*pga*A and *pga*B encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*

Lucie PAŘENICOVÁ, Jacques A. E. BENEN, Harry C. M. KESTER and Jaap VISSER[1]

Section of Molecular Genetics of Industrial Micro-organisms, Wageningen Agricultural University, Dreyenlaan 2, 6703 HA Wageningen, The Netherlands

*pga*A and *pga*B, two genes encoding endopolygalacturonases (PGs, EC 3.2.1.15) A and B, were isolated from a phage genomic library of *Aspergillus niger* N400. The 1167 bp protein coding region of the *pga*A gene is interrupted by one intron, whereas the 1234 bp coding region of the *pga*B gene contains two introns. The corresponding proteins, PGA and PGB, consist of 370 and 362 amino acid residues respectively. Northern-blot analysis revealed that *pga*A- and *pga*B-specific mRNA accumulate in mycelia grown on sucrose. mRNAs are also present upon transfer to media containing D-galacturonic acid and pectin. Recombinant PGA and PGB were characterized with respect to pH optimum, activity on polygalacturonic acid, and mode of action and kinetics on oligogalacturonates of different chain length (n = 3–7). At their pH optimum the specific activities in a standard assay for PGA (pH 4.2) and PGB (pH 5.0) were 16.5 μkat·mg^{-1} and 8.3 μkat·mg^{-1} respectively. Product progression analysis, using polygalacturonate as a substrate, revealed a random cleavage pattern for both enzymes and indicated processive behaviour for PGA. This result was confirmed by analysis of the mode of action using oligogalacturonates. Processivity was observed when the degree of polymerization of the substrate exceeded 6. Using pectins of various degrees of methyl esterification, it was shown that PGA and PGB both preferred partially methylated substrates.

Key words: ascomycetes, enzyme kinetics, gene expression, multiple attack, pectinase.

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 hetero-polysaccharides 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 homogalacturonan part of the pectin molecule include several pectin lyases [2–4], a single pectate lyase (J. A. E. Benen, H. C. M. Kester, L. Pařenicová and J. Visser, unpublished work), pectin methylesterase [5] and a family of endopolygalacturonases (EC 3.2.1.15) (PGs) [6–9].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 gene families 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 *Colleto-trichum lindemuthianum* [12] and *A. flavus* [13], and the analysis of secreted PG isoforms during the growth of *S. sclerotiorum* on polygalacturonic acid [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 *pga*I [7], *pga*II [6], *pga*C [8] and *pga*E [9] as well as the corresponding enzymes [9,15] have been characterized. In the present study, two novel *pga* genes of *A. niger*, *pga*A and *pga*B, 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 (*csp*A1, *pyr*A6, *leu*A1, *prt*F28, *gox*C17), 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 *pga*II 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 *pyr*A gene, which encodes orotidine-5′-phosphate decarboxylase from *A. niger* [21], served as a selection marker for restoration of uridine prototrophy.
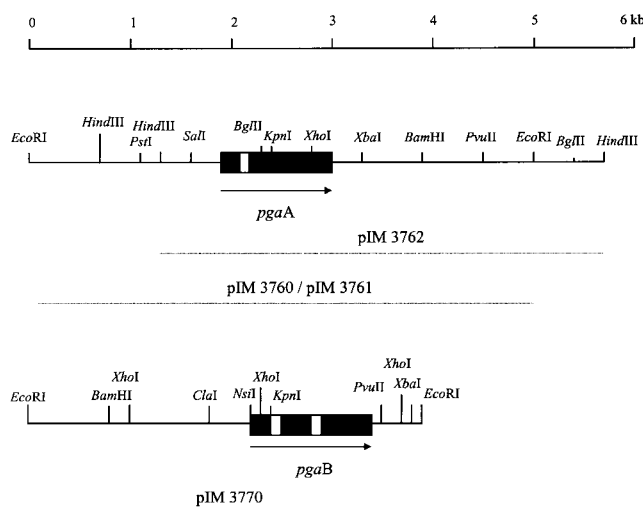

Figure 1   Restriction map of the 5.8 kb *Eco*RI/*Hin*dIII λA43 and the 3.8 kb *Eco*RI λB4 fragments

The black (exons) and white (introns) blocks depict the position of the *pga*A and *pga*B coding region. The arrows indicate the 5′ → 3′ orientation of the genes. The fine dotted lines beneath the restriction maps indicate those parts of the λ phage resulting in pIM plasmids (pIM3762, pIM3760/pIM3761 and pIM3770).

pGW1800 [6], encoding the *pga*II gene, and pIM2130 [22], containing a part of the *A. niger* 18 S gene, were used for isolation of fragments for probes.

The plasmids pIM3760, pIM3761, pIM3762 and pIM3770 (Figure 1) carry the originally subcloned *pga*II hybridizing fragments of the *pga*A and *pga*B genes. pIM3760 contains the 5.0 kb *Eco*RI *pga*A fragment in pEMBL19 with the 5′ of the gene oriented towards the *lacZ* translational start. In pIM3761 the orientation is reversed. pIM3762 contains the 4.5 kb *Hin*dIII *pga*A fragment in pBluescriptSK⁺. pIM3770 carries the 3.8 kb *Eco*RI *pga*B fragment in pBluescriptSK⁺.

Promoter–gene fusions

The expression plasmids containing the *pki*A promoter–gene fusion were constructed in the following way. The 0.7 kb *Bam*HI–*Nsi*I fragment of the *pki*A promoter was isolated from pUC18HN [9]. In the next step, pIM3760 served as a template to introduce the *Nsi*I restriction site at the translational start of *pga*A by PCR using *Taq* polymerase (Pharmacia Biotech., Uppsala, Sweden). For the PCR synthesis of the *Nsi*I–*Kpn*I *pga*A fragment (see Figure 1), two primers were designed: primer *pga*A-*Nsi*, a nineteen-residue long oligonucleotide, 5′CGCAAT-CATGCATTCTGCC 3′, localized at position 1907–1925 bp, and primer *pga*A-*Kpn*, a twenty-residue long oligonucleotide, 5′AGGTACCGCCGGAGAAGTAG 3′, annealing at position 2640–2621 bp of the *pga*A 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 *pga*A PCR fragment was checked by sequencing for PCR errors. The *pki*A promoter *Bam*HI–*Nsi*I fragment and the *pga*A *Nsi*I–*Kpn*I fragments were ligated into pGEM-7. In the final ligation, the 2.5 kb *Kpn*I–*Eco*RI fragment (see Figure 1), containing the remainder of the *pga*A gene and its downstream sequence, was cloned into pGEM-7 harbouring the *pki*A–*pga*A fragment, resulting in pIM3763. For construction of pIM3773, the *pki*A–-

*pga*B expression plasmid, the presence of the *Nsi*I site localized at the translational start of the *pga*B gene was exploited. The 1.6 kb *Xho*I fragment (see Figure 1) containing the *pga*B promoter sequence and the 5′ end of the *pga*B coding sequence was subcloned into pBluescriptSK⁺. In the next step, the *pga*B promoter part of this subclone was replaced by the 0.7 kb *Bam*HI–*Nsi*I fragment of the *pki*A promoter from pUC18HN. The final construct was obtained after ligation of the 1.3 kb *Xho*I fragment, consisting of the *pga*B gene and an additional 170 bp of sequence downstream of the 3′ end of *pga*B.

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 form-amide. For *pga*A the 335 bp *Xho*I–*Xba*I fragment, localized in the 3′ part of the gene, and the 3′ non-translated region was used as a probe, and for *pga*B the *Nsi*I–*Kpn*I 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 pGW635 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 leukeamia virus reverse transcriptase under the conditions described by the supplier (Gibco BRL). Gene-specific primers were used. In the case of *pga*A, primer *pga*A-*Kpn* was used in the first reaction (see above), and for *pga*B a new primer was designed, a nineteen-residue long oligonucleotide, 5′AGTGCCAGTCTTGCCGTAG 3′, anneal-ing 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 *pga*A, a twenty-residue long oligonucleotide, 5′ TGTTCTGCCTTGCG-ACCCTG 3′, annealing at position 1933–1952 bp, and for *pga*B, an eighteen-residue long oligonucleotide, 5′ GCATTTCCTCC-AGAACGC 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 *pga*A a temperature of 43.5 °C was used and for *pga*B 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 NaNO$_3$, 1.5 g KH$_2$PO$_4$, 0.5 g MgSO$_4$·7 H$_2$O, 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 10^6 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/l 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 poly-galacturonic 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 0–0.8 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) NaN$_3$. 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 ab-sorption 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 esteri-fication, 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 *pga*A and *pga*B genes

Phages λA43 and λB4, representatives of the classes A and B [8] respectively, were selected for Southern blotting using the 1.3 kb *Bam*HI/*Bgl*II *pga*II-specific probe (see the Experimental section).

The λA43 analysis revealed a 4.5 kb *Hin*dIII and a 5.0 kb *Eco*RI fragments, both hybridizing strongly (Figure 1). For subcloning of the 3.8 kb *Eco*RI-hybridizing fragment from λB4 (Figure 1) the same strategy was employed, which resulted in pIM3770.

Analysis of nucleotide sequences of *pga*A and *pga*B

The entire 5.8 kb *Eco*RI–*Hin*dIII subclone derived from λA43 contains 1167 bp of coding sequence and the 1913-bp upstream and the 2700-bp downstream sequence. The *pga*B nucleotide sequence includes 2224 bp of the 5′ non-coding region, 1234 bp of the *pga*B coding sequence and 330 bp of the 3′ non-coding region (Figure 1).

Based on the sequence alignment of *pga*A and *pga*B with other fungal *pga* genes present in the EMBL Gene Database, introns of 54 bp in length for *pga*A and 70 and 74 bp for *pga*B 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 *pga*A and *pga*B 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 *pga*A promoter, a TATATAA 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 *pga*B 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 fulfil 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 exopolygalacturonase from *A. tubingensis* [33] that (poly-)galac-turonic acid had a positive effect, whereas glucose had a negative effect on the expression. The promoters of both the *pga*A and *pga*B genes were analysed for the presence of specific DNA sequences, such as consensus CREA (carbon catabolite re-pressing protein A) binding sites [34–36] and the 5′ TYATTG-GTGGA 3′ sequence. The latter was proposed [8,37] to play a role in the induced expression of the *pga*II gene from *A. niger*.

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 *pga*A and *pga*B promoters respectively. Furthermore, in the *pga*A 5′ promoter region (−1525 bp from the ATG), the sequence 5′ TGATTGGT 3′, resembling the *pga*II 5′ TYATTGGTGGA 3′ sequence [37], was found on the opposite strand.

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

Influence of the various carbon sources on *pga*A and *pga*B expression

*pga*A- and *pga*B-specific probes were checked for cross-hybridi-zation with other *A. niger pga* genes by a dot-blot analysis, under
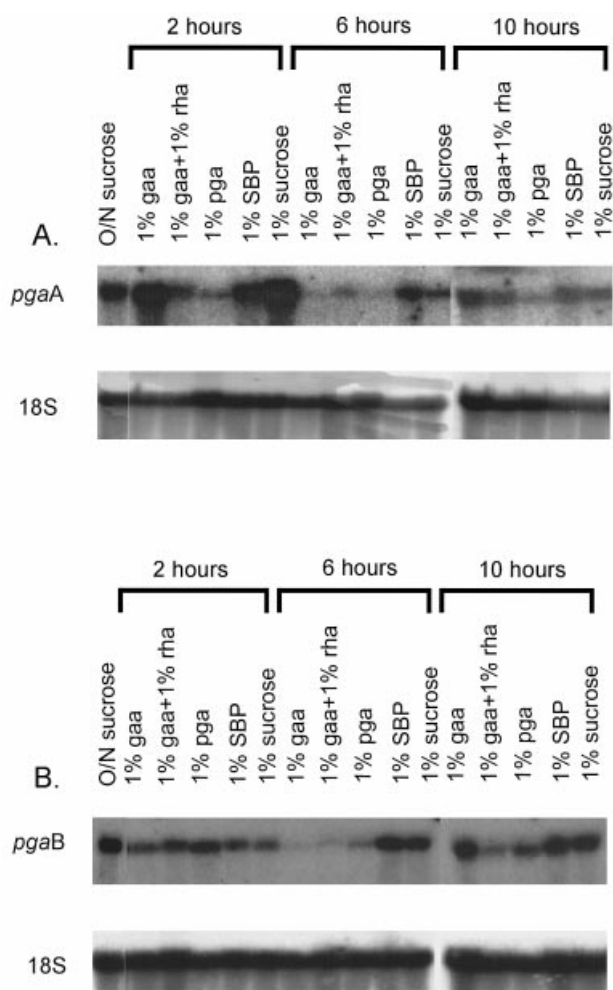
Figure 2   Northern-blot analysis of the effect of the carbon source on *pga*A and *pga*B expression

The mycelium was pre-grown overnight for 20 h in minimal medium containing 1% (w/v) sucrose (O/N sucrose) and the medium was then changed to a minimal medium containing 1% (w/v) of D-galacturonic acid (gaa), 1% (w/v) D-galacturonic acid and 1% (w/v) L-rhamnose (gaa+rha), 1% (w/v) polygalacturonic acid (pga), 1% (w/v) sugar beet pectin (SBP) or 1% (w/v) sucrose. The mycelium was harvested at 2, 6 and 10 h after the change of medium. As a loading control, the 18 S rDNA fragment was used for reprobing the blots.

stringent hybridization conditions, using 5 ng of plasmid DNA containing those genes. Although some background hybridization was detected, the intensity of the signal corresponding to the specific gene was much higher.

For the expression study several carbon sources were selected, which included the monomeric sugars present in the backbone of pectin, D-galacturonic acid and L-rhamnose, as well as the polymeric substrates, polygalacturonic acid and sugar beet pectin. Sucrose was used in order to test whether *pga*A and *pga*B gene expression was affected by carbon catabolite repression. The results of this analysis are presented in Figure 2.

It is clear from the results shown in Figure 2 that *pga*A- and *pga*B-specific mRNA were both already present in mycelia of *A. niger* pre-grown overnight on sucrose. After the medium was replaced with fresh sucrose-containing medium the signal of the *pga*A and *pga*B mRNA remained high or even increased. Using the various pectic substrates, the expression patterns were only

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 *pga*A and *pga*B is probably similar to the expression on sucrose. The same feature, the constitutive expression on glucose and pectin, was previously reported for the *pec*A 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 *pga*A and *pga*B genes differ depending on the substrate and time of induction. Whereas the level of the *pga*B transcript 2 h after the change of medium was similar on all carbon sources tested, there was an increase in the level of *pga*A 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 *pga*II 5′ TYATTGG-TGGA 3′ upstream-activating sequence [8,37] in the 5′ part of the *pga*A promoter. This sequence is not present in the *pga*B promoter. The *pga*A 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 *pga*A 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 *pga*A expression. The high level of *pga*A 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 *pga*A and *pga*B 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 *pga*A and *pga*B 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 *pga*A specific.

The presence of both the *pga*A and *pga*B transcripts on all carbon sources tested demonstrates the constitutive expression of these genes.

PGA- and PGB-derived amino acid sequences

The *pga*A and *pga*B 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[19] and Ala[20], 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-
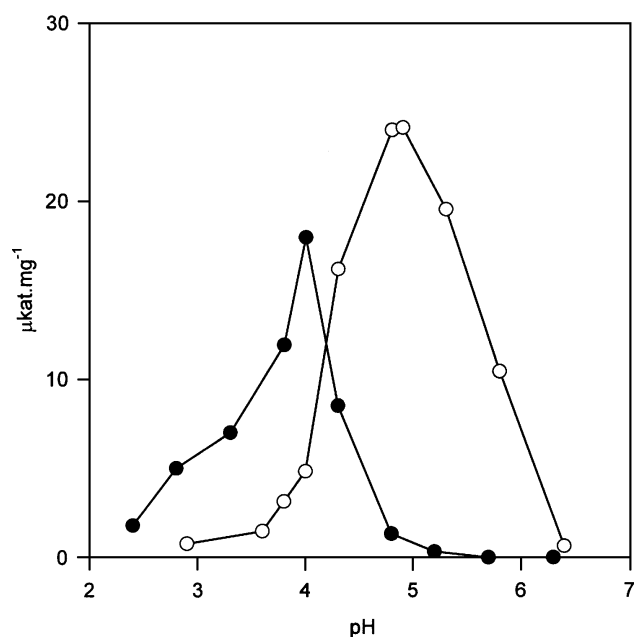
Figure 3   pH optima for PGA and PGB with polygalacturonate as substrate

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.

Table 1   Effect of the degree of methylation of pectin on the specific activity of PGA and PGB

The rate of hydrolysis was determined during the initial linear stages of the enzyme reaction by the determination of reducing-end groups. As substrate, 0.25 % (w/v) of lemon pectin was used in 50 mM sodium acetate buffer, pH 4.2, at 30 °C. *, 200 mM NaCl was added to the assay mixture.

		Relative specific activity					
PG	Degree of esterification (%) …	0	7	22	45	60	75
A		100	125	135	52	7	6
B		100	150	168	131	62	27
B*		100	98	92	45	18	16

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^{214} of the mature protein and, in mature PGB, these sites were found at Asn^{265} and Asn^{308} 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, V_{max} (app) and K_m (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
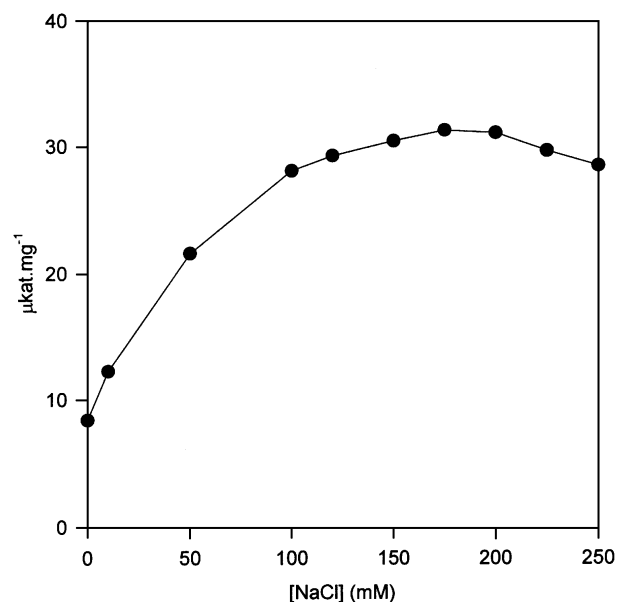
strength, the effect of the addition of NaCl to the standard assay mixture on the specific activity of PGB was studied (Figure 4). The specific activity of PGB increased to 30.9 μkat·mg^{-1} at 175 mM NaCl.

Due to the sensitivity limits of the assay for reducing end groups, the K_m (app) value could not be accurately determined for PGA, but was < 0.15 mg·ml^{-1}. The V_{max} (app) for PGA was calculated to be 18 μkat·mg^{-1}. In view of the low K_m (app), the pH optimum for PGA represents V_{max} (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 K_m (app) and V_{max} (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
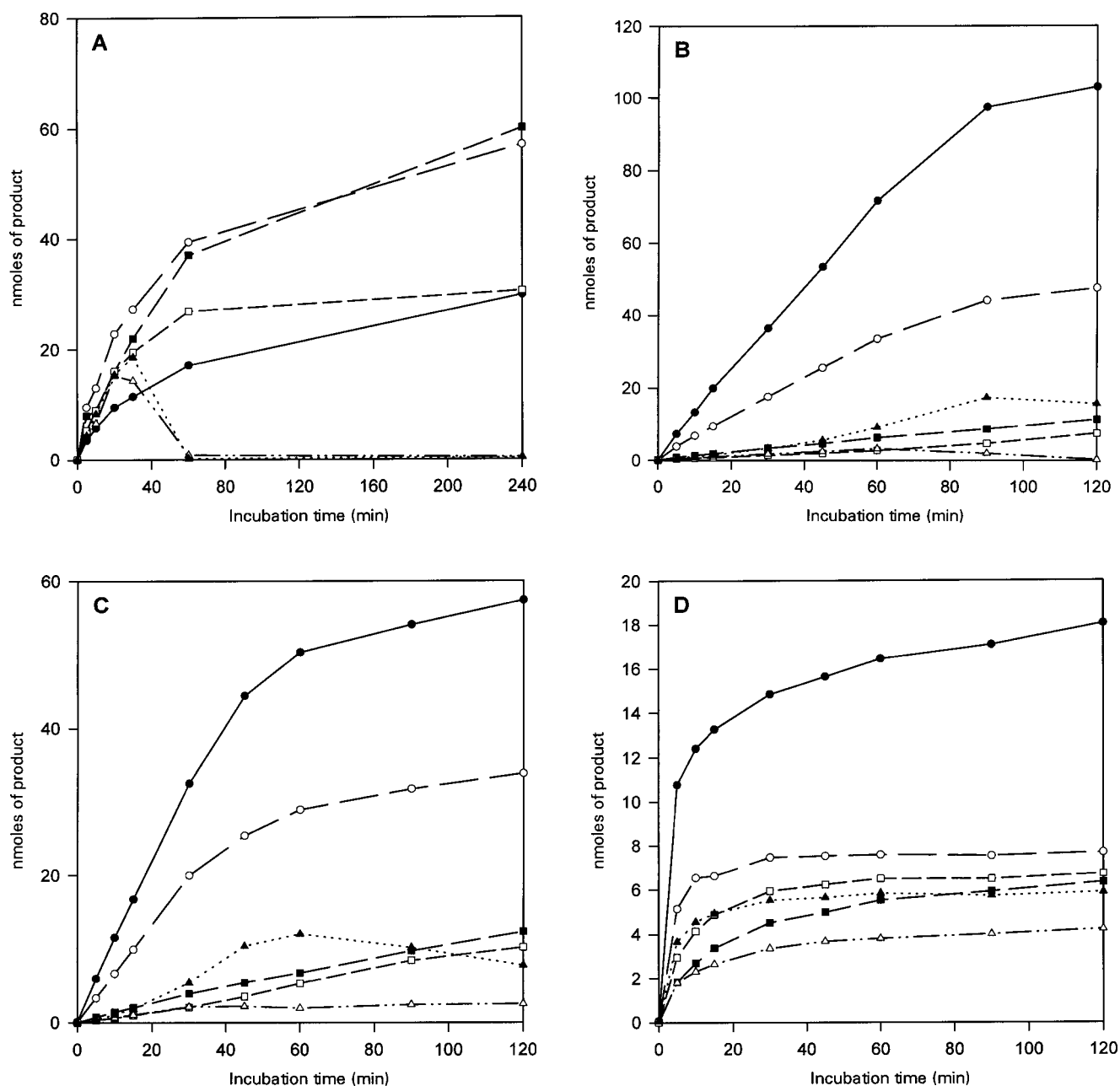
Figure 5   Progression of products formed by PGA and PGB

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. ●, (GalpA)$_1$; ○, (GalpA)$_2$; ■, (GalpA)$_3$; □, (GalpA)$_4$; ▲, (GalpA)$_5$; △, (GalpA)$_6$.

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_{max} (app) for PGB using lemon 7 was calculated as 14.1 μkat·mg^{-1} and the K_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 con-

centration. 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 $(GalpA)_1$ from the start of the reaction. It was shown previously that this type of product progression originated from processive 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 well 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 exopolygalacturonase attacked the substrate from the non-reducing end [33]. Similarly, using reduced $(GalpA)_6$ as a substrate, and comparing the bond-cleavage frequencies (BCFs) with those obtained for non-reduced $(GalpA)_6$, it was inferred that PGA and PGB also hydrolysed the oligogalacturonates from the reducing end (Table 2). As for the other *A. niger* PGs, $(GalpA)_3$ was the smallest substrate hydrolysed.

Inspection of the BCFs for PGA revealed that, except for $(GalpA)_5$, the first glycosidic linkage from the reducing end was

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

Assay conditions: 500 μM (reduced) oligogalacturonates were incubated with PGA or PGB in 0.5 ml of 50 mM sodium acetate buffer, at pH 4.2 or pH 5.0 respectively. At timed intervals, 50 μl aliquots were withdrawn and mixed with 50 μl of stopmix (2.0 mM Tris/HCl, 50 mM NaOH) to raise the pH to 8.3–8.5. Products were analysed and quantified by HPAEC-PAD, as described in the Experimental section. G indicates each galacturonate residue with the BCFs (in percentages) given below the relevant bond. The boldface typescript (**G**) indicates the reducing end of the polymer and the asterisk (*) indicates the reduced end (galactonate).

$(GalpA)_n$	Galacturonate polymer	Rate (μkat·mg^{-1})
PGA		
6	G—G—G—G—G—**G*** 51 49	1.07
3	G—G—**G** 100	0.0245
4	G—G—G—**G** 3 97	1.32
5	G—G—G—**G** 46 54	1.18
6	G—G—G—G—**G** 1 3 96	6.54
7	G—G—G—G—G—G—**G**	7.22
PGB		
6	G—G—G—G—G—**G*** 6 94	0.0022
3	G—G—**G** 100	0.0058
4	G—G—**G** 2 98	1.14
5	G—G—G—**G** 90 10	1.96
6	G—G—G—G—**G** 50 27 23	3.74
7	G—G—G—G—G—**G** 46 47 7	5.05
8	G—G—G—G—G—G—G—**G** 15 29 53 3	7.19

preferentially hydrolysed. For $(GalpA)_7$ and $(GalpA)_8$, 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 $(GalpA)_5$ when compared with $(GalpA)_4$. 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 $(GalpA)_5$ 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 $(GalpA)_6$. Likewise, $(GalpA)_5$ may be a competitive inhibitor for PGA. This is corroborated by the fact that, for $(GalpA)_6$, 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 $(GalpA)_5$ most likely occurs.

For PGA, processive behaviour occurred only from $(GalpA)_7$ onward, as shown in Figure 6, where a ratio plot for $(GalpA)_7$ hydrolysis according to Robyt and French [40] is presented. This plot shows the non-coincidence for $(GalpA)_6$ and $(GalpA)_1$ 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
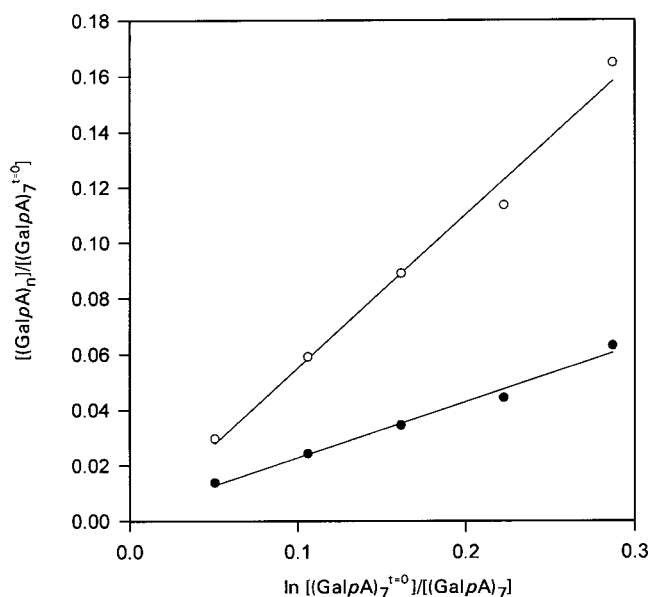
Figure 6   Ratio plot for $(GalpA)_7$ hydrolysis by PGA

Diagnostic plot for the occurrence of processivity according to Robyt and French [40]. The ratio of product formation, $[(GalpA)_1]$ ($\bigcirc$) and $[(GalpA)_6]$ ($\bullet$) relative to the initial substrate $[(GalpA)_7]$, is plotted against the progress of the hydrolysis reaction represented as $\ln [(GalpA)_7^{t=0}]/[(GalpA)_7]$.

extreme bias for $(GalpA)_6$ hydrolysis in $(GalpA)_5–(GalpA)_1$ mode. Those properties make a shift of $(GalpA)_6$-bound unproductively at sites -6 to -1 (resulting from $(GalpA)_7$ 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 PGE 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.

REFERENCES

1   Carpita, N. C. and Gibeaut, D. M. (1993) Plant J. **3**, 1–30
2   Harmsen, J. A. M., Kusters-van Someren, M. A. and Visser, J. (1990) Curr. Genet. **18**, 161–166
3   Kusters-van Someren, M. A., Harmsen, J. A. M., Kester, H. C. M. and Visser, J. (1991) Curr. Genet. **20**, 293–299
4   Kusters-van Someren, M. A., Flipphi, M. J. A., de Graaff, L. H., van den Broek, H. C., Kester, H. C. M., Hinnen, A. and Visser, J. (1992) Mol. Gen. Genet. **234**, 113–120
5   Khanh, N. Q., Albrecht, H., Ruttkowski, E., Loffler, F., Gottschalk, M. and Jany, K. D. (1990) Nucleic Acids Res. **18**, 4262
6   Bussink, H. J. D., Kester, H. C. M. and Visser, J. (1990) FEBS Lett. **273**, 127–130
7   Bussink, H. J. D., Brouwer, K. B., de Graaff, L. H., Kester, H. C. M. and Visser, J. (1991) Curr. Genet. **20**, 301–307
8   Bussink, H. J. D., Buxton, F. P., Fraaye, B. A., de Graaff, L. H. and Visser, J. (1992) Eur. J. Biochem. **208**, 83–90
9   Pařenicová, L., Benen, J. A. E., Kester, H. C. M. and Visser, J. (1998) Eur. J. Biochem. **251**, 72–80
10   Fraissinet-Tachet, L., Reymond-Cotton, P. and Févre, M. (1995) Curr. Genet. **29**, 96–99
11   Wubben, J. P., Mulder, W., ten Have, A., van Kan, J. A. L. and Visser, J. (1999) Appl. Environ. Microbiol. **65**, 1596–1602
12   Centis, S., Guillas, I., Séjalon, N., Esquerré-Tugayé, M.-T. and Dumas, B. (1997) Mol. Plant–Microbe Interact. **10**, 769–775
13   Whitehead, M. P., Shieh, M. T., Cleveland, T. E., Cary, J. W. and Dean, R. A. (1995) Appl. Environ. Microbiol. **61**, 3316–3322
14   Martel, M.-B., Létoublon, R. and Févre, M. (1998) FEMS Microbiol. Lett. **158**, 133–138
15   Benen, J. A. E., Kester, H. C. M. and Visser, J. (1999) Eur. J. Biochem. **159**, 577–585
16   Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S. S., Michael, M. Z. and Graham, M. W. (1989) Nucleic Acids Res. **17**, 3469–3478
17   Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18   Short, J. M., Fernandez, J. M., Sorge, J. A. and Huse, W. D. (1988) Nucleic Acids Res. **16**, 7583–7600
19   Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103–119
20   Dente, L., Cesareni, G. and Cortese, R. (1983) Nucleic Acids Res. **11**, 1645–1655
21   Goosen, T., Bloemheuvel, G., Gysler, C., de Bie, D. A., van den Broek, H. W. J. and Swart, K. (1987) Curr. Genet. **11**, 499–503
22   Melchers, W. J. G., Verweij, P. E., van den Hurk, P., van Belkum, A., de Pauw, B. E., Hoogkamp-Korstanje, A. A. and Meis, J. F. G. M. (1994) J. Clin. Microbiol. **32**, 1710–1717
23   Gilliland, G., Perrin, S. and Bunn, H. F. (1990) in PCR Protocols: A Guide to Methods and Applications, Competitive PCR for Quantitation of mRNA (Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J., eds.), pp. 60–69, Academic Press Inc., San Diego
24   Vishniac, V. and Santer, M. (1957) Bactiol. Rev. **21**, 195–213
25   Edelhoch, H. (1967) Biochemistry **6**, 1948–1954
26   Unkles, S. E. (1992) in Applied Molecular Genetics of Filamentous Fungi, Gene Organization in Industrial Filamentous Fungi (Kinghorn, J. R. and Turner, G., eds.), pp. 28–53, Chapman & Hall, London
27   Gurr, S. J., Unkles, S. E. and Kinghorn, J. R. (1987) in Gene Structure in Eukaryotic Microbes. The Structure and Organization of Nuclear Genes of Filamentous Fungi (Kinghorn, J. R., ed.), pp. 93–139, IRL Press, Oxford
28   Bussink, H. J. D., Buxton, F. P. and Visser, J. (1991) Curr. Genet. **19**, 467–474
29   Kitamoto, N., Kimura, T., Kito, Y., Ohmiya, K. and Tsukagoshi, N. (1993) FEMS Microbiol. Lett. **111**, 37–42
30   Cary, J. W., Brown, R., Cleveland, T. E., Whitehead, M. and Dean, R. A. (1995) Gene **153**, 129–133
31   Kozak, M. (1984) Nucleic Acids Res. **12**, 857–872
32   Ho, M.-C., Whitehead, M. P., Cleveland, T. E. and Dean, R. A. (1995) Curr. Genet. **27**, 142–149
33   Kester, H. C. M., Kusters-van Someren, M. A., Müller, Y. and Visser, J. (1996) Eur. J. Biochem. **240**, 738–746
34   Cubero, B. and Scazzocchio, C. (1994) EMBO J. **13**, 407–415
35   Kato, M., Sekine, K. and Tsukagoshi, N. (1996) Biosci. Biotechnol. Biochem. **60**, 1776–1779
36   Takashima, S., Iikura, H., Nakamura, A., Masaki, H. and Uozumi, T. (1996) FEMS Microbiol. Lett. **145**, 361–366
37   Bussink, H. J. D., van den Hombergh, J. P. T. W., van den IJssel, P. R. L. A. and Visser, J. (1992) Appl. Microbiol. Biotech. **37**, 324–329
38   von Heijne, G. (1986) Nucleic Acids Res. **14**, 4683–4690
39   Jeenes, D. J., Marczinke, B., MacKenzie, D. A. and Archer, D. B. (1993) FEMS Microbiol. Lett. **107**, 267–272
40   Robyt, J. F. and French, D. (1970) Arch. Biochem. Biophys. **138**, 662–670