Characterization of Pectic Enzymes from the Chickpea Pathogen *Ascochyta rabiei*

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The chickpea pathogenic fungus *Ascochyta rabiei* when growing in a pectin containing mineral medium secretes an exo-polygalacturonase (PG) and a pectin methyl esterase into the culture medium. The induction of the PG is controlled by catabolite repression. The PG was purified to apparent homogeneity from the culture fluid by acetone precipitation and chromatography on a Mono S and a Mono Q column. The molecular weight of the purified glycoprotein enzyme in SDS-PAGE was about 70 kDa, whereas the *M*r by gelfiltration it was estimated to be 53 kDa. Polygalacturonic acid is the only substrate of the PG whereas pectin is not degraded. The PG releases only galacturonic acid from polygalacturonic acid and no oligo-galacturonides which might elicitate chickpea plants and trigger plant defense response. The enzyme is not inhibited by pectin, the released product galacturonic acid or by chickpea proteins or phenolics.

Introduction

Ascochyta rabiei is the main fungal pathogen on chickpea *(Cicer arietinum),* causing blight disease on leaves and stems. Histological studies revealed rapid disintegration of leaf tissue after successful infection [1]. The fungal hyphae penetrate the plant cell wall and spread beneath the epidermal layer before this collapse of tissue occurs. It has been assumed [1] that during this process cell wall degrading enzymes are being produced by the penetrating pathogen. Pectin degrading enzymes seem to be widely involved in this process as has been shown for several plant-pathogen interactions [2, 3]. Digestion of pectin appears to be an important strategy to establish fungal infections in plants. On the other hand, the oligomeric pectin fragments released during this process are often able to trigger plant defense response because they act as elicitors [4, 5]. The balance between these two contrasting effects can be influenced either by the cleavage pattern of the fungal pectic enzymes (exo- *versus* endo-cleavage) or by plant compounds acting as inhibitors for the pectic hydrolases.

As part of a comprehensive research program of the chickpea *A. rabiei* interaction [1, 6] he have now purified and characterized from the culture filtrate an exo-polygalacturonase (PG) from this fungus and investigated the possible occurrence of further pectic enzymes. These investigations aim at determing pathogenicity factors in *A. rabiei.*

Abbreviations: BSA, bovine serum albumine; DP, degree of polymerization; FPLC, fast protein liquid chromatography; GalA, galacturonic acid; PG, polygalacturonase; pGalA poly-galacturonic acid; PGIP, polygalacturonase inhibiting protein; SDS-PAGE, sodium dodecylsulfate polyacrylamide gelelectrophoresis; Tris, tri-(hydroxymethyl)-aminomethane.

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Materials and Methods

Fungal strains

Ascochyta rabiei strain III (CBS 534.65) was obtained from the Centralbureau voor Schimmelcultures in Baarn and *A. rabiei* strain X (Atareb-edleb) comes from Dr. M. C. Saxena, ICARDA, Aleppo, Syria. The fungus was grown in modified Richard's medium [7] approximate 500 ml/flask containing an appropiate carbon source. In these studies, 5 to 10 g of either pectin, polygalacturonic acid (pGalA), galacturonic acid (GalA), sucrose or glucose was used as nutrient in the growth medium.

Buffers

The buffers were: A, 10 mM potassium phosphate pH 7.0, 3 mM dithioerythritol; buffer B, 15 mM sodium acetate pH 4.2, 5% (v/v) ethanol; buffer C, 15 mM Tris/HCl pH 8.5, 5% (v/v) ethanol; buffer D, 50 mM sodium acetate pH 4.7; buffer

E, 50 mM Tris/HCl pH 8.5, 1 mM $CaCl_2$; buffer F, 20 mM Tris/HCl pH 7.5; buffer G, 50 mM sodium acetate pH 5.0, 50 mM NaCl. Determination of the pH optimum of the enzyme was carried out in McIlvane widerange buffer.

Protein isolation

The culture filtrate was cooled on ice and the PG was precipitated with an equal volume of precooled acetone (−20 °C). The protein containing pellet was collected by centrifugation (15 min, 20,000 × *g*) and resuspended in buffer A. Most of the precipitate did not redissolve and was removed by centrifugation (15 min, 20,000 × *g*). The crude extract was dialysed against buffer B over night with a single buffer exchange and then used for further enzyme purification.

Enzyme purification

The protein preparation was applied to a Mono S column equilibrated with buffer B and the PG was eluted with a NaCl gradient up to 0.5 M in the same buffer. Fractions with pronounced enzyme activity were pooled and transferred to buffer C using a Sephadex G25 (PD 10) column. The resulting protein preparation was loaded onto a Mono Q column and eluted with a linear gradient up to 0.5 M NaCl in buffer C. Enzymatically active fractions were pooled and used for further characterization of the enzyme. All chromatographic runs were performed with a FPLC-apparatous (Pharmacia, Freiburg, F.R.G.).

Enzyme assays

The PG was measured with 0.2% sodium pGalA in buffer D. The standard incubation assay consisted of 0.4 ml sodium pGalA (0.5%), 0.5 ml buffer D, and 0.1 ml enzyme preparation. Enzyme activity was determined using the increase of reducing groups from released GalA with the para-hydroxybenzoate-hydrazide method [8] which was measured photometrically at 410 nm. The enzyme assay for pectate-lyase was performed in buffer E, and activity was either measured by recording the increase of reducing groups or by increase of absorption at 235 nm. The pectin methyl esterase was measured with 1% pectin C as substrate in 10 mM sodium acetate buffer (pH 6.5) and methylenred (0.01%) as pH-indicator. The enzyme activity was quantified by titration of the enzyme assay with 0.1 N NaOH, measuring the pH with a microelectrode in 1.5 ml reaction tubes.

Product analysis

A 100 µl aliquot of an enzyme assay was boiled for 10 min to stop the enzyme activity, diluted 5-fold with distilled water and then analyzed for released products from the pGalA substrate. The aliquot was supplied to a Mono Q column equilibrated with buffer F and $(GalA)_n$-fragments were separated according to their degree of polymerization (DP) by elution with a linear gradient up to 0.5 M NaCL in buffer F. Each fraction was analyzed for galacturonides with the *o*-phenylphenol method [9].

Protein determination

Protein concentrations were determined according to the method of Bradford [10] with BSA as standard.

Molecular weight determination

The *M*r of the native PG was determined by gel-chromatography on a Superose 12 column with buffer G. The *M*r was compared with a standard protein mixture consisting of hexokinase (100 kDa), BSA (67 kDa), β-lactoglobuline (36.5 kDa) and cytochrome *c* (12.5 kDa). SDS-PAGE of the purified PG was performed under denaturating conditions according to Lämmli [11]. Gels were stained either with Coomassie blue or the silver method.

Chemicals

All chromatographic materials for the FPLC-system were supplied by Pharmacia (Freiburg, F.R.G.), belonging to the FPLC-system. Pectin, pGalA and *o*-phenylphenol were purchased from Serva (Heidelberg, F.R.G.) and para-hydroxybenzoate-hydrazide came from Sigma (Munich, F.R.G.).

Results

Secretion and production of the polygalacturonase

A. rabiei readily grows in a minimal medium with pectin as the only carbon source. Under this condition the fungus produces PG and pectin

methyl esterase activities for degrading pectin. The expression of PG activity was found to be inducible by pectin, pGalA or GalA, respectively. No PG activity was detected when *A. rabiei* was grown in a glucose or sucrose containing minimal medium. The enzyme production in pectin containing medium could be repressed by adding sucrose or glucose to the culture medium, indicating catabolite repression of PG production by the fungus. The PG is secreted into the culture fluid and no enzyme activity was found inside the mycelium or in cell wall fragments of the fungus. The level of PG activity increases concomitantly with mycelium biomass and reaches maximal amounts in the stationary phase of the growth cycle some 7 to 9 days after inoculation.

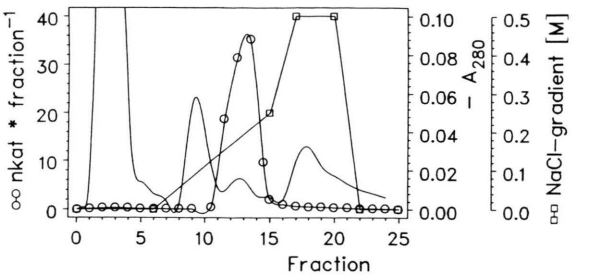

Fig. 1. Chromatography of the PG on a Mono S column (FPLC-system). The PG was supplied to the column in 15 mM sodium actate buffer pH 4.2 with 5% (v/v) ethanol and eluted with a gradient up to 0.5 M NaCl. Fractions of 1 ml were collected and tested for enzyme activity. The PG eluted at approximately 150 mM NaCl in fraction 11 to 14.

Enzyme purification

The PG from the culture fluid was purified to apparent homogeneity in a three step procedure. First, the PG was precipitated by adding to the culture fluid precooled acetone up to 50% (v/v) and collected by centrifugation. The large pellet could only be partially resuspended but most of the PG activity was recovered after solubilization over night at 4 °C. After centrifugation, the clear solution was applied to a Mono S column (Fig. 1) where most of the protein did not bind. The PG was eluted with approximately 150 mM NaCl in buffer B. Fractions with pronounced PG activity were collected and transferred to buffer C. The subsequent chromatography on a Mono Q column removed further minor protein contaminations. During this purification procedure (Table I) the PG was enriched 23-fold and seems to be apparently homogeneous as judged by SDS-PAGE.

Kinetic properties

The hydrolytic depolymerization of pGalA catalyzed by the purified PG was linear up to at least 1 h at 30 °C. The K_m value and the maximal velocity of the enzyme reaction were determined with pGalA (average DP = 170) in the range from 0.001% to 0.75% substrate concentration under standard incubation conditions.

The enzyme activity follows Michaelis-Menten kinetics with a K_m value of 0.009% pGalA (approximately 2.7 μM) and a maximal velocity of 856 U/mg protein (= 14.3 kat/kg protein). No product inhibition by GalA was observed even when more than 50% of the glycosidic bonds of pGalA were cleaved by the PG. The enzyme did not degrade pectin instead of pGalA.

The pH optimum of the PG (Fig. 2) in McIlvaine widerange buffer shows a broad plateau with high enzyme activity between pH 3.0 and 5.5. The enzyme was active for at least 1 h at pH 2.5 without any loss of activity. PG activity increases linear with temperature up to 50 °C but above 60 °C the PG was rapidly denaturated. Under standard enzyme assay conditions at 30 °C approximately 75% of the maximal PG activity were obtained.

Table I. Purification of the exo-polygalacturonase from the culture medium of *A. rabiei*.

	Volume [ml]	Protein [mg]	Activity [kat/kg protein]	Yield [μkat]	[%]	Purification fold
Culture filtrate	1600	49.6	0.62	30.6	100	1
Acetone precipitation	65	27.3	0.74	20.3	66	1.2
Mono S	46	1.14	6.66	7.6	24	10.8
Mono Q	66	0.33	14.3	4.7	15	23.1

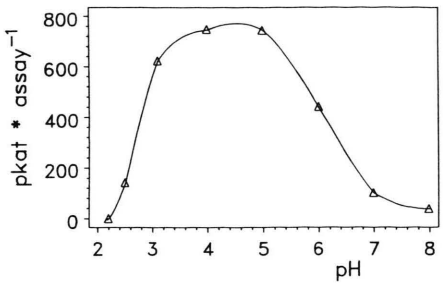

Fig. 2. pH-optimum of the PG. The enzyme assays were carried out in McIlvaine widerange buffer and stopped after 30 min. The enzyme reaction was linear at pH 2.5 for at least 1 h. The PG showes a broad optimum between pH 3.5 to 5.5 but no enzyme activity was found above pH 8.

Enzyme inhibition

Though pGalA appears to be the only substrate of the PG, the structurally similar macromolecule pectin might act as a competitive inhibitor of the enzyme activity. To investigate this assumption various concentrations of pGalA and pectin were tested but no such inhibitory effect was found. Even with a 50-fold excess of pectin over pGalA no change in the kinetic data of the PG could be measured.

Certain plant proteins or phenolics are known inhibitors of fungal PG [12, 13]. During our search for such plant compounds we have tested protein extracts and phenolics from the chickpea plant and also used the described inhibitor epicatechin. Addition of 1 mM epicatechin or 1 mM of the chickpea isoflavones biochanin A or formononetin did not lower PG activity. Furthermore, identical results were obtained by adding chickpea protein extracts (up to 1 mg in a 1 ml assay) to the standard enzyme assays. The enzyme was not inhibited by SH-group blocking reagents (100 μM $CuSO_4$, $AgNO_3$ or *para*-chloromercuribenzoate) or by chelators of divalent cations (1 mM EDTA, 1.10 phenanthroline or diethyldithiocarbamate). The PG requires no divalent cations for enzyme activity because the addition of Ca-, Mg-, Mn- or Co-ions (0.05 to 1 mM) did not affect the enzyme reaction.

Product analysis

The products of pGalA degradation by the PG were analyzed with regard to their degree of polymerization (DP) to distinguish between exo- and endotype cleavage pattern. The only product released from the PG reaction was the monomer GalA. Furthermore, the residual substrate still showed a rather high DP indicating that an exo-PG had been isolated from *A. rabiei* (Fig. 3).

Isoenzymes

Screening for isoenzymes showing PG-, pectinlyase or pectate-lyase activity was performed by chromatographing the culture filtrate of the two investigated *A. rabiei* strains. Each fraction was tested for the three different possibly occurring pectic enzymes. Culture filtrate was transferred to buffer B with a PD10 column and 10 ml of the eluate were applied to a Mono S column using the same conditions as for the purification of the exo-PG. In both investigated strains of *A. rabiei* activity of pectin methyl esterase was detected. The esterase activity was rather low and 2 to 12 h incubationtime were necessary to detect pectin methyl esterase activity by colour change of the pH indicator. No other enzyme acting on pectin or pGalA was found than the described exo-PG and the pectin methyl esterase. To corroborate these results, dialyzed aliqouts of the culture filtrate from both *A. rabiei* isolates were incubated with pGalA and the products of each enzyme assay were analyzed by Mono Q chromatography. Again, GalA was the only detectable product from the PG degradation reaction and the high DP of the residual substrate was preserved. These results clearly indicate that no other pectic enzymes than the exo-PG and the pectin methyl esterase were present in the culture filtrate.

Molecular mass of the enzyme

The purified PG revealed a single band in SDS-PAGE showing a molecular weight of approximately 70 kDa. By gelfiltration, representing genuine conditions a molecular weight of 53 kDa was measured (Fig. 4). In this case only one absorption signal at 280 nm was obtained which clearly coincidated with PG enzyme activity.

Glycoprotein

A gel from SDS-PAGE was blotted on a nitrocellulose membrane for the detection of glycoproteins. For this experiment, the lectin concanavalin

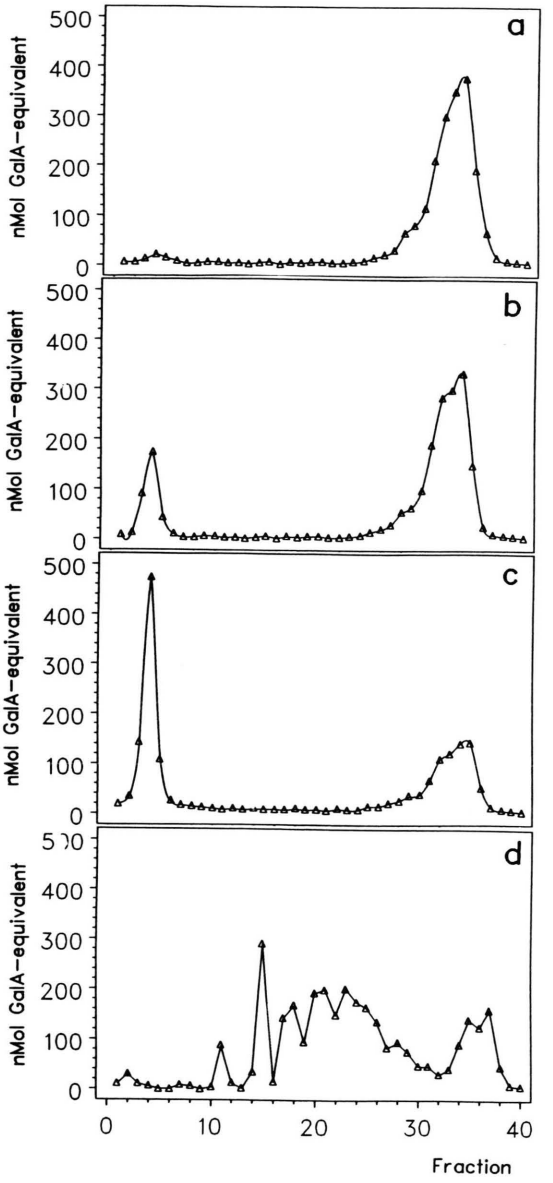

Fig. 3. Product analysis after pGalA digestion with the PG from *A. rabiei* (a to c) and for comparison with the PG from *Aspergillus niger* (d). A 100 µl aliquot of each enzyme assay was boiled for 10 min to inactivate the enzyme, diluted 5-fold with water and chromatographed on a Mono Q column (FPLC-system) in 20 mM Tris/HCl buffer pH 7.5. Galacturonides were eluted with a linear gradient up to 0.5 M NaCl in starting buffer within 40 ml. The amount of galacturonides was measured with the *o*-phenylphenol method. The monomer GalA eluted in fraction 2 to 4 whereas the polymeric GalA eluted in-fractions 30 to 38. The digestion of pGalA with the PG from *A. rabiei* was stopped after 0 min (a), 5 min (b) and after 20 min (c). The results show an exo-type cleaving of pGalA. The endo-PG from *A. niger* releases oligo-galacturonides because after 5 min (d) a rapid breakdown of the highly polymeric pGalA was observed.

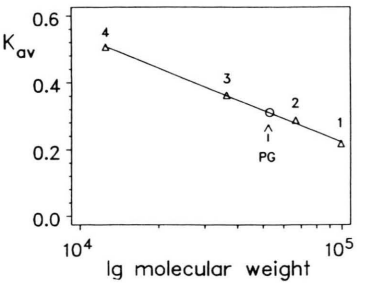

Fig. 4. Determination of the molecular weight of the PG by gelfiltration on a Superose 12 column (FPLC-system). The purified PG was applied to a Superose 12 column equilibrated with 50 mM sodium acetate buffer, pH 5.0, and 50 mM NaCl. The adsorption at 280 nm correlated with the PG-activity and corresponded to a molecular weight of 53 kDa when compared with the marker proteins (**1:** hexokinase 100 kDa; **2:** BSA 67 kDa, **3:** β-lactoglobulin 36.5 kDa; **4:** cytochrome *c* 12.5 kDa).

A was linked to the glycoportion of the PG and to horse-radish peroxidase as reporter enzyme. The PG was labelled very sensitively with the peroxidase essentially as the ovalbumine used as molecular weight marker. However BSA did not show any labelling, indicating the glycoprotein character of the PG from *A. rabiei*.

Discussion

Pectin degrading enzymes are widely produced by plant pathogenic fungi, but to our knowledge nothing is known about the pectic enzymes of the chickpea pathogen *A. rabiei*. Therefore, we have investigated in this fungus the formation of pectic enzymes and purified an exo-PG from the culture filtrate. The expression of PG activity was strongly induced by monomers or polymers of GalA and this induction could be completely repressed by adding glucose to the growth medium. The PG induction in *A. rabiei* shows a typical catabolite repression of gene regulation as shown for *Aspergillus nidulans* [14]. In contrast to the *Aspergillus* system, the monomer GalA is also a good inducer of exo-PG in *A. rabiei*. This is of interest, because the exo-PG releases only GalA during the degradation of pectin.

The purification of the PG was achieved by acetone precipitation and subsequent chromatogra-

phy on a Mono S column. Thereby the PG was separated from almost all other proteins. The following chromatography on a Mono Q column removed minor contaminating proteins so that the resulting PG preparation was homogeneous as judged by SDS-PAGE. The molecular weight of the enzyme in SDS-PAGE was about 70 kDa, whereas gelfiltration with a Superose 12 column showed a molecular weight of 53 kDa. Probably the glycoprotein character of the exo-PG accounts for the unusually high *M*r in SDS-PAGE. The same phenomenon has been described for a glycoprotein PG from *Cochliobolus carbonum*, where the *M*r differs from 27 kDa determined by gelfiltration to 41 kDa found in SDS-PAGE [15].

The kinetic properties of the *A. rabiei* PG demonstrate a high affinity of the enzyme to the substrate pGalA with a K_m value of 0.009% pGalA that corresponds to approximately 2.7 μM substrate in the assay. The enzyme is stable up to 50 °C and shows optimal activity in the acidic range from pH 3.5 to 5.5 that reflects the pH value in plant cell walls. The only substrate of the PG is pGalA whereas pectin itself is not degraded. The similar structure of these two biopolymers might suggest an inhibitory effect of pectin for the exo-PG as it is reported for the PG of *Botrytis cinerea* [16]. Such a competitive inhibition of the *A. rabiei* PG could not be detected. On the other hand, this lack of competitive inhibition possibly increases the efficiency of pGalA degradation in plant cell walls, where only a part of the pectin becomes demethylated by the activity of pectin methyl esterase.

The pectin methyl esterase activity in the culture fluid was rather low. Because of the insensitivity of the enzyme assay it was difficult to determine esterase activity during chromatographic procedures. Nevertheless the activity of pectin methyl esterase is sufficient to demethylate pectin to pGalA indicated by fungal growth on pectin as nutrient source.

We have not found any protein or phenolic compound from the chickpea plant acting as an inhibitor on the exo-PG of *A. rabiei*. The failure to detect such a polygalacturonase-inhibiting protein (PGIP) in chickpea does not imply the absence of such a protein in this plant, because PGIP normally acts on fungal endo-PG only [17].

Although we have extensively looked for further isoenzymes of PG or pectin/pectatelyase, in *A. rabiei* no other pectinolytic activity has been found. This implies a sufficient efficiency of the exo-PG in collaboration with the pectin methyl esterase to degrade pectin and to macerate chickpea leaves and stems. In most phytopathogenic fungi, the cleavage pattern of PG is of the endo-type or a mixture of exo-PG and endo-PG is formed by the fungus [18, 19]. The production of only one exo-PG by *A. rabiei* permits cell wall degradation without the release of oligo-galacturonides, which are well known signal molecules for triggering plant defence responses [4, 5]. *Sclerotia sclerotiorum* for instance produces PG isoenzymes of the endo- and exo-type, but elicitor-active oligo-galacturonides were released from soybean hypocotyls by the endo-PGs only [20].

Therefore we regard the exo-PG of *A. rabiei* as a virulence factor during plant-pathogen interaction. *A. rabiei* appears to be a good system for analyzing the role of pectic enzymes for pathogenicity in phytopathogenic fungi by molecular biological tools. The presence of only one enzyme provides reliable chances to disrupt or replace this PG gene without the difficulties encountered in other fungi due to the expression of several isoenzymes for pectin degradation. Such investigations will be the objects of future studies in this laboratory.

Acknowledgements

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