Characterization of a novel endopolygalacturonase from *Aspergillus niger* with unique kinetic properties

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Abstract We isolated and characterized a new type of endopolygalacturonase (PG)-encoding gene, *pga*D, from *Aspergillus niger*. The primary structure of PGD differs from that of other *A. niger* PGs by a 136 amino acid residues long N-terminal extension. Biochemical analysis demonstrated extreme processive behavior of the enzyme on oligomers longer than five galacturonate units. Furthermore, PGD is the only *A. niger* PG capable of hydrolyzing di-galacturonate. It is tentatively concluded that the enzyme is composed of four subsites. The physiological role of PGD is discussed.

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Key words: Endopolygalacturonase; Processivity; Methylated oligogalacturonate; *Aspergillus niger*

1. Introduction

Polygalacturonases (E.C. 3.2.1.15) are known as enzymes mainly hydrolyzing the demethylated backbone of the pectin molecule (viz. pectate) consisting of 1,4-α-linked D-galacturonic acid units.

In the saprophytic fungus *Aspergillus niger*, a multigene family encoding endopolygalacturonases (PGs) was identified [1]. Thus far, six out of seven members of the PG-encoding gene family have been sequenced [1–5] and the corresponding enzymes biochemically characterized [4–6]. Although the extracellular mature *A. niger* PGs share overall 66–85% of amino acid identity, they exhibit quite different enzymatic properties. The specific activity of the *A. niger* PGs on polygalacturonate ranges from 415 nkat/mg for PGC to 36.5 μkat/mg for PGII. Furthermore, PGA and PGB appeared to prefer partially methylated substrates (viz. pectin with 22–45% degree of esterification (DE)) in contrast to the other PGs, which tolerate methylation to various extents. The six *A. niger* PGs further differ in their pH optima (ranging from 3.9 to 5.0) and their mode of action on oligogalacturonates of defined chain length.

The presence of a polygalacturonase family suggests that the enzymes have complementary activities and that each one fulfills a unique role during the growth of the fungus on pectic substrates.

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Abbreviations: PG, endopolygalacturonase; $(GalpA)_n$, oligogalacturonate with degree of polymerization (*n*); $r(GalpA)_n$, reduced oligogalacturonate with degree of polymerization (*n*); DE, degree of esterification; BCF, bond cleavage frequency

Here, we present the study of PGD, a novel PG from *A. niger* with unique properties.

2. Materials and methods

2.1. Strains and plasmids

The plasmids were isolated as described by Sambrook et al. [7] and propagated in *Escherichia coli* DH5α [8]. *A. niger* NW188 (*csp*A1, *pyr*A6, *leu*A1, *prt*F28, *gox*C17) was used for transformation. PGD was purified from the *A. niger* NW188::pIM3793/30 multicopy transformant. pGW635 containing the *A. niger pyr*A [9] served as a co-transforming plasmid to restore the uridine prototrophy. The transformation of *A. niger* was performed as described by Kusters-van Someren et al. [10]. Subcloning of phage λD11 [1] yielded pIM3790. This plasmid contains the PGD gene, *pga*D (Fig. 1), localized on a 5.0 kb *Xho*I fragment. pIM3790 was used for sequencing. pIM3793 is a *pga*D expression construct and contains the translational fusion of the *pki*A promoter [11] with *pga*D. It was constructed following a similar strategy as described previously [4–6].

2.2. Purification of PGD

Seven 1 l Erlenmeyer flasks with 300 ml of minimal medium supplemented with leucine (0.02% (w/v)) were inoculated with 10^6 spores/ml of *A. niger* NW188::pIM3793/30 and cultivated at 30°C for 22 h at 250 rpm in an orbital shaker. The composition of the minimal media was based on Pontecorvo et al. [12], except that $NaNO_3$, serving as the nitrogen source, was replaced by NH_4Cl (4 g/l). 2% (w/v) fructose served as the carbon source. The culture fluid was collected upon harvesting of the mycelia by filtration through a Büchner funnel with nylon gauze and diluted 5-fold with water. The pH was adjusted to 6.0. In the next step, batchwise adsorption of PGD to DEAE Streamline (Pharmacia Biotech, Uppsala, Sweden) was followed by elution with a pulse of 0.01 M piperazine/HCl buffer, pH 6.0, containing 1 M NaCl. The fractions with PG activity were pooled and extensively dialyzed against the same buffer without NaCl. The dialysate was loaded onto a Source 30Q column (1.5×8 cm, Pharmacia Biotech) equilibrated with 0.01 M piperazine/HCl, pH 6.0. Elution was performed with a 300 ml linear gradient (0–1 M NaCl) in the same buffer and 5 ml fractions were collected. PG containing fractions were pooled, diluted 5-fold with 0.01 M piperazine/HCl, pH 6.0, buffer and re-chromatographed on the same column. Elution was performed with a 250 ml linear gradient (0–0.5 M NaCl) in 0.01 M piperazine/HCl, pH 6.0. Fractions containing pure PGD, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), were pooled and dialyzed against 0.05 M sodium acetate buffer, pH 5.0. The final yield from a 2.1 l culture was 19 mg of PGD. The enzyme was stored at 4°C in the presence of 0.02% (w/v) sodium azide.

2.3. Enzyme assays and analytical methods

PGD activity on polygalacturonate, the determination of the pH optimum, the enzymatic activity on lemon pectins with 7, 22, 45, 60 and 75% DE (Copenhagen Pectin A/S, Lille Skensved, Denmark), the product progression on polygalacturonate, determination of bond cleavage frequencies (BCFs) on (reduced) oligogalacturonates were all performed as described before [4,6]. Assays on specifically methylesterified di- and tri-galacturonates, preparation of substrates and product analysis were described by Kester et al. [13]. The molar extinction coefficient for PGD, 38070 M^{-1} cm^{-1}, was calculated by the method of Edelhoch [14]. The purity of PGD was monitored by SDS–

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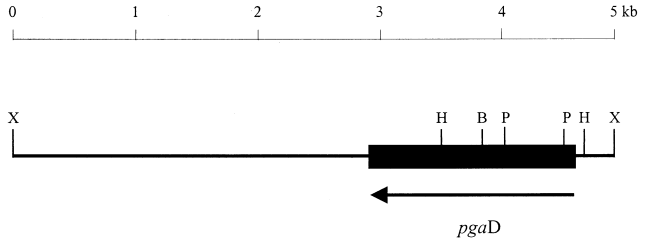

Fig. 1. Partial restriction map of the 5.0 kb *Xho*I insert of pIM3790. The black bar depicts the position of *pga*D and the arrow indicates the orientation of the gene. The 2371 bp sequence from the 3′ *Xho*I site containing 1716 bp of the protein coding region and 394 bp and 261 bp of the 5′ and 3′ non-coding sequences, respectively, was determined over two strands. The capital letters correspond to the following restriction enzymes: X = *Xho*I, B = *Bam*HI, P = *Pst*I and H = *Hin*dIII. The novel nucleotide sequence is available in the EMBL database under the accession number Y18806.

PAGE. Gels were stained with Coomassie brilliant blue R250 and the molecular mass was estimated using molecular weight markers, test mixture 4 (Serva). The N-terminal sequencing was done at Eurosequence, Groningen, The Netherlands, using automated Edman degradation.

3. Results and discussion

3.1. The primary amino acid sequence of PGD

Based on the *pga*D nucleotide sequence, the derived PGD consists of 495 amino acid residues. The calculated relative molecular mass is 50 788 and the isoelectric point approximately 4.1. A signal peptide cleavage site was predicted in PGD after the first 16 N-terminal amino acid residues [15] and confirmed by the N-terminal sequencing of the purified enzyme. PGD shares the highest sequence similarity with the *Fusarium moniliforme* PG [16] and BcPG3 from *Botrytis cinerea* [17]. This is demonstrated also at the molecular level by the similar exon and intron organization and the presence of the additional exon in the 5′ part of *pga*D, when compared to the *F. moniliforme* PG-encoding gene and to *Bcpg*3 from *B. cinerea*, respectively (Fig. 2). Thus, the PGD primary structure, which contains the 136 N-terminal amino acid extension, differs from the other members of the PG-encoding gene family from *A. niger* [1–5]. So far, out of more than 30 fungal PGs present to date in the database, only the *A. niger* PGD and BcPG3 of *B. cinerea* are characterized by this N-terminal extension and therefore they can represent a specific type of PG in fungi (see below).

3.2. Biochemical characterization of PGD

The pH optimum of the purified PGD is around 4.2. However, this enzyme retains more than 30% of its activity over a broad pH interval, from 2.4 to 5.2. The specific activity of PGD, determined at 30°C and pH 4.2, is 1.55 μkat/mg. The kinetic parameters $K_{m\,app}$ and $V_{max\,app}$ measured under identical reaction conditions are 0.2 ± 0.03 mg/ml and 1.57 ± 0.05 μkat/mg, respectively. The relatively low specific activity of PGD on polygalacturonate suggests that polygalacturonic acid is not the optimal substrate for this enzyme.

3.3. Mode of action of PGD on polymeric and oligomeric substrates

Generally endolytic enzymes either attack a polymer chain only once every encounter, the so-called single-attack enzymes, or they may attack the same polymer chain repeatedly during one encounter, the multiple-attack or processive enzymes. The product progression of single-attack enzymes typically shows the formation of long oligomers, which are gradually converted into smaller oligomers upon increased reaction time. In contrast, multiple-attack enzymes accumulate small oligomers or even monomers from the start of the reaction and the formation of longer oligomers is reduced [18]. The degree of processivity is a result of the number of subsites and their individual binding affinities [5,6]. The product progression for PGD, using polygalacturonate as a substrate (Fig. 3), is characterized by a very small increase of oligogalacturonides ($(GalpA)_n$) with longer chain length ($n > 3$) and a prominent increase of Gal*p*A and $(GalpA)_2$. Thus, PGD is an endolytic enzyme with a strong processive behavior [4,6] and the processivity significantly contributes to the overall activity of this enzyme. Of the four processive

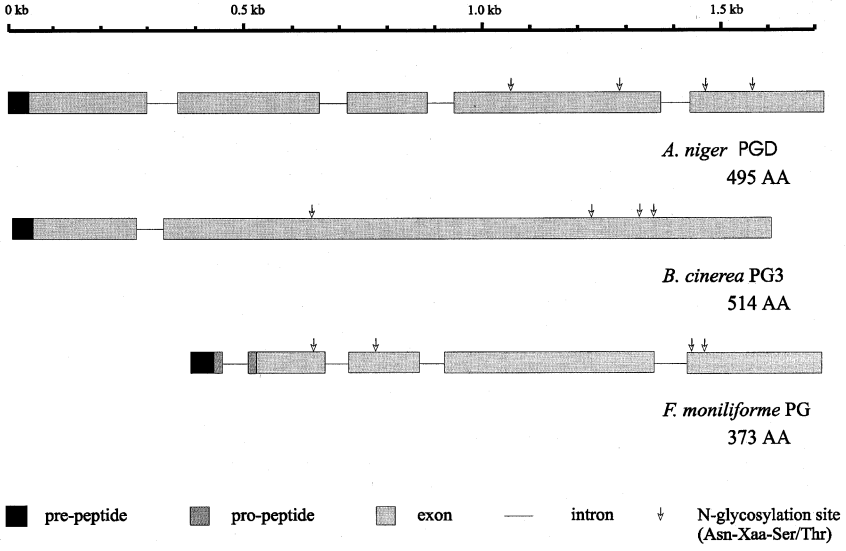

Fig. 2. Comparison of PGD and the structurally closely related PG from *F. moniliforme* and PG3 from *B. cinerea*.

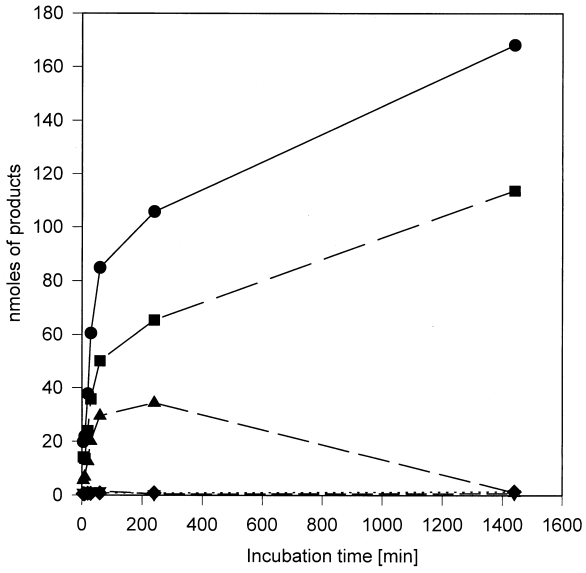

Fig. 3. Progression of products formed by PGD on polygalacturonate. Hydrolysis of 0.25% (w/v) polygalacturonate in 1 ml 50 mM sodium acetate pH 4.2 (0.76 μg PGD) at 30°C. 50 μl samples were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection. ●, (Gal*p*A)$_1$; ■, (Gal*p*A)$_2$; ▲, (Gal*p*A)$_3$; ▼, (Gal*p*A)$_4$; ◆, (Gal*p*A)$_{5–7}$.

A. niger PGs (I, A, C and D), the enzyme described here is the most profound in this respect. This is also reflected by the BCFs using (Gal*p*A)$_n$ ($n=2$–7) (see below). In Table 1, the BCFs on the (reduced) oligogalacturonate (r(Gal*p*A)$_n$) are listed. Cleavage of r(Gal*p*A)$_n$ occurred exclusively at the second glycosidic bond from the reduced end up to $n=5$, suggesting that subsite +1 cannot accommodate a reduced galacturonate moiety. For r(Gal*p*A)$_6$, the third glycosidic linkage was the preferred bond hydrolyzed. The preference to hydrolyze the second glycosidic bond from the reduced end suggests that, like the other *A. niger* PGs, PGD attacks the chain from the reducing end of the substrate. Interestingly, in contrast to the other *A. niger* PGs [4–6], PGD is able to hydrolyze the r(Gal*p*A)$_3$, although at a very low rate (0.17 nkat/mg).

Hydrolysis of (Gal*p*A)$_2$ is also unique to PGD. Thus, unlike the other *A. niger* PGs which all require subsites −2 to +1 to be filled for catalysis, PGD only requires subsites −1 and +1 to be occupied. Comparison of the rates of hydrolysis of (Gal*p*A)$_n$ at 50 and 500 μM reveals that, except for the dimer, the $K_{m\ app}$ values are below 50 μM. In fact, the rates at 50 μM are even somewhat higher than at 500 μM, suggesting substrate inhibition as was observed for PGI [6]. At 50 μM (Gal*p*A)$_n$, the rate of hydrolysis of (Gal*p*A)$_n$ increased up to $n=4$, and decreased for (Gal*p*A)$_5$ and (Gal*p*A)$_6$. At 500 μM substrate, a similar tendency was observed. At both substrate concentrations, the strongest rate increase (15-fold) was recorded upon occupying subsite −3, viz. (Gal*p*A)$_4$. The same rate increase was also recorded for r(Gal*p*A)$_5$ and r(Gal*p*A)$_6$ which also cover subsite −3. Moreover, binding of r(Gal*p*A)$_6$ at subsite −4 appears not favorable, this in contrast to PGI, II, A, C and E [4,6]. From these data and the product progression on polygalacturonic acid, it is tentatively concluded that PGD is composed of only four functional subsites stretching from −3 to +1. At present, we cannot satisfactory explain the higher rates of hydrolysis of the reduced oligomers.

The mode of action analysis for (Gal*p*A)$_5$ to (Gal*p*A)$_7$ revealed deviation from stoichiometry of product pairs as expected for a processive enzyme. A ratio plot according to

Table 1
BCFs and hydrolysis rates of PGD on (reduced) oligogalacturonates of defined length

n												μkat/mg	
												500 μM	50 μM
2									G		**G**	0.67×10^{-3}[a]	0.67×10^{-4}[a]
										100			
3						G			G		**G**	0.28×10^{-1}[a]	0.33×10^{-1}[a]
										100			
4				G		G			G		**G**	0.43[a]	0.53[a]
							1			99			
5			G	G		G			G		**G**	0.38[b]	0.46[b]
							58			42			
6		G	G	G		G			G		**G**	0.42[b]	0.33[b]
					processive								
7	G	G	G	G		G			G		**G**	0.26[b]	
					processive								
2									G		**G**[d]	0	
3						G			G		**G**[d]	0.17×10^{-3}[c]	
							100						
4				G		G			G		**G**[d]	0.53×10^{-1}[c]	
							100						
5			G	G		G			G		**G**[d]	0.78[c]	
							100						
6		G	G	G		G			G		**G**[d]	0.75[c]	
					84		16						

BCFs are given in percentages and were determined from the 500 μM data. PGD is also processive on (Gal*p*A)$_5$ as explained in the text. The reducing end of the oligogalacturonides is shown in bold.
[a]Rate calculation based on product formation.
[b]Rate calculation based on substrate consumption.
[c]Rate calculation based on reduced oligogalacturonide formation.
[d]Reduced end.

Table 2
Hydrolysis rates for *A. niger* PGD on mono-methylesterified di- and tri-galacturonates

Substrate	nkat/mg	Relative rate (%)
(Gal*p*A)$_2$	0.83×10^{-1}	100
1-Methyl (Gal*p*A)$_2$	0.12×10^{-1}	14
2-Methyl (Gal*p*A)$_2$	0.08×10^{-1}	10
(Gal*p*A)$_3$	33.8	100
1-Methyl (Gal*p*A)$_3$	0.25×10^{-1}	0.07
2-Methyl (Gal*p*A)$_3$	0.03×10^{-1}	0.01
3-Methyl (Gal*p*A)$_3$	0.45	1.3

Substrates (0.05 mM) dissolved in 300 μl 50 mM NaAc buffer pH 4.2 were incubated with 15 μg of PGD at 30°C. The reaction rates were determined as described before [13].

Robyt and French [18] for (Gal*p*A)$_5$ hydrolysis (data not shown) indeed demonstrated processive behavior on (Gal*p*A)$_5$. Due to the fact that processive behavior was observed already on (Gal*p*A)$_5$ and the strong preference for hydrolysis of (Gal*p*A)$_4$ at the first glycosidic linkage from the reducing end allowed for the calculation of the initial binding modes for (Gal*p*A)$_5$ as presented in Table 1. (Gal*p*A)$_2$ formation from (Gal*p*A)$_5$ served as the identifier of the first event in that particular mode and thus for the calculation of that particular frequency (58%). From this, the frequency of the binding mode that in a first event would result in the formation of Gal*p*A and (Gal*p*A)$_4$ logically followed (42%). Next, the difference between the amount of (Gal*p*A)$_2$ and (Gal*p*A)$_3$ served for the calculation of the degree of processivity. This learned that 66% of the binding modes of (Gal*p*A)$_5$ in (Gal*p*A)$_4$−Gal*p*A mode resulted in processive attack. The decrease of hydrolysis observed for (Gal*p*A)$_{5-7}$ compared to (Gal*p*A)$_4$ may be caused by the temporal tying up of the enzyme in the unproductive enzyme-product complex prior to the multiple attack.

3.4. Pectin degradation and specific activities on mono-methylesterified di- and tri-oligogalacturonates

The enzyme exhibits a relatively high activity on pectins with methylesterification up to 45%, viz. 1.05 μkat/mg. However, it appears that PGD prefers unmethylesterified substrate like PGI, II, C and E, although, compared to these enzymes, PGD shows the highest tolerance towards methylesterification.

Recently, the use of synthetic mono-methylesterified di- and tri-galacturonates allowed us to study the tolerance for a methylesterified galacturonate binding to the subsites around the catalytic site of the individual *A. niger* PGs [13]. In contrast with the other *A. niger* PGs, PGD is able to hydrolyze all mono-methylesterified di- and tri-galacturonates (Table 2). This demonstrates that PGD can accommodate a methylesterified galacturonic acid at subsite −1 whereas the other enzymes were unable to do so [13]. It should however be noted that PGD was not able to split the fully methylesterified di- and tri-galacturonates. These data may explain the ability of PGD to still hydrolyze efficiently highly methylesterified pectins.

The biological function of PGD is most likely to generate galacturonic acid from the oligogalacturonates released from the pectin by the other PGs. This is based on the ability to hydrolyze the galacturonic acid dimer, the processivity of the enzyme to become evident starting from (Gal*p*A)$_5$ onwards, the likely presence of only four subsites, and the tolerance for a methylesterified galacturonic acid residue around the active site. We therefore propose that PGD is in fact an oligogalacturonate hydrolase.

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