), and for pgaB a new primer was designed, a nineteen-residue long oligonucleotide, 5'AGTGGCACGTTCTGCGCCG 3', annealing at position 3161–3143 bp. In the second step, the cDNA synthesis was completed by PCR, employing forward primers annealing in the 5' region of the respective gene. For pgaA, a twenty-residue long oligonucleotide, 5'TGTTCGCTGGCCG-ACCTTG 3', annealing at position 3193–3152 bp, and for pgaB, an eighteen-residue long oligonucleotide, 5'GCGTCTCCGAGAACGC 3', annealing at position 2227–2244 bp, was used. The amount used of each primer was 100 pmoles. The PCR cycler was programmed as described above with the following
changes in the annealing temperature. For pgaA a temperature of 43.5 °C was used and for pgaB the temperature was 50 °C. The resulting PCR products were checked by sequencing.

Induction studies

A. niger N400 mycelium was pre-grown for 20 h in Erlenmeyer flasks with 200 ml of minimal medium containing per litre: 6.0 g NaNO₃, 1.5 g KH₂PO₄, 0.5 g MgSO₄·7 H₂O, 0.5 g KCl, trace elements [24], 0.1% (w/v) dialysed yeast extract [9] and 1% (w/v) sucrose. The flasks were inoculated with 10⁶ spores ml⁻¹ and the cultures were grown at 30 °C in an orbital shaker at 250 rev./min. The pre-grown mycelium was harvested using a Büchner funnel with nylon gauze, extensively washed with sterilized saline (9 g/1 NaCl) and water. Aliquots (3 g) of wet mycelium were then transferred to 250 ml Erlenmeyer flasks containing 50 ml of minimal medium and 1% (w/v) of a carbon source, as specified in Figure 2. Sucrose, sugar beet pectin (Copenhagen Pectin A/S, Lille Skensved, Denmark) and polygalacturonate acid were added to the media before autoclaving, whereas d-galacturonic acid and l-rhamnose were filter-sterilized and added after autoclaving. The starting pH of the cultures was adjusted to pH 6.0.

Enzyme purification and characterization

The PGA and PGB overproducing transformants were selected as described previously [9]. The transformants, 617.42-1 and 617.42-15, producing the highest amount of PGA and PGB respectively, were used for a large scale purification under the same culture conditions as applied for overproduction of PGE [9]. The PGA was purified using the same chromatographic steps as applied for the purification of PGE [9]. For the isolation of PGB, the culture fluid was diluted three-fold with water and the pH was adjusted to 4.0. Next, pre-swollen CM-Sephadex C-50 (100 g) were added and stirred for 16 h at 4 °C. Bound PGB was eluted from the matrix by a pulse of 1M NaCl in 20 mM sodium acetate buffer, pH 4.0, followed by extensive dialysis against the same buffer. For final purification, the enzyme was loaded on to a S-Sepharose Fast Flow column (2.6 cm × 20 cm) and eluted with a 1200 ml linear 0-8 M NaCl gradient in buffer. Enzyme-containing fractions were pooled, dialysed against 50 mM sodium acetate buffer, pH 5, and stored at 4 °C in the presence of 0.02% (w/v) NaN₃. The purity of both enzyme preparations was monitored by SDS/PAGE and the gels were stained with Coomassie Brilliant Blue R 250. The molecular masses were estimated by SDS/PAGE; the gel was calibrated with protein test mixture 4 (Serva-Boehringer, Ingelheim, Germany). The protein concentrations were calculated from the molecular absorption coefficients using the method of Edelhoch [25].

PG activity was routinely assayed as described for PGE [9], using 50 mM sodium acetate buffer pH 4.2 or pH 5.0 for PGA and PGB respectively. The pH optima and the effect of the degree of methylation of the substrate on activity were determined as described previously [15]. Product-progression analysis on polygalacturonate and pectins with various degrees of esterification, as well as the determination of bond-cleavage frequencies on oligogalacturonates with different degrees of polymerization (DP = 2-8), were performed as described previously [9].

RESULTS AND DISCUSSION

Subcloning of the pgaA and pgaB genes

Phages λA43 and λB4, representatives of the classes A and B [8] respectively, were selected for Southern blotting using the 1.3 kb BamHI/BglII pgaI-specific probe (see the Experimental section). The λA43 analysis revealed a 4.5 kb HindIII and a 5.0 kb EcoRI fragments, both hybridizing strongly (Figure 1). For subcloning of the 3.8 kb EcoRI-hybridizing fragment from λB4 (Figure 1) the same strategy was employed, which resulted in pIM3770.

Analysis of nucleotide sequences of pgaA and pgaB

The entire 5.8 kb EcoRI–HindIII subclone derived from λA43 contains 1167 bp of coding sequence and the 1913-bp upstream and the 2700-bp downstream sequence. The pgaB nucleotide sequence includes 2224 bp of the 5’ non-coding region, 1234 bp of the pgaB coding sequence and 330 bp of the 3’ non-coding region (Figure 1).

Based on the sequence alignment of pgaA and pgaB with other fungal pga genes present in the EMBL Gene Database, introns of 54 bp in length for pgaA and 70 and 74 bp for pgaB were predicted, which were confirmed by cDNA sequencing of the regions indicated in Figure 1. The intron boundaries in both genes follow the consensus sequences found for filamentous fungal introns [26,27].

When compared with other genes, the A. niger pga genes are highly identical in structural organization of introns and exons with those of A. tubingensis [28], A. oryzae [29], A. flavus [13] and A. parasiticus [30].

5’ and 3’ non-transcribed regions of pgaA and pgaB genes

According to Gurr et al. [27], the transcription start point of filamentous fungal genes is often preceded or followed by CT-rich motifs, and contains TATAAA and CAAT boxes. In the case of the pgaA promoter, a TATAAA sequence, a modified version of a TATAAA box, was found at position -160 bp upstream from the translational start point, and three CAAT boxes were found between -192 and -4 bp. In the case of the pgaB promoter, no CAAT or TATAAA boxes could be detected close to the translation start point, but a CT-rich region was present at the position -91 to -44 bp [27]. Both of the genes fulfill the Kozak rule [31] in that a purine (adenosine) is present at position -3 bp from the AUG translation initiation codon.

It was shown previously for pectinolytic genes from other fungi, like a pectate lyase from A. nidulans [32] and an exopolypgalacturonase from A. tubingensis [33] that (poly-)galacturonidase had a positive effect, whereas glucose had a negative effect on the expression. The promoters of both the pgaA and pgaB genes were analysed for the presence of specific DNA sequences, such as consensus CREA (carbon catabolite repressing protein A) binding sites [34-36] and the 5’ non-transcribed regions of fungal promoters is often preceded or followed by CT-rich motifs, and contains TATAAA and CAAT boxes. In the case of the pgaA promoter, a TATAAA sequence, a modified version of a TATAAA box, was found at position -160 bp upstream from the translational start point, and three CAAT boxes were found between -192 and -4 bp. In the case of the pgaB promoter, no CAAT or TATAAA boxes could be detected close to the translation start point, but a CT-rich region was present at the position -91 to -44 bp [27]. Both of the genes fulfill the Kozak rule [31] in that a purine (adenosine) is present at position -3 bp from the AUG translation initiation codon. Ten and six consensus CREA binding sites were found at a distance of -325 and -185 from the translation start codon to the far upstream sequence of the pgaA and pgaB promoters respectively. Furthermore, in the pgaA 5’ promoter region (-1525 bp from the ATG), the sequence 5’TGATTGGT 3’, resembling the pgaI5’TGYATTGGTGA 3’ sequence [37], was found on the opposite strand.

The search for a (truncated) polyadenylation signal, (A)AUAA [27], revealed the sequence, AUAAA, 253 bp downstream of the pgaA-gene translation stop codon, but a similar motif was not observed in the pgaB sequence.

Influence of the various carbon sources on pgaA and pgaB expression

pgaA- and pgaB-specific probes were checked for cross-hybridization with other A. niger pga genes by a dot-blot analysis, under
straightforward when the complex sugar beet pectin, which contains high amounts of neutral sugars such as l-arabinose, d-galactose and d-xylose, was used. The two pentoses constitute more easily metabolized substrates than d-galacturonic acid and l-rhamnose, and therefore the expression of pgaA and pgbB is probably similar to the expression on sucrose. The same feature, the constitutive expression on glucose and pectin, was previously reported for the pecA genes from A. flavus [13] and A. parasiticus [30], which also encode a PG. Interestingly, the derived amino acid sequences of these PGs share the highest sequence similarity and phylogenetic relatedness with the A. niger PGA and PGB [11].

From the results presented in Figure 2, it is obvious that, using defined pectic constituents, such as d-galacturonic acid, d-galacturonic acid in combination with l-rhamnose, and polygalacturonic acid, the expression levels of both pgaA and pgbB genes differ depending on the substrate and time of induction. Whereas the level of the pgbB transcript 2 h after the change of medium was similar on all carbon sources tested, there was an increase in the level of pgaA transcription not only on sucrose but also in the presence of d-galacturonic acid. The high transcription signal detected on d-galacturonic acid might be related to a DNA sequence resembling the pgaII 5′ TYATTGG-TGGA 3′ upstream-activating sequence [8,37] in the 5′ part of the pgaA promoter. This sequence is not present in the pgbB promoter. The pgaA transcript levels on d-galacturonic acid were apparently lower when l-rhamnose was present simultaneously. Only when polygalacturonic acid was used as a substrate, was pgaA transcription reduced 2 h after the change of medium. High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis of the culture medium (results not shown) indicated that the concentration of d-galacturonic acid released was low and thus probably not sufficient to sustain proper growth and pgaA expression. The high level of pgaA mRNA on pectin could be the combined effect of the induction by d-galacturonic acid and the release of other sugars, such as l-arabinose and d-xylose, during pectin degradation, thus providing a better carbon source for growth.

The transcription of both pgaA and pgbB was barely detectable 6 h after the change of medium to d-galacturonic acid, d-galacturonic acid and l-rhamnose and to polygalacturonic acid. At the present time we do not have a satisfactory explanation for these results. The fact that pgaA and pgbB transcription was affected simultaneously suggests that the fungus had not yet adapted its metabolism to the utilization of d-galacturonic acid. An increase in mRNA levels after 10 h is in agreement with such a growth-related response, whereas modulation of expression 2 h after the change of medium seems to be pgaA specific. The presence of both the pgaA and pgbB transcripts on all carbon sources tested demonstrates the constitutive expression of these genes.

**PGA- and PGB-derived amino acid sequences**

The pgaA and pgbB genes encode proteins of 370 and 362 amino acids respectively. As observed earlier for the PGI and PGE from A. niger [7,9], both enzymes are most likely synthesized as prepro-enzymes. The cleavage of the signal peptide probably occurs at Ala.superscript{14} and Ala.superscript{28}, based on the ‘(−3,−1)-rule’ [38], in both PGA and PGB. The pro-sequences are most likely cleaved by a KEX2-like dibasic peptidase [39] after the Lys-Arg residues at positions 32 or 26 of PGA and PGB respectively. The mature PGA consists of 338 amino acid residues, a calculated relative molecular mass of 35497 and an isoelectric point of 3.43. The N-
terminal processing of PGB leads to a 336 amino-acid-long protein with a predicted relative molecular mass of 35188 and an isoelectric point of 6.19. Both enzymes were analysed for the presence of putative N-glycosylation sites (Asn-Xaa-Thr/Ser). In PGA such a site is located at Asn of the mature protein and, in mature PGB, these sites were found at Asn and Asn respectively. The N-glycosylation site of PGA is strictly conserved in all four *A. niger* PGs previously characterized [6–9]. However, the first N-glycosylation site of PGB can only be found in PGI [7], whereas the second site is unique.

**Biochemical characterization of PGA and PGB**

**Specific activities**

A standard assay performed at pH 4.2 for PGA and pH 5.0 for PGB, resulted in specific activities of 16.5 μkat·mg⁻¹ and 8.3 μkat·mg⁻¹ for PGA and PGB respectively. These specific activities were in the same order as those reported for PGI and PGII, 13.8 μkat·mg⁻¹ and 36.5 μkat·mg⁻¹ respectively [15], but much higher than those reported for PGC, 0.42 μkat·mg⁻¹ [15] and PGE, 0.5 μkat·mg⁻¹ [9]. This indicates that, like PGI and PGII, PGA and PGB prefer homogalacturonan as a substrate.

**pH optima, Vₘₐₚ (app) and Kₘ (app) for PGA and PGB**

The pH optima for PGA and PGB were 4.0 and 5.0 respectively (Figure 3). Since PGA and PGB are constitutively expressed in *A. niger*, their distinct complementary pH optima seem to ensure pectin degradation by the fungus over a wider pH range. Comparison of the specific activities at the pH optima in McIlvaine buffer with those in sodium acetate buffer (standard assay) revealed a much higher specific activity (27.5 μkat·mg⁻¹) for PGB in McIlvaine buffer, whereas for PGA and PGs I, II, [15] and E [9] comparable values were found in McIlvaine and sodium acetate buffer. Since McIlvaine buffers are high in ionic strength, the effect of the addition of NaCl to the standard assay mixture on the specific activity of PGB was studied (Figure 4). The specific activity of PGB increased to 30.9 μkat·mg⁻¹ at 175 mM NaCl.

Due to the sensitivity limits of the assay for reducing end groups, the Kₘ (app) value could not be accurately determined for PGA, but was < 0.15 mg·ml⁻¹. The Vₘₐₚ (app) for PGA was calculated to be 18 μkat·mg⁻¹. In view of the low Kₘ (app), the pH optimum for PGA represents Vₘₐₚ (app) versus pH plot (Figure 3). The narrow profile of the plot suggests that only one ionizing group determines effective catalysis.

For PGB, the formation of reducing end groups, routinely determined at 10, 20, 30 and 40 min in a standard assay procedure, was not linear at low substrate concentrations (< 1 mg·ml⁻¹), precluding accurate determination of the reaction rate. Thus Kₘ (app) and Vₘₐₚ (app) values for PGB using polygalacturonic acid could not be calculated. This indicated that PGB is likely to be a true endo-acting enzyme, which prefers a polymer substrate and thus depletes the polymeric substrate during the time of the assay. The degree of polymerization of the

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**Table 1**

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<tr>
<th>Degree of esterification (%)</th>
<th>0</th>
<th>7</th>
<th>22</th>
<th>45</th>
<th>60</th>
<th>75</th>
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<td>125</td>
<td>135</td>
<td>52</td>
<td>7</td>
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<td>B</td>
<td>100</td>
<td>150</td>
<td>168</td>
<td>131</td>
<td>62</td>
<td>27</td>
</tr>
<tr>
<td>B*</td>
<td>100</td>
<td>98</td>
<td>92</td>
<td>45</td>
<td>18</td>
<td>16</td>
</tr>
</tbody>
</table>

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**Figure 3** pH optima for PGA and PGB with polygalacturonate as substrate

The pH optima for PGA (○) and PGB (△) were determined in McIlvaine buffers using 0.25% (w/v) polygalacturonic acid as the substrate at 30 °C.

**Figure 4** The effect of [NaCl] on the activity of PGB

PGB activity was assayed using the standard assay procedure with NaCl added as indicated.
Figure 5  Progression of products formed by PGA and PGB

Products produced during the first 2 h of hydrolysis, at 30 °C, of: (A) 1% (w/v) polygalacturonate in 1 ml of 50 mM sodium acetate buffer, pH 5.0, containing 490 ng PGB; (B) 1% (w/v) polygalacturonate in 1 ml of 50 mM sodium acetate buffer, pH 4.2, containing 220 ng PGA; (C) 1% (w/v) lemon pectin with a degree of esterification of 22% in 1 ml of 50 mM sodium acetate buffer, pH 4.2, containing 220 ng PGA; (D) 1% (w/v) lemon pectin with a degree of esterification of 45% in 1 ml 50 mM sodium acetate buffer, pH 4.2, containing 220 ng PGA. Samples (50 μl) were analysed by HPAEC-PAD.

A particular polygalacturonic acid preparation was 150 [33]. When a substrate with a degree of polymerization of 215, such as lemon pectin with 7% methylesterification (lemon 7), was used formation of reducing end groups was linear over the 40 min interval. The $V_{\text{max}}$ (app) for PGB using lemon 7 was calculated as 14.1 μkat·mg⁻¹ and the $K_{\text{m}}$ (app) as 0.9 mg·ml⁻¹. At 2.5 mg·ml⁻¹ lemon 7, the activity in a standard assay was 10.8 μkat·mg⁻¹. This activity was 1.2-fold higher than the activity observed when polygalacturonic acid, which also gave a linear response in reducing end groups, was used at the same substrate concentration. It is therefore possible that other factors, besides the higher degree of polymerization, contributed to the higher activity and linear progression. Those factors may be the presence of small amounts of salt(s) or the methylation itself (see below).

Activity of PGA and PGB with methylated substrates

It appeared that PGA and PGB were more active on partly methylated pectin (Table 1). However, for PGB the preference
for methylated pectin changed into a tolerance for methylation when 200 mM NaCl was included in the assay. The preference/tolerance of the constitutively produced PGA and PGB for partially methylated substrate in combination with their complementary pH optima would allow the fungus to quickly and effectively respond to the presence of pectic substances once more favourable carbon sources are depleted. Both PGA and PGB differ, with respect to their sensitivity for methylation, from PGs I, II, C and E [15], which showed a clear preference for non-methylated substrate.

**PGA and PGB are endo-acting enzymes**

The product progression during polygalacturonic acid hydrolysis (Figures 5A and 5B) showed that both enzymes are endo-acting, since a transient accumulation of oligogalacturonates with a degree of polymerization $> 5$ was observed. These were gradually converted into oligogalacturonates with a degree of polymerization $< 5$. For PGB, the transient accumulation of higher oligogalacturonates was more pronounced than for PGA. The product progression of PGB resembled the product progression of PGII and PGE, both randomly acting PGs [9,15]. However, PGA behaved more like PGI and PGC in this respect, displaying a strong increase in (GalpA)$_n$, from the start of the reaction. It was shown previously that this type of product progression originated from progressive behaviour on oligogalacturonates, with $n > 5$ or $n > 6$ for PGI and PGC respectively [15].

For PGB, the effect of the addition of 200 mM NaCl to the reaction mixture on the product progression was investigated, and lemon pectins with 7, 22, 45, 60 and 75% esterification were also included in the study. Apart from the expected increased rate of hydrolysis upon addition of 200 mM NaCl, which was corrected for by higher dilution of PGB, no effect on the product progression with polygalacturonic acid as a substrate was observed (results not shown). The same observation was made for the series of lemon pectins. However, differences in product progression were observed, depending on the pectin used (results not shown). Generally, the higher the degree of esterification, the slower the formation of the oligogalacturonates with $n < 5$. Based on these results, it can be concluded that an increase in ionic strength for PGB results in a general increase in the rate of hydrolysis, probably by increasing the affinity for the substrate without increasing the affinity at a particular subsite.

Comparison of the product progression for PGA, using polygalacturonic acid, lemon pectin 22 and 45, revealed a more even accumulation of oligogalacturonates for the partially methylated substrates, particularly lemon pectin 45 (Figures 5C and 5D). Thus the processivity of PGA is lessened when methylated substrates are used, indicating that not all subsites can equally accommodate a methylated galacturonate moiety.

**Mode of action of PGA and PGB**

Using reduced (GalpA)$_n$, chain-length $n = 3–8$, it was plausible that PGs I, II, C and E attacked oligogalacturonates from the reducing end [9,15], whereas exopolypgalacturonase attacked the substrate from the non-reducing end [33]. Similarly, using reduced (GalpA)$_n$ as a substrate, and comparing the bond-cleavage frequencies (BCFs) with those obtained for non-reduced (GalpA)$_n$, it was inferred that PGA and PGB also hydrolysed the oligogalacturonates from the reducing end (Table 2). As for the other A. niger PGs, (GalpA)$_n$ was the smallest substrate hydrolysed.

**Table 2** BCFs and hydrolysis rates for PGA and PGB acting on (reduced) oligogalacturonates of defined length

<table>
<thead>
<tr>
<th>(GalpA)$_n$</th>
<th>Galacturonate polymer</th>
<th>Rate ($\mu$kat·mg$^{-1}$)</th>
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<tbody>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>G – G – G – G – G</td>
<td>0.0245</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>G – G – G – G – G</td>
<td>1.18</td>
</tr>
<tr>
<td>7</td>
<td>G – G – G – G – G</td>
<td>7.22</td>
</tr>
<tr>
<td>PGB</td>
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<td></td>
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<tr>
<td>6</td>
<td>G – G – G – G – G – G*</td>
<td>0.0022</td>
</tr>
<tr>
<td>3</td>
<td>G – G – G – G – G</td>
<td>0.0058</td>
</tr>
<tr>
<td>4</td>
<td>G – G – G – G – G</td>
<td>1.14</td>
</tr>
<tr>
<td>5</td>
<td>G – G – G – G – G</td>
<td>1.96</td>
</tr>
<tr>
<td>7</td>
<td>G – G – G – G – G</td>
<td>5.05</td>
</tr>
</tbody>
</table>

preferentially hydrolysed. For (GalpA)$_n$ and (GalpA)$_{n-1}$ BCFs could not be determined because of deviation from stoichiometry of product pairs. A similar observation was made for PGI and PGC [15]. Furthermore, PGA and PGI displayed exactly the same BCFs up to $n = 5$. Also, both PGA and PGI showed a decreased rate of hydrolysis for (GalpA)$_n$ when compared with (GalpA)$_{n-1}$. The opposite would be expected since the rates increased again for $n = 6$, which indicates that more than five subsites are present on the enzymes (seven subsites were estimated for PGI). For PGI the decreased rate of hydrolysis of (GalpA)$_n$ was ascribed to competitive inhibition due to non-productive binding from subsites $5$ to $1$. This was also in agreement with the observed processive behaviour for PGI on (GalpA)$_n$.

Likewise, (GalpA)$_n$ may be a competitive inhibitor for PGA. This is corroborated by the fact that, for (GalpA)$_n$, an extreme preference for binding from subsites $5$ to $1$ was observed, thus highlighting the high affinity of subsites $5$ to $1$ where non-productive binding of (GalpA)$_n$ most likely occurs.

For PGA, processive behaviour occurred only from (GalpA)$_n$ onward, as shown in Figure 6, where a ratio plot for (GalpA)$_n$ hydrolysis according to Robyt and French [40] is presented. This plot shows the non-coincidence for (GalpA)$_n$ and (GalpA)$_{n-1}$ formation at the early stages of the reaction, which demonstrates the processive behaviour. The origin of this processivity resides in the high affinity of subsites $5$ to $1$ and the
extreme bias for (Galp)_n hydrolysis in (Galp)_5–(Galp)_1 mode. Those properties make a shift of (Galp)_n-bound unproductively at sites -6 to -1 (resulting from (Galp)_7 hydrolysis) to sites -5 to +1 very likely.

Inspection of the BCFs for PGB shows that the pattern is typical for an endo-acting enzyme. The observed increased hydrolysis rate upon increasing chain length is also in agreement with this and suggests that the number of subsites is at least seven. Of all A. niger PGs studied in this respect [9,15], PGB shows the strongest preference for the second and third glycosidic linkage from the reducing end, which is indicative of high affinity at subsites +2 and +3.

The BCFs and rates for PGA and PGB presented in Table 2 can well account for the observed product progression on polygalacturonate.

We now have characterized six out of seven A. niger PGs. Previously it was suggested that PGI and PGII, being the most active enzymes, would be responsible for the major substrate supply for the fungus, whereas PGC and PGF would attack regions in the pectin molecule that are inaccessible to PGI and PGII. The constitutive expression of PGA and PGB, in combination with their preference for a methylated substrate and the complementary pH optima, identifies these enzymes as ‘scouting’ enzymes which allow rapid adaptation of the fungus to pectic substrates.

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REFERENCES


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