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An essential tyrosine residue of *Aspergillus* polygalacturonase

Eva Stratilová[a], Mária Dzúrová[a], Oskar Markovič[a], Hans Jörnvall[b,*]

[a]*Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, SK-842 38 Bratislava, Slovakia*
[b]*Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden*

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Abstract **Based on strict conservation of a tyrosine residue in 24 polygalacturonases, tyrosine modification was assessed in two different forms of the *Aspergillus* enzyme. The second subform was unknown in structure but submitted to sequence analysis and was found also to have the conserved tyrosine residue. Results of chemical modifications are consistent in showing inactivation of the proteins with all tyrosine-reactive agents tested, acetic anhydride, *N*-acetyl imidazole, and tetranitromethane. Furthermore, after acetylation, regeneration of enzyme activity was possible with hydroxylamine. Spectrophotometric pH titration showed that one accessible tyrosine residue is ionized at pH 9.3–9.5, whereas the remaining, masked residues are all ionized at pH 10.5. It is concluded that one tyrosine residue is catalytically important, in agreement with the inactivation and reactivation data, that this residue is accessible, and that it is likely to correspond to the strictly conserved residue observed in all forms.**

Key words: Polygalacturonase; Multiple forms; Tyrosine; Modification; Homology

1. Introduction

Polygalacturonase [poly (1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15] catalyzes hydrolysis of internal α-(1–4) glycosidic linkages in D-galacturonans, resulting in a pronounced decrease of substrate viscosity already at a low extent of degradation. The enzyme is, together with exopolygalacturonase [poly (1,4-α-D-galacturonide) galacturonohydrolase, EC 3.2.1.67], present in higher plants, and in plant-pathogenic fungi and bacteria. Amino acid sequence information of many forms of both enzymes is available (most recent additions in [1–10]).

Structural comparisons of known polygalacturonases from different sources show that one histidine residue in a GHGXSIGS structure, three aspartic acids (in NTD and DD structures), an RIK structure and one tyrosine, are all conserved and have been ascribed catalytic functions [1,11]. These residues are in a segment with localized, inter-species homology in the C-terminal half of the polygalacturonase molecule (cf. [1]). Based on sensitivity to inhibitors, it has been suggested that polygalacturonases have amino groups, sulfhydryls, tyrosine-hydroxyls [12], histidine imidazoles [13] or carboxyls of acidic residues [14] that are essential to the activity. In this study, the important role of a single tyrosine residue in the catalytic action of *Aspergillus* polygalacturonase was ascertained by chemical modification and spectrophotometric pH titration.

2. Materials and methods

For spectrophotometric pH titrations, two homogeneous *Aspergillus* sp. polygalacturonase forms were purified from a commercial preparation (Rohament P; Rohm GmbH, Germany) as described [1]. For enzyme activity determinations during chemical modifications, the Rohament P polygalacturonase preparation was dissolved in water, submitted to two precipitation steps with ammonium sulfate (90% saturation) and ethanol (1:4, v/v) and desalted on Sephadex G-25 Medium (Pharmacia, Sweden).

Amino acid compositions were determined with a 451 AlphaPlus amino acid analyzer (LKB, Sweden) after hydrolysis in evacuated tubes for 24 h at 110°C in 6 M HCl, 0.5% phenol. Carboxymethylation was performed as described [1]. For Lys-specific cleavage, the carboxymethylated polygalacturonase (3 nmoles) was dissolved in 9 M urea (50 μl), diluted to 1 M urea immediately before protease addition (endoproteinase Lys-C, 0.5 μg; Boehringer-Mannheim, Germany) in 0.1 M ammonium bicarbonate, pH 8.0, and digested for 10 h at 37°C. Asp-specific cleavage was performed similarly with endoproteinase Asp-N, 1.1 μg (Boehringer-Mannheim, Germany) for 48 h. The digests were separated by HPLC on Vydac C4 (Separations Group, Hesperia, California) with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Amino acid sequences were analyzed with an ABI 470A sequencer.

Polygalacturonase activity was assayed at 30°C, pH 4.6, in 0.1 M acetate buffer, by measurement [15] of the increase of reducing groups (μmol liberated per min and mg enzyme preparation), using 0.5% sodium pectate as substrate and D-galactopyranuronic acid as standard.

Acetylation with acetic anhydride [16] was conducted in 1 M acetate buffer, pH 5.8, or in 0.05 M phosphate buffer, pH 7.0, and in 1.65 M NaCl at 0°C. Final concentration of acetic anhydride was 4.0×10^{-3}–1.5×10^{-2} M and of polygalacturonase 1.4 mg per ml. The reaction was stopped at 30 min by passing the reaction mixture through a PD 10 column (Pharmacia) equilibrated with 0.1 M acetate buffer, pH 4.6. Acetylation with *N*-acetylimidazole [16] (Fluka) was carried out at room temperature in 0.01 M Tris-HCl, pH 7.5. The final concentrations were 0.9–6.8 mM *N*-acetylimidazole and 1.4 mg polygalacturonase per ml. Deacetylation was effected by treatment with hydroxylamine HCl (Fluka) at a final concentration of 28 mg per ml, pH 7.5, and room temperature for 2 h. Excess reagent was removed by PD 10 chromatography.

Nitration [16] was effected with tetranitromethane (Fluka). The agent (1.04×10^{-3}–8.32×10^{-3} M final concentration) was added to the enzyme (1.4 mg per ml) dissolved in 0.1 M Tris-HCl, pH 8.0. After 60 min, the reaction mixture was passed through a PD column equilibrated with 0.1 M acetate buffer, pH 4.6, to remove unreacted reagent.

Spectrophotometric pH titrations were conducted in glycine/NaOH solutions of pH 8.6–12.2, with the enzyme concentration at 0.5 mg per ml. The change in absorptivity at 295 nm associated with ionization of one tyrosine residue was calculated from the relation: 2540 $M^{-1}\times cm^{-1}$ [17], where M is the molecular mass of the enzyme.

3. Results and discussion

The major polygalacturonase forms, III and IV [1] of *Aspergillus* Pectinase Rohament P, were submitted to structural analysis after cleavage with Asp-specific and Lys-specific endoproteinases. Six protein segments have been identified before by analysis of form III and alignment with other distantly

*Corresponding author. Fax: (46) (8) 33 74 62.

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A. sp. Rohament form IV	D Y - A Y T - - G K E P - - C
A. sp. Rohament form III	D T - N Y C - - R T N V A - C
A. niger I	E Q - D Y E N - G S - P T - G
A. niger II	Q Q - D Y E D - G K - P T - G
A. niger III	E Q - N Y D D T S K F P T T G
A. tubigensis	Q Q - D Y E D - G K - P T - G
A. oryzae	E Q - D Y E N - G S - P T - G
A. flavus	E - L D Y K N - G - G P T - G
A. parasiticus	E Q - D Y E N - G S - P T - G
C. carbonum	E Q - D Y L N - G - G P T - G
P. solanacearum	D P - F Y S - S V K - - - - G
E. carotovora 1	D T V - Y E - - K K E - - - G
E. carotovora 2	D T V - Y E - - K K E - - - G
E. chrysanthemi	G T I D Y T P A K V - P A - -
Tomato	D Q - N Y C D - R V E P - - C
Peach	D Q - N Y C D - H K N K D - C
Oenothera	D Q - G Y C - - P Y N Q - - C
Maize	D M - K Y C - - P - N K L - C
Kiwi	D Q - N Y C D Q D K - P - - C
Brassica	D Q - E Y C - - P W N Q - - C
Tobacco	D Q V - Y C - - P F N K - - C
Avocado	D Q - Y Y C D - S K D P - - C
Apple	D Q - N Y C D - H K T K D - C
Cotton	D Q - Q Y C - - P W N K - - C

Fig. 1. Comparison of 24 characterized polygalacturonases around a strictly conserved tyrosine residue (at the arrow, corresponding to position 297 in the *Aspergillus niger* I structure [18]). In spite of this conservation, a remarkable variation in both residues and gap positions is noticed, in agreement with a general variable polygalacturonase structures as already observed [1]. Structures from [1–11,18] and references therein.

related polygalacturonases [1]. The peptide segment covering positions 294–308 contains the strictly conserved tyrosine residue. We now completed this comparison by analysis of form IV also, and inclusion of it and thirteen more polygalacturonases in the scheme (Fig. 1). The strictly conserved Tyr-297 is present in these additional sequences, too.

Chemical modifications of purified *Aspergillus* sp. polygalacturonase with 4.0×10^{-3} M acetic anhydride in 1 M acetate buffer led to 90% decrease of the enzyme activity, and with 1.5×10^{-2} M reagent concentration the enzymes were completely inactivated. A low extent of deacetylation with hydroxylamine was achieved (less than 15% recovery of enzyme activity) after acetylation at the lowest acetic anhydride concentration used (4.0×10^{-3} M), while acetylation in 0.05 M phosphate buffer, pH 7.0, 1.65 M NaCl, led to complete inactivation already at that reagent concentration. This non-reversible modification with acetic anhydride is assumed to involve amino groups [16] and polygalacturonase has reactive lysine [12], where the strictly conserved Arg-Ile-Lys at positions 262–264 constitutes a likely candidate for ionic interactions with substrate carboxylate groups [11]. The hydroxylamine-reversible part of this inactivation under strong acetylating conditions is interpreted to reflect tyrosine O-deacetylation, and motivated further studies with a mild acetylation reagent and other tyrosine modifications.

N-acetylimidazole generally gives milder and more selective acetylation [16], and this was found to be true for the purified *Aspergillus* sp. polygalacturonase (Fig. 2) At 2.3×10^{-3} M *N*-acetylimidazole, corresponding to a 60-fold molar excess, 50% inactivation of polygalacturonase was achieved within 60 min, and at 6.8×10^{-3} M, corresponding to 170-fold molar excess, 80% inactivation was achieved within 10 min. In the latter case, hydroxylamine treatment reactivated the enzyme to about 50% activity, while the enzyme inactivated to 50% at the low *N*-acetylimidazole concentration was completely reactivated by hydroxylamine treatment. These results strongly suggest selective Tyr modification interpreted to represent the single Tyr residue strictly conserved at position 297.

The action of tetranitromethane on polygalacturonase also indicated a role of Tyr in the catalytic function of the enzyme

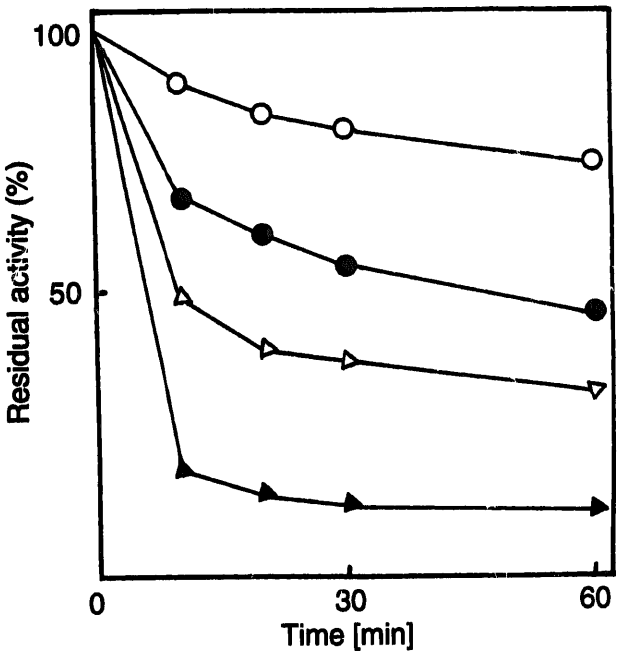

Fig. 2. Inactivation of *Aspergillus* sp. polygalacturonase at different *N*-acetylimidazole concentrations (○, 9.1×10^{-4} M; ●, 2.3×10^{-3} M; △, 4.4×10^{-3} M; ▲, 6.8×10^{-3} M). The enzyme concentration was 1.4 mg per ml in 0.01 M Tris-HCl, pH 7.5.

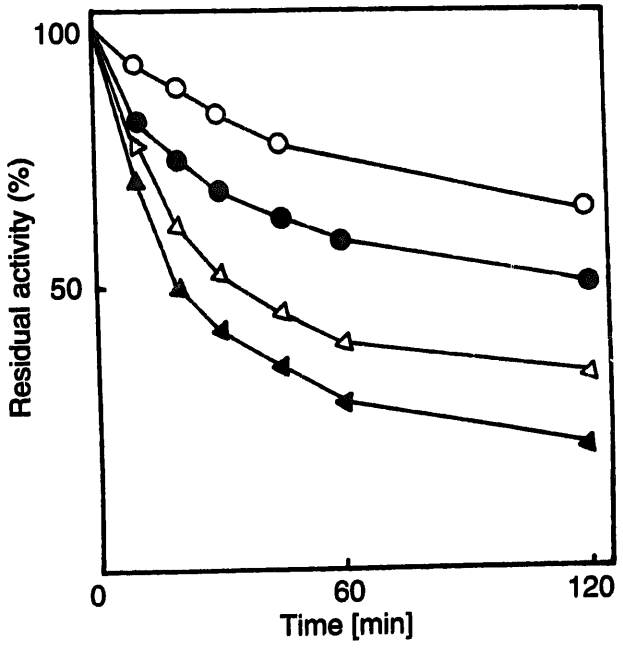

Fig. 3. Inactivation of *Aspergillus* sp. polygalacturonase at different tetranitromethane concentrations (○, 1.04×10^{-3} M; ●, 2.08×10^{-3} M; △, 4.16×10^{-3} M; ▲, 8.32×10^{-3} M). The enzyme concentration was 1.4 mg per ml in 0.1 M Tris-HCl, pH 8.0.

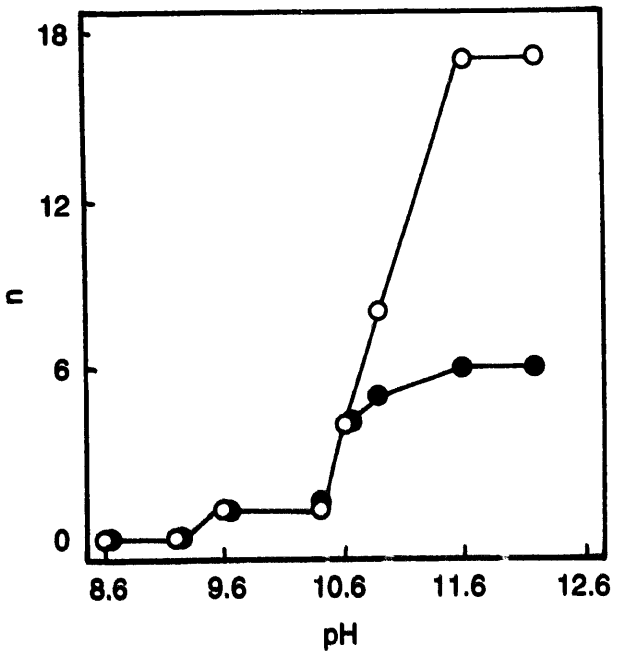

Fig. 4. Spectrophotometric pH titration of tyrosine residues of homogeneous *Aspergillus* sp. polygalacturonase III (○) and IV (●) forms, respectively. Measurements at 295 nm with *n* denoting the number of ionized tyrosine residues.

(Fig. 3). At a 50-fold molar excess of tetranitromethane a 40% inactivation of polygalacturonase activity was obtained, and at a 100-fold molar excess the activity decrease was 60% within 60 min. It was not possible to follow the rate of nitration of polygalacturonase by measurement of the absorbance of the nitro-form anion at 350 nM, because of development of turbidity even at the lowest tetranitromethane concentration used.

Finally, the reactivity of tyrosine residues in relation to protein conformation was monitored by spectrophotometric pH titration of homogeneous form III containing 17 tyrosine residues, and form IV containing 6 tyrosine residues. For both enzymes, the tyrosine content was determined by amino acid analysis. The course of titration showed (Fig. 4) that for both form III and form IV, one tyrosine residue is ionized at pH 9.4–9.5, indicating an accessible tyrosine residue, whereas the remaining residues are all ionized at pH 10.5, indicating masked tyrosine residues.

In conclusion, the results obtained suggest that polygalacturonases contain a catalytically essential tyrosine residue. In all known primary structures of polygalacturonases a strictly conserved tyrosine residue is present, and in the two major *Aspergillus* polygalacturonases, nitration, reversible acetylation, and spectrophotometric pH titration indicate the presence of an accessible and enzymatically important tyrosine residue.

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