Changing a Single Amino Acid Residue Switches Processive and Non-processive Behavior of Aspergillus niger Endopolygalacturonase I and II*

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Processivity, also known as multiple attack on a single chain, is a feature commonly encountered only in enzymes in which the substrate binds in a tunnel. However, of the seven Aspergillus niger endopolygalacturonases, which have an open substrate binding cleft, four enzymes show processive behavior, whereas the other endopolygalacturonases are randomly acting enzymes. In a previous study (Benen, J.A.E., Kester, H.C.M., and Visser, J. (1999) Eur. J. Biochem. 259, 577–585) we proposed that the high affinity for the substrate of subsite −5 of processive endopolygalacturonase I constitutes the origin of the multiple attack behavior. Based on primary sequence alignments of A. niger endopolygalacturonases and three-dimensional structure analysis of endopolygalacturonase II, an arginine residue was identified in the processive enzymes at a position commensurate with subsite −5, whereas a serine residue was present at this position in the non-processive enzymes. In endopolygalacturonase I mutation R95S was introduced, and in endopolygalacturonase II mutation S91R was introduced. Product progression analysis on polymer substrate and bond cleavage frequency studies using oligogalacturonides of defined chain length for the mutant enzymes revealed that processive/non-processive behavior is indeed interchangeable by one single amino acid substitution at subsite −5, Arg→Ser or Ser→Arg.

Polygalacturonases (PGs), which belong to family 28 in the general classification of the glycosyl hydrolases (1, 2), hydrolyze the 1,4-α-L-galacturonic acid linkages in the homogalacturonan regions of pectin. The PGs that hydrolyze the homogalacturonan are either endo-acting enzymes (EC 3.2.1.15) or exo-acting enzymes (EC 3.2.1.67). Endo-enzymes attack randomly along the chains of the polymeric substrate in contrast to exo-enzymes that attack the chains from the extremities. In Aspergillus niger, Sclerotinia sclerotiorum, and Botrytis cinerea families of genes encoding endoPGs were discovered (3–8); but actually little is known about the precise role of the different endoPGs in these organisms. However, the characterization of the seven highly homologous endoPGs secreted by A. niger (PGI, PGII, PGA-E) suggested that individual enzymes harbor specific enzymic and physiological functions. One of the enzymatic characteristics is the processive behavior observed for endoPG, A, C, and D (9–11). Processivity, first described by Robyt and French for several α-amylases (12, 13) is characterized by multiple attack on a single substrate chain. The initial cleavage is followed by the release of only one part of the substrate, and the retained polymer fragment can be reattacked one or more times. A number of processes were proposed to explain the mechanism of multiple attack (12–14). All tend to merge into a common mechanism permitting the sliding of the substrate fragment retained at the enzyme after the initial cleavage.

In a previous study comparing endoPGI, II, and C we proposed that the origin of the processive behavior of endoPGI resides in the relatively high affinity at subsite −5 that is responsible for retaining the product at the enzyme after cleavage of the substrate (9). Although no subsite affinities were calculated for the processive endoPGAs and C, the striking similarity in bond cleavage frequencies and relative rates of cleavage of oligogalacturonides of defined length (9, 10) also indicated a high affinity for the substrate at subsite −5 for these enzymes. For the processive endoPGD the estimated number of subsites is much smaller and does not extend to subsite −5. In fact, it was proposed that this enzyme is an oligogalacturonide hydrolase (11), and it was therefore not included in further analyses. Recently, the three-dimensional structure for non-processive endoPGII has become available (15), which, in combination with site-directed mutagenesis studies, allowed us to establish the orientation of binding of the substrate and location of several subsites (16). Based on this knowledge and the biochemical properties of the A. niger endoPGs we analyzed the primary sequences and the endoPGII three-dimensional structure for an amino acid(s) responsible for the difference in processive behavior. Mutant enzymes were prepared and characterized with respect to product progression and mode of action to verify the role of the amino acid identified.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—Escherichia coli** DH5α F− (F′/endA1, hsdR17, rK− , mK−), supE44, thi-1, recA1, gyrA (NaF), relA1, ΔlacZYA-argFU169, deoR, F′80lacΔ(lacZ/M15) was used for cloning experiments. A. niger NW188 (carA1, pyrA6, leu-13, prfF28, gosC) was used for transformation (17).

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The abbreviations used are: PG, polygalacturonase; BCF, bond cleavage frequency; (GalpA)n, oligogalacturonate with degree of polymerization (n).
Plasmids pIM3700 and pIM3710 (9) were used as templates for the introduction of the mutations. Both plasmids contain a promoter-gene fusion of the constitutive pyruvate kinase A promoter (pki) (18) and the pgaI gene (pIM3700) or the pgaII gene (pIM3710) encoding PGI or PGII, respectively. Plasmid pGW635 containing the ppga gene (accession number X96734) was used for co-transformation of A. niger NW188.

Site-directed Mutagenesis of pgaI and pgaII and Transformation of A. niger—Appropriate mutations were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA). The pki-pgaI and pki-pgaII genes were sequenced entirely to check for the introduction of the desired mutation and for the absence of undesired mutations. A. niger NW188 was co-transformed as described previously (17), using 1 μg of pGW635 and 20 μg of the plasmid carrying the appropriate mutation.

Selection of Mutated Endopolygalacturonase-producing Transformants, Production, Purification of the Enzymes, and Enzyme Assay—Transformants producing the highest amount of mutated PGI or PGII were selected as described before (6). Large scale cultivation and purification of the enzymes was carried out as outlined in (19). The specific activity, the kinetic parameters V_{max} and K_{m} on polygalacturonic acid (USB, Cleveland, OH) and the mode of action of each mutant on oligogalacturonides of defined length (USB) were determined.
dues Gly-87-Glu-95, whereas those on the fourth turn comprise Lys-127-Asp-135. To identify residues that can explain the affinity difference at subsite $/H_{11002}$, a high affinity in processive enzymes (endoPGI, A, and C) or low affinity in non-processive enzymes (endoPGII, B, and E), several straightforward criteria should be met. Firstly, they should be able to interact with the substrate (face out into the cleft) thus eliminating Ile-90, Met-92, Phe-129, and Ala-131 that are part of PB1. Secondly, they should not be identical in all enzymes or conservatively changed, thus ruling out Gly-87, Pro-88, Leu-89, Ser-93, Gly-94, Lys-127, Phe-128, Tyr-130, His-132, and Leu-134. Thirdly, the biochemical properties of the side chains should be compatible with a low or high affinity depending on the enzyme. Of the remaining amino acid residues, Ser-91, Glu-95, Gly-133, and Asp-135, only Ser-91 meets this criterion as it is present in the three non-processive enzymes, whereas it is replaced by an arginine in the processive enzymes. The presence of the charged arginine to interact with the carboxylate of a substrate residue provides an attractive and simple hypothesis to account for the high affinity at subsite $/H_{11002}$. To test this hypothesis mutant enzymes endoPGI-R95S and endoPGII-S91R were prepared and characterized. In view of the strong sequence conservation among the A. niger endoPGs and the fact that Ser-91 is located on PB1 facing into the cleft, minimal if any disturb-

![Progression of products formed by the mutated enzymes endoPGI-R95S and endoPGII-S91R and the wild type enzymes.](image)

**Fig. 3.** Progression of products formed by the mutated enzymes endoPGI-R95S and endoPGII-S91R and the wild type enzymes. Products produced during the first hours of hydrolysis of 0.8% (mass/vol) polygalacturonic acid in 1 ml of 50 mM sodium acetate, pH 4.2, at 30°C. Samples (50 μl) were analyzed by HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection). A, products formed by wild type endoPGI; B, products formed by wild type endoPGII; C, products formed by endoPGI-R95S; D, products formed by endoPGII-S91R. ○, (GalpA)$_2$; □, (GalpA)$_3$; ■, (GalpA)$_4$; △, (GalpA)$_5$; ◇, (GalpA)$_6$. A and B were taken from Ref. 9, Benen, J. A. E., Kester, H. C. M and Visser, J. (1999) *Eur. J. Biochem.* 259, 577–585 and are reprinted with permission by the *Eur. J. Biochem*. 

Interchanging Processive Behavior between A. niger endoPGs
Interchanging Processive Behavior between A. niger endoPGs

Table I
Bond cleavage frequencies on (GalpA)n (2 < n < 8) for wild type endoPGI and endoPGII and mutated forms of endoPGI-R95S and endoPGII-S91R

| Enzyme      | Oligogalacturonide (GalpA)n | Rate  |
|-------------|------------------------------|-------|
|             | unit/mg                      |       |
| endoPGI     |                              |       |
| wild type   | G — G ↓ G                    | 1.6   |
| R95S        | G — G ↓ G                    | 2.3   |
| wild type   | G — G ↓ G                    | 150   |
| R95S        | G — G ↓ G                    | 114   |
| wild type   | G — G ↓ G                    | 96    |
| R95S        | G — G ↓ G                    | 315   |
| wild type   | G — G ↓ G                    | 244   |
| R95S        | G — G ↓ G                    | 179   |
| wild type   | G — G ↓ G                    | 52    |
| R95S        | G — G ↓ G                    | 305   |
| wild type   | G — G ↓ G                    | 83    |
| R95S        | G — G ↓ G                    | 513   |
| wild type   | G — G ↓ G                    | 1200  |
| S91R        | G — G ↓ G                    | 510   |
| wild type   | G — G ↓ G                    | 15    |
| S91R        | G — G ↓ G                    | 83    |
| wild type   | G — G ↓ G                    | 132   |
| S91R        | G — G ↓ G                    | 513   |
| wild type   | G — G ↓ G                    | 1200  |
| S91R        | G — G ↓ G                    | 510   |

Bond cleavage frequencies on (GalpA)n (2 < n < 8) for wild type endoPGI and endoPGII and mutated forms of endoPGI-R95S and endoPGII-S91R

0.5 mm of the oligogalacturonide substrates were incubated in 0.5 ml 50 mM sodium acetate buffer pH 4.2 at 30 °C. At timed intervals aliquots were withdrawn and mixed with stopmix (2.0 mM Tris, 50 mM NaOH) to raise the pH to 8.3–8.5. Products were analysed and quantified by HPAEC-PAD as previously described (6). Bond cleavage frequencies are given in percentages. The bold face typescript signifies the reducing end.

The onset of the reaction without a substantial accumulation of lacturonides of different chain length. The mode of action of the enzyme on the individual oligogalacturonides of different chain length. The mode of action analysis of the enzyme on the individual oligogalacturonides is presented in Table I. The data for the wild type enzymes (9) are included in this Table. For endoPGI and endoPGI-R95S the BCFs for hydrolysis of (GalpA)3 and (GalpA)5 remained the same, and only a small change in BCFs for (GalpA)5 was observed. For wild type endoPGI BCFs on (GalpA)5 could actually not be determined as a result of processive behavior on this substrate, which resulted in deviation from stoichiometry for product pairs (9). Therefore the BCFs of the wild type enzyme for this substrate were deduced from the data on reduced (GalpA)n. These BCFs revealed a strong preference of (GalpA)5 for binding from subsites −5 to +1, which reflects the high affinity at subsite −5. For the mutant endoPGI-R95S stoichiometric product pairs were observed during (GalpA)5 hydrolysis, and the BCFs reflect a preferred binding from subsites −4 to +2. From this we conclude that Arg-95 is indeed located at subsite −5. A second significant effect of the mutation is a 3-fold higher rate of hydrolysis of (GalpA)5 in the mutant enzyme. As a result of the high affinity at subsite −5 in the wild type enzyme, (GalpA)5 can bind in a non-productive mode from subsites −5 to −1. Because the mutation R95S obviously reduced the affinity at subsite −5, as evidenced by the change in the BCFs, the likelihood to form a non-productive complex has decreased. This is also reflected in the Vmax on the polymer substrate (polylacturonate acid), which increased from 957 units/mg for the wild type enzyme to 1930 units/mg for the mutant enzyme; Km values are <0.15 mg/ml and 0.5 mg/ml, respectively.

Mode of Action Analysis to Demonstrate Processive Behavior in endoPGI-S91R—For wild type endoPGI and enzyme endoPGII-S91R the following Vmax and Km values were calculated on polylacturonate acid: 4000 units/mg, <0.15 mg/ml and 1400 units/mg, 1.2 mg/ml, respectively. This effect on Vmax in the mutant enzyme is most likely caused by the very strong reduction of (GalpA)5 hydrolysis and to a lesser extent of (GalpA)4 hydrolysis compared with the wild type enzyme (Table I). Because the mutation involved subsite −5 it has to be concluded that the reduced hydrolysis rates are due to unproductive complex formation as a result of increased affinity at subsite −5 (see below).

As for endoPGI-R95S, for endoPGII-S91R the effect of the mutation on the BCFs was most evident for (GalpA)5 with a smaller effect on the BCFs for (GalpA)3. This is again compatible with the location of the mutation at subsite −5 with a small contribution to subsite −4. Unlike wild type endoPGI, for endoPGII-S91R stoichiometric product pairs were observed during (GalpA)5 hydrolysis. This demonstrates that this mutant enzyme is not yet processive on (GalpA)5. The deviation from stoichiometry as a result of processivity for this mutant was evident during (GalpA)5 hydrolysis, which precluded the determination of BCFs. Although the possibility of secondary cleavage of this substrate cannot entirely be excluded, we conclude that mutation S91R rendered the enzyme processive for the following reasons: 1) the observed product progression on polymeric substrate is typical for a processive enzyme, and 2) a strong non-productive complex formation is observed (19-fold decreased hydrolysis rate for (GalpA)5) which is a prerequisite for processive behavior. For endoPGII-S91R processivity became evident using (GalpA)5 as a substrate. For wild type endoPGI processive behavior was already observed on (GalpA)5. This discrepancy can be explained by taking the affinity differences in the total subsite array, including subsites −4 to +1, into account. In endoPGI the affinity distribution of the individual subsites is such that the product retained at subsites −5 to −1 as a result of (GalpA)5 hydrolysis can easily slide to subsites −4 to +1 to become hydrolyzed again as the total affinity of subsites −5 to −1 is lower than the total affinity of subsites −5 to −1.
than the total affinity of subites $-4$ to $+1$ (primarily as a result of high affinity of subites $-3$ and $+1$) (9). The high affinity at subite $-5$ as result of the introduction of the arginine in endoPGII-S91R may well result in an affinity higher than subite $+1$ and thus disfavor the sliding of the product retained at $-5$ to $-1$ to $-4$ to $+1$. For $(\text{GalpA})_n$, $n > 6$, the product retained from $-5$ to $-1$ extends beyond subite $-5$. Sliding of the product retained will lead to a complete covering of the subites $-5$ to $-1$ resulting in a higher total binding energy and thus permitting the processive behavior when the substrate is longer than $(\text{GalpA})_6$. For endoPGC and endoPGA processivity became evident as well using $(\text{GalpA})_7$ as a substrate (9, 10) indicating the same underlying principle.

**Conclusion**—From primary sequence alignments, three-dimensional structural information, and kinetic analyses, it was deduced that the presence or absence of an arginine or serine residue at subite $-5$ might be the pivotal criterion for processive or non-processive behavior of an *A. niger* endoPG. The detailed characterization of the two mutated enzymes described illustrates that it is possible to switch a processive endoPG into a non-processive one and vice versa. The data presented form solid evidence for the crucial role of the serine and arginine for processive/non-processive behavior.

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