The Active Site Topology of Aspergillus niger Endopolygalacturonase II as Studied by Site-directed Mutagenesis*

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Pectic polysaccharides are among the most complex plant cell wall polysaccharides. In the homogalacturonan part, the so-called smooth regions, the 1,4-α-D-galacturonic acid backbone is partly esterified. These smooth regions are interspersed by the rhamnogalacturonan parts consisting of repeating stretches of 1,2-α-L-rhamnose-1,4-α-D-galacturonic acid dimers. Other sugar residues can be attached to the rhamnose residues (1). Because of this complexity, a wide range of enzymes, the so-called pectinases, is necessary for the complete degradation of pectic substances. Two main classes of depolymerizing enzymes act on these polysaccharides: the hydrolases (endopolygalacturonases) and the lyases (pectin lyase, pectate lyase, and rhamnogalacturonan lyase).

Endopolygalacturonases (PGs; EC 3.2.1.15) catalyze the random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectates. They have been isolated from a variety of organisms (eukaryotae and prokaryotae). Furthermore, over 40 genes encoding PGs have been cloned and sequenced. The corresponding enzymes have been grouped in family 28 of the general classification of glycosyl hydrolases based on amino acid sequence similarities (2, 3).

The gene encoding the endopolygalacturonase II (PGII) from Aspergillus niger has been previously cloned, sequenced, and expressed in A. niger (4). The enzyme hydrolyses the glycosidic linkages with inversion of configuration (5). Recently, PGII was extensively characterized with respect to activity on polygalacturonic acid, mode of action, and kinetics on oligogalacturonates (6).

Two different mechanisms have been identified for glycosyl hydrolases: one resulting in retention and the other in inversion of the configuration at the anomeric carbon of the scissile bond (7, 8). Despite this difference, in most glycosidases two residues are directly involved in catalysis: a nucleophile and a proton donor. The average distance between the two catalytic residues has been shown to be about 5.5 Å in retaining glycosidases and about 9.5 Å in inverting enzymes, irrespective of whether α- or β-glycosidic bonds are hydrolyzed (9, 10). Moreover, crystallographic studies revealed that the catalytic amino acids are always aspartates and/or glutamates (11). However, site-directed mutagenesis experiments remain important in the identification of amino acids involved in catalysis.

Of the family 28 enzymes, the rhamnogalacturonase A from Aspergillus aculeatus and the polygalacturonase from Erwinia caratovora are the only members for which three-dimensional structures have been described (12, 13). Even though the two enzymes do not act on the same region of the pectic molecule, their structures, as well as the structure of A. niger PGII, do indeed show similar topologies, and many of the conserved residues throughout family 28 are located in the active sites of rhamnogalacturonase A and the polygalacturonases.

There are two aspartate residues strictly conserved within family 28, which could have catalytic roles. Moreover, other charged amino acids conserved among polygalacturonases are likely to play important roles such as maintaining the structure of the enzyme and establishing hydrogen bonding and hydrophobic interactions between the enzyme and the substrate.

For a better understanding of the mode of action and function of A. niger PGII in the degradation of pectins, site-directed mutagenesis has been carried out on the six charged amino acids (Asp-180, Asp-201, Asp-202, His-223, Arg-256, and Lys-258; sequence numbers according to PGII) that are conserved among polygalacturonases. Based on our results, we propose two aspartic acids to act together to activate the water.

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¶ The abbreviations used are: PG, polygalacturonase; BCF, bond cleavage frequency; GalpA, galacturonic acid; HPACE-PAD, high performance anion exchange chromatography with pulsed amperometric detection; HPLC, high pressure liquid chromatography.

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whereas a third aspartic acid serves as the general acid in PGII. The corresponding residues in the polygalacturonase from *E. carotovora* were identified in the recently solved crystal structure (13).

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—The strains of *Escherichia coli* used in this study were JM109 (14) and *E. coli* BMH 71-18 mutS (mutS::Tn10 Δ(lac-pro) thi supE [F' proA A- blac ZF23 M15])]. The *A. niger* strain used was NW188 (pyrA6, pyrF28, goxC17, lenA1), which is derived from *A. niger* N400 (CBS 12040). The original pyrF28 mutation was described previously (15). *E. coli* strains containing recombinant plasmids were cultured in LB supplemented with 0.5 µg/ml ampicillin at 37 °C. *A. niger* co-transformants containing the desired recombinant plasmids were cultured in a minimal medium containing 0.15% (w/v) KH2PO4, 0.05% (w/v) KCl, 0.05% (w/v) MgSO4·7H2O and 0.4% (w/v) NH4Cl (pH 6.0) supplemented with 0.1% (w/v) yeast extract, trace metal solution (16), 3% (w/v) fructose, and 0.02% (w/v) leucine.

**Site-directed Mutagenesis**—Site-directed mutagenesis was carried out using the Altered Sites II kit (Promega, Madison, WI) and synthetic oligonucleotides (Isogen, Maaren, The Netherlands). Reactions were done according to the supplier recommendations with the following modifications. First, single-stranded DNA of the phi-pga promoter gene fused into pALTER I was used as a template for double-stranded DNA. The phi-pgaII promoter gene fusion was recovered as an EcoRI-HindIII insert from pM3710 (6) and cloned into EcoRI-HindIII restricted pALTER I. Second, following *E. coli* BMH 71-18 mutS cells transformation with the mutagenesis mixture, plasmids were isolated instead of phagemids. *E. coli* JM109 competent cells were transformed with the potential mutated plasmids. To establish the expected mutation and to check for undesired mutations, the complete mutated pgaII gene was sequenced. *A. niger* NW156 was then co-transformed with the plasmids showing the expected mutations and the pGW635 carrying the pyrA gene as a selection marker (17). Mutated PGII producers were selected as the same way as described for ppgaE (18).

**Expression and Purification of the Enzymes**—Asp-180, Asp-201, Asp-202, His-223, Arg-256, and Lys-258 were changed to asparagine, glutamate, histidine, arginine, lysine, and histidine, respectively, in *A. niger* Endopolygalacturonase II Site-directed Mutagenesis

**HPLC Analysis of the Products Distribution after the Hydrolysis of Polygalacturonic Acid**—Each mutant enzyme was incubated with 1 ml of 1% (w/v) polygalacturonic acid in 50 mM sodium acetate buffer (pH 4.2) at 30 °C. After different incubation times (0–24 h), the enzymatic hydrolysis was stopped, and the reaction products were analyzed and quantified by HPAEC-PAD as described previously (18).

**RESULTS**

**Expression and Purification of the Enzymes**—Asp-180, Asp-201, Asp-202, His-223, Arg-256, and Lys-258 were changed to asparagine, glutamate, histidine, arginine, lysine, and histidine, respectively, in *A. niger* Endopolygalacturonase II Site-directed Mutagenesis

**Determination of the pH Optimum**—Incubations were carried out as for the standard enzyme assay on 0.25% (w/v) polygalacturonic acid using McIlvaine buffers ranging from pH 2.5 to pH 6. For the K258N mutant, the reactions were carried out in pH adjusted distilled water containing 50 mM NaCl, and the pH of the reaction mixture was carefully measured at the end of the reaction.
Specific activities of PGII mutants were determined in 50 mM sodium acetate buffer (NaAc) (pH 4.2), 20 mM methyl-piperazine/HCl buffer (MePIP) (pH 4.2), and McIlvaine buffer (McIlv) (pH 4.2). Kinetic parameters were determined in 50 mM sodium acetate buffer (pH 4.2), pH optima (pH opt) were determined using McIlvaine buffers. Polygalacturonic acid was used as a substrate. For specific activities 2.5 mg ml⁻¹ polygalacturonic acid was used. Temperature throughout 30 °C. ΔΔG values signify the transition state energy difference between wild type and mutant enzyme as calculated from the kinetic parameters. ND, not determined.

### Table I

| Enzyme        | pH opt (McIlv) | Specific activities | Kinetic parameters (NaAc) | ΔΔG  |
|---------------|----------------|--------------------|--------------------------|------|
|               |                | NaAc               | MePIP                    | McIlv| Kₘ | Vₘₙₙ | ΔΔG  |
| Wild type     | 4.2            | 2000               | 2270                     | 2140 | 0.17 | 0.15 | 22.5 |
| D180A         | 4.1            | 0.18               | 0.28                     | 0.27 | 0.17 | 0.15 | 22.5 |
| D180E         | 4.2            | 0.15               | 0.20                     | 0.14 | 0.24 | 0.3  | 23.3 |
| D180N         | 3.9            | 0.95               | 1.25                     | 0.95 | 1.4  | 1.5  | 23.0 |
| D201E         | 4.2            | 0.05               | 0.06                     | 0.04 | 0.04 | 0.3  | 27.6 |
| D201N         | 3.9            | 0.15               | 0.38                     | 0.22 | 0.19 | 0.3  | 23.9 |
| D202E         | 4.2            | 9.4                | 15.4                     | 10.2 | 12.7 | 0.7  | 15.9 |
| D202N         | 4.1            | 0.17               | 0.26                     | 0.12 | 0.3  | 1.5  | 26.7 |
| D180E/D201E   | 4.2            | 0.04               | 0.01                     | 0.02 | 0.04 | <0.15| 26.0 |
| H223A         | 4.1            | ND                 | ND                       | ND   | 10.0 | 0.15 | 15.6 |
| H223C         | 3.8            | 21.1               | 18.9                     | 18.0 | 21.5 | 0.80 | 14.9 |
| H223Q         | 3.9            | 0.19               | 0.38                     | 0.21 | 0.36 | 1.1  | 25.5 |
| H223S         | 4.1            | 1.68               | 1.65                     | 1.2  | 1.7  | 1.5  | 22.5 |
| R256Q         | 3.8            | 130                | 326                      | 129  | 127  | 1.7  | 10.6 |
| K258N         | 3.8            | 12.7               | 8.9                      | 0.00 | 16.2 | 2.8  | 18.6 |
| R258N         | 3.8            | 12.7               | 8.9                      | 0.00 | 16.2 | 2.8  | 18.6 |

* Determined in water.

**Acetate, but the specific activities of all the Asp-180 mutated forms of PGII in McIlvaine buffer were comparable. Finally, the amino acid introduced to replace Asp-201 only played a minor role because the specific activities between D201E and D201N only varied by a factor of 3–5, depending on which buffer was used. The reason why the nature of the amino acid introduced leads to a difference in the remaining specific activity of the enzyme is still unclear, but detailed crystallographic studies may provide further insights.**

**Kinetic Parameters**—The kinetic parameters Kₘ and Vₘₙₙ for the hydrolysis of polygalacturonic acid in 50 mM sodium acetate buffer (pH 4.2) were determined for the wild type (6) and the mutated enzymes by measuring the initial reaction rates at different substrate concentrations. The results are listed in Table I. D180(A/E), D201(E/N), D180E/D201E, and H223A displayed values of Kₘ (between 0.15 and 0.3 mg ml⁻¹) in the same range as the Kₘ calculated for the wild type enzyme (<0.15 mg ml⁻¹). All the other mutated forms of PGII exhibited higher values of Kₘ ranging from 0.7 mg ml⁻¹ for D202E to 2.8 mg ml⁻¹ in the case of K258N.

As already observed with the specific activities, the effect on Kₘ appears to depend on the side chain engineered as well as on the residue replaced. For instance, mutagenesis of His-223 resulted in enzymes with Kₘ values ranging from 0.15 mg ml⁻¹ (H223A) to 1.5 mg ml⁻¹ (H223S). Changes in Kₘ values were also observed among the different Asp-180 mutated forms of PGII. The only exceptions were enzymes D201E and D201N for which the Kₘ was found to be 0.3 mg ml⁻¹ in both cases.

**pH Optimum**—The pH optima for the activity on polygalacturonic acid were determined using McIlvaine buffers in the pH range from 2.5 to 6.0, except for K258N. As this enzyme was not active in McIlvaine buffers, its activity was measured in pH adjusted nonbuffered solutions. The pH optima of the mutated enzymes varied only slightly between pH 3.8 and pH 4.1. Thus, the mutations only produced a minimal effect on the pH optimum of the mutated forms of PGII.

**Bond Cleavage Frequencies on Oligogalacturonates**—By studying the hydrolysis reactions of the wild type PGII on reduced and nonreduced oligogalacturonates, it was previously shown that the cleavage of the glycosidic bond in the oligomers occurs from the reducing end (6). In a similar way, the cleavage patterns for each mutated form of the polygalacturonase were determined using (GalpA)₃₋₆ as substrates. The hydrolysis re-
actions were conducted in 50 mM sodium acetate buffer (pH 4.2) at an oligomer concentration of 0.5 mM. The BCFs thus calculated are presented in Table II.

None of the enzymes, including the wild type PGII, appeared to hydrolyze (GalpA)₄ under these conditions. The hydrolysis of (GalpA)₄ by each enzyme resulted only in (GalpA)₁ and (GalpA)₃, as already observed for the wild type PGII (6). A minor production (<0.5%) of (GalpA)₂ was, however, observed in some cases, when the reaction was allowed to proceed beyond 50% of conversion of the substrate.

Ben et al. (6) showed that the hydrolysis of (GalpA)₆ by native polygalacturonase II produced (GalpA)₁ and (GalpA)₄ with a BCF of 67%, whereas the formation of (GalpA)₂ and (GalpA)₃ occurred with a BCF of 33%. For the majority of the mutated forms of PGII (D180A/E/N), D201E/N, D202N, D180E/D201E, H223A, and H223S, the observed BCFs of the pentamer are comparable with those of native PGII. D180E, D202E, H223Q, R256Q, and K258N did not show any preference for the linkage hydrolyzed because the cleavage at the first and second glycosidic bond counting from the reducing end occurred with the same frequency. H223C displayed the most striking effect compared with the wild type enzyme because this enzyme hydrolyzed (GalpA)₇ preferentially producing (GalpA)₂ and (GalpA)₃ with BCFs of 80% and 33% for the native enzyme.

The hydrolysis of (GalpA)₆ revealed stronger effects on the BCFs because none of the mutated enzymes displayed an hydrolysis pattern comparable with that of the wild type PGII (Table II). Each mutated form of PGII exhibited a shift in BCFs in favor of the second and/or third linkage, counting from the reducing end. The most remarkable effect was once again observed with H223C, which hydrolyzed the hexamer producing (GalpA)₁ and (GalpA)₃ with a BCF of 84%, and only attacked the first glycosidic bond sporadically (BCF of 2%). For the majority of the other mutated enzymes, the cleavage at the first glycosidic linkage also occurred less often than in the case of the wild type PGII, favoring the attack of the second glycosidic bond, whereas the BCF at the third bond was unchanged. D180A, H223S, R256Q, and K258N were the only enzymes to display a clear increase in the hydrolysis of the hexamer into (GalpA)₄, even if the release of (GalpA)₂ and (GalpA)₃ remained predominant as is the case for the native polygalacturonase II.

The shift in BCFs for the mutated enzymes was also reflected in the product progression curves during the hydrolysis of polygalacturonic acid. For the wild type enzyme a product progression curve typical for endo-acting enzymes was observed (6). The mutated enzymes accumulated higher oligomers (n > 5) in a way similar to that of the wild type PGII. However, the rate of accumulation of smaller oligomers (n < 5) was different depending on the enzyme. Enzymes D180A/E/N, D201E/N, and D202N showed only small differences compared with the wild type enzyme with respect to the ratio of oligomers formed. More striking differences were observed with H223A/S/C, D202E, R256Q, and K258N, where a strong accumulation of (GalpA)₂ was monitored (not shown). These data correlate with the observed changes in the BCFs on pentaa- and hexaoigosaccharides.

**DISCUSSION**

The data presented in this paper describe the biochemical characterization of several site-specific mutants of PGII from A. niger. The primary objective of this study was to investigate the role of the highly conserved residues in the active site of PGII.

**General Considerations**—All the residues mutated appeared to be very critical for catalysis. For each residue, except for His-223, a counterpart is present in the rhamnogalacturonase from A. aculeatus (12). The basic difference between the polygalacturonases and the rhamnogalacturonases resides in their substrate specificity. Whereas polygalacturonases hydrolyze the α-1,4 glycosidic linkage between galacturonic acid residues, rhamnogalacturonases hydrolyze the α-1,2 glycosidic linkage between galacturonic and rhamnose. The common part of the substrate, the galacturonic moiety, will be accommodated at subsite −1. This subsite −1 is expected to display the highest sequence conservation. As a consequence, residues Asp-180, Asp-201, Asp-202, Arg-256, and Lys-258 should constitute residues of subsite −1 and the catalytic machinery between subsites −1 and +1. It is therefore assumed that the mutations do not affect subsites −4, −3, −2, +2, and +3.

The bond cleavage frequencies for a particular (mutated) enzyme do not necessarily reflect the real binding energy distribution over the subsites covered because the rates of hydrolysis of each particular binding mode may be different (22). Indeed, the bond cleavage frequencies only take productive complexes into account, thus the substrate must always cover subsites −1 and +1. Therefore any change of affinity at subsites −1 or +1 would affect any binding mode covering subsites −1 and +1 with the same ΔG change. However, changes in BCFs were observed that could be explained by the following. At subsite −1, the substrate moiety is generally thought to be bound in a particular distorted configuration to facilitate the
Table II
Bond cleavage frequencies for wild type and mutated endopolygalacturonase II

| Enzyme          | Gn |
|-----------------|----|
|                 | G  | G  | G  | G  | G  | G  | G  | G  |
| Wild type       | 8  | 57 | 35 |
| D180A           | 20 | 58 | 22 |
| D180E           | 9  | 73 | 18 |
| D180N           | 10 | 68 | 22 |
| D201E           | 9  | 76 | 15 |
| D201N           | 11 | 65 | 24 |
| D202E           | 9  | 81 | 10 |
| D202N           | 11 | 62 | 27 |
| D180E/D201E     | 11 | 63 | 26 |
| H223A           | 10 | 65 | 25 |
| H223C           | 14 | 84 | 2  |
| H223Q           | 6  | 72 | 22 |
| H223S           | 16 | 64 | 20 |
| R256Q           | 15 | 74 | 11 |
| R258N           | 18 | 70 | 12 |

Assay conditions: 0.5 mM oligogalacturonates were incubated with (mutated) endopolygalacturonase II in 0.5 ml of 50 mM sodium acetate (pH 4.2). At timed intervals 50-μl aliquots were withdrawn and mixed with 50 μl of stopmix (2.0 mM Tris, 50 mM NaOH) to raise the pH to 8.3–8.5. Products were analyzed and quantified by HPAEC-PAD as described under “Experimental Procedures.” The bold type indicates the reducing end. Bond cleavage frequencies are given in percentages. On signifies (GalpA)₄₆.

Glycosidic bond cleavage (31), which results in a net negative affinity at this subsite. To compensate for this negative affinity and to properly align the scissile bond, subsite +1 binds the substrate with high affinity and thus allows effective catalysis. Any decrease of the affinity at subsite +1 may not allow for compensation for the negative affinity at subsite −1, and this would therefore result in less effective catalysis. Only by invoking subsite +2 would there be enough binding energy to compensate for the low affinity at subsite −1, and hence this would result in a shift of the BCFs. For affinity changes at subsite −1, the reasoning follows the same lines. Any change at this site involved in the binding of the substrate would decrease the rate of hydrolysis, because the ideal distortion will be changed. To compensate for this and to provide enough strain on the scissile bond to allow an effective catalysis, additional binding at subsite +2 would be required. Thus, the bond cleavage frequencies become meaningful when combining both the affinity and the rate of hydrolysis in terms of effectiveness.

Importance of Arg-256 and Lys-258—To evaluate the role of these two residues, they were substituted for glutamine and asparagine respectively, and the biochemical properties of the resultant proteins were analyzed. Among all the mutated enzymes studied, R256Q and K258N displayed the highest specific activities and highest $K_m$ values on polygalacturonic acid. Despite the high $K_m$ values, the mutated enzymes revealed the smallest effect on the transition state energy. It should be noted that for the calculation of the transition state energy for the wild type enzyme a $K_m$ value of 0.15 mg ml⁻¹ was used, which may result in an underestimation of the effect of the mutations on the transition state energy. In the crystal structure Arg-256 and Lys-258 are about 4 Å apart. This suggests that the residues may interact with adjacent galacturonate residues, occupying subsite −1 (Lys-258) and +1 (Arg-256). This is corroborated by the transition state energy difference, which is for both mutants in the order of an ionogenic bond. Furthermore, mutant K258N was severely inhibited by the negatively charged ions citrate and phosphate. Also, both mutations led to enzymes with a mode of action on oligogalacturonates clearly different from the wild type PGII, which is compatible with a mutation at subsite −1 or +1. It can easily be envisaged that the absence of the interaction between the substrate and residue Arg-256 or Lys-258 will change the geometry of the ideal distortion of the substrate, which will result in the observed decreased $V_{max}$. Taken together, these data strongly suggest that Arg-256 and Lys-258 are primarily involved in the interactions with the substrate.

Role of His-223—The (in)direct involvement of a histidine residue in the activity of PGII has been proposed several times on the basis of chemical modifications (23–25) and site-directed mutagenesis (26). However, there is no example of such a residue being one of the catalytic amino acids in glycosyl hydrolases. In addition, although this histidine is strictly conserved among the family 28 polygalacturonases, it is not present in the rhamnogalacturonase A from A. aculeatus, which belongs to the same family. As the catalytic residues are always strictly conserved within a family, this latter point definitely rules out His-223 to be the proton donor in the reaction catalyzed by PGII. The remaining activity of the His-223 mutated enzymes was extremely dependent on the nature of the amino acid introduced, which is in contradiction with His-223 being a catalytic residue. Inspection of the His-223 mutated enzymes revealed that all enzymes were severely affected in catalysis but that the bond cleavage frequencies did not change dramatically for enzymes H223A and H223S, when a small residue is
engineered. This implies that the relative effectiveness of the wild type enzyme and enzymes H223A and H223S has not changed, and thus the disturbance at subsites 1 and 1 + 1 is minimal, which indicates that His-223 plays an indirect role in catalysis. This role may be to maintain the proper ionization state of a carboxylate involved in catalysis by sharing a proton. Based on the crystal structure, this carboxylate could be Asp-201, which is the closest. The presumed role of His-223 is corroborated by the fact that enzyme H223C, whose sulfhydryl group is capable of sharing a proton, despite its interference with the substrate, still retains the highest activity. Moreover, the rhamnogalacturonases A and B from A. niger, which lack an His-223 counterpart have specific activities in the same range as the one found for enzyme H223C viz. 30–40 units mg\(^{-1}\) (27).

Importance of Asp-180, Asp-201, and Asp-202—Polygalacturonases have been shown to hydrolyze glycosidic bonds with an inverting mechanism (5) that requires two carboxylic groups at a distance of 9–9.5 Ǻ from each other (9, 10). Sequence alignment of polygalacturonases revealed three strictly conserved Asp residues among these enzymes (Asp-180, Asp-201, and Asp-202). However, in the rhamnogalacturonase that belongs to the same family, Asp-202 is replaced by a Glu. Our data show that the mutation of each of these residues led to a dramatic decrease of the specific activity of PGII. All the mutated enzymes indeed retained between 0.002 and 0.07% of the wild type activity, except D202E, for which the remaining activity was 0.64%.

To understand the role of the acidic residues, we compared the PGII structure\(^2\) with the similar phage 22 tailspike rhamnosidase (28). This latter enzyme also shows a β-helix fold and, moreover, has three acidic residues in the active site (Glu-359, Asp-392, and Asp-395) in a geometry similar to that of the polygalacturonase and rhamnogalacturonase. In accordance with our results, mutagenesis of those residues also resulted in severe reduction of enzymatic activity in each case (29). Comparison of the rhamnosidase structures with and without the Salmonella typhimurium 2637β nonsaccharide bound revealed that the water bound between Glu-359 and Asp-395 (Asp-180 and Asp-202 in endopolygalacturonase II) is in an ideal position for nucleophilic attack in an inverting mechanism. Asp-392 (Asp-202 in endopolygalacturonase II), at hydrogen bonding distance from the O-1 atom of the rhamnose at subsite 1, may be the general acid. In PGII, the structure indeed revealed that a water molecule is bound between Asp-180 and Asp-202. Moreover, a galacturonate residue could be modelled into the structure of PGII based on the superposition of the catalytically important oxygen atoms resembling the position of the rhamnose in the rhamnosidase.\(^2\)

Based on our results and in analogy to the phage 22 tailspike rhamnosidase, we propose that Asp-180, with the assistance of Asp-202, acts as a base to activate the bound water molecule. The fact that the mutation of Asp-180 into either Ala or Gln or Glu leads to an inactive enzyme supports the possibility that this amino acid is much more critical for the catalysis than Asp-202. Indeed, the mutation of Asp-202 into Gln (a conservative change that retains the anionic property) leads to an enzyme that displays the highest activity among the Asp mutants.

Asp-201 would then be the general acid that protonates the product when it deparures. Three arguments are in favor of this proposal: (i) the mutation of this residue led to an inactive enzyme; (ii) its replacement revealed the smallest effect on the BCPs on oligogalacturonates, which suggests that Asp-201 does not directly interact with the substrate; and (iii) His-223, which is important for catalysis most likely shares a proton with Asp-201, allowing this latter amino acid to be in the proper ionization state to protonate the product. These three aspartic residues in the polygalacturonase from \textit{Erwinia carotovora} were identified in the recently solved crystal structure, and Pickersgill et al. (13) the authors also proposed that the amino acids corresponding to Asp-180 and Asp-201 in PGII are directly involved in catalysis.

A careful inspection of both rhamnogalacturonase and polygalacturonase structures (12, 13) revealed that the three acidic residues are very close to each other and that the distance in PGII between Asp-180 and Asp-201 (4.1 Ǻ), Asp-201 and Asp-202 (4.9 Å), and Asp-180 and Asp-202 (5.7 Ǻ) is not compatible with an inverting mechanism. Our data and the data obtained for the phage 22 tail spike rhamnosidase strongly indicate that nucleophilic attack and protonation can occur from the same side of the glycosidic bond in an α-linked carbohydrates. It can therefore be stated that family 28 glycosyl hydrolases diverge with respect to their active site configuration from the generally observed active site architecture found in inverting enzymes.

To firmly establish the exact role of the individual amino acids, it is important to obtain an enzyme-substrate complex with polygalacturonase II. The mutant D180E/D201E prepared in this study would be the protein of choice for the study of the enzyme-substrate complex structure.