Characterization and partial purification of pectinesterase, a differentiation-specific enzyme of *Uromyces viciae-fabae*

ALEXANDER K. FRITTRANG, HOLGER DEISING and KURT MENDGEN*

Lehrstuhl für Phytopathologie, Fakultät für Biologie, Universität Konstanz, Postfach 5560, 7750 Konstanz, Federal Republic of Germany

(Received 13 April 1992; revised 2 July 1992; accepted 10 July 1992)

The differentiation-specific formation of three isoforms of pectinesterase by the broad bean rust fungus *Uromyces viciae-fabae* is described. Activity becomes detectable when substomatal vesicles are formed. In crude extracts isoform A contributed 78% of the total pectinesterase activity, and isoforms B and C contributed 20% and 2%, respectively. All three isoforms were found extracellularly in ratios identical to those in extracts. The isoelectric points of the pectinesterase isoforms were 8.4 (A), 5.7 (B), and 4.7 (C) as determined by chromatofocusing. Isoform A had an M_r of 33500 and showed a distinct pH optimum at 6.0. The M_r of isoform B was 40000; its pH optimum ranged from 5.5 to 7.5. Due to its extremely low activity, isoform C was not further characterized. The possible role of pectinesterases in the infection process of the broad bean rust fungus is discussed.

Introduction

Plant cell walls function as physical defence barriers to the ingress of pathogens. Consequently, a large variety of hemibiotrophic and necrotrophic fungi are thought to produce cell-wall-degrading enzymes in order to breach the host cell wall (Keon *et al.*, 1987). Since pectin is the predominant carbohydrate polymer in primary cell walls, significant efforts have been made to elucidate the role of pectic enzymes in plant–pathogen interactions. Pectic enzymes are able to cause intensive tissue maceration (Bateman, 1976), and pectinesterase and pectinase are considered as virulence factors in *Fusarium solani* f. sp. *pisi* (Köller *et al.*, 1982).

The production of cell-wall-degrading enzymes by obligate biotrophs such as powdery mildews and rust fungi has not been conclusively demonstrated. In an early study of cell-wall-degrading enzymes from *Puccinia graminis* var. *tritici*, a substrate-inducible pectinase was detected from uredosporelings after germination on an agar containing pectin (Van Sumere *et al.*, 1957). However, the significance of this report remains questionable, since microbial contamination cannot be excluded on the basis of the data presented. Other investigators found limited amounts of cell-wall-degrading enzymes but no pectic enzymes in rusts and powdery

mildews. They concluded that growth of biotrophs through host walls is facilitated by glycosidases and glycanases rather than by pectic enzymes (Cooper, 1983, 1984; Keon *et al.*, 1987). In studies dealing with rust fungi, only dormant spores, or structures that are formed on the leaf surface (i.e. germ tubes and appressoria), have been examined. Infection structures formed within the host mesophyll have not been analysed, although it has been shown that mRNA and protein synthesis change dramatically when appressoria and subsequent structures are formed (Deising *et al.*, 1991; Hoch & Staples, 1987).

Uredosporelings of the broad bean rust fungus *Uromyces viciae-fabae* form a series of infection structures in order to invade the leaf through stomata (Mendgen *et al.*, 1988). When grown on inductive polyethylene sheets, germ tubes, appressoria, substomatal vesicles, infection hyphae and haustorial mother cells are formed in a time-dependent and synchronous fashion (Deising *et al.*, 1991). The availability of specific stages of morphogenic differentiation of this fungus provides a tool for the investigation of physiological parameters involved in host–parasite interactions. For example, using this system (Deising *et al.*, 1991), a polygalacturonate lyase was found when haustorial mother cells of *U. viciae-fabae* were differentiated (Deising & Mendgen, 1992). The activity of this enzyme decreased with increasing degrees of methylation of the polygalacturonate substrate (H. Deising, unpublished results). These

* Author for correspondence. Tel. 07531 883680; fax 07531 883035.

Abbreviation: DTE, dithioerythritol.

data indicate the potential importance of pectinesterases in wall degradation caused by the broad bean rust fungus. The present report describes the partial purification and properties of different pectinesterases (EC 3.1.1.11) of *U. viciae-fabae*. The characteristics of the enzyme isoforms are compared with pectinesterases of non-biotrophic fungi.

Methods

Spore production. Uredospores of *U. viciae-fabae* (Pers.) Schroet. originated from a single spore line isolated from naturally infected plants. Uredospores were propagated on *Vicia faba* cv. *con amore* as described previously (Deising *et al.*, 1991). Uredospores were immediately used or stored at -70°C for subsequent analyses.

Production of infection structures. Approximately 150 mg uredospores of *U. viciae-fabae* were dusted onto a scratched polyethylene sheet (55×34 cm) (Deising *et al.*, 1991). For controls (i.e. non-differentiated uredosporelings) polyethylene sheets were replaced by glass plates. Inoculated sheets or glass plates were misted with sterile distilled water and incubated at room temperature in the dark at 100% relative humidity. Sheets sprayed with a cycloheximide solution (1 or $10\ \mu\text{g ml}^{-1}$) were used to ensure the fungal origin of pectinesterase activity. Structures were harvested 24 h after inoculation unless otherwise indicated.

Preparation of enzyme extracts. *Preparation of crude extracts.* Prior to preparing extracts, germination rates and differentiation of infection structures were checked microscopically. Crude extracts were produced by homogenizing fungal germ tubes and infection structures (Potter-Elvehjem homogenizer; Kummer, Freiburg, FRG) or dormant spores (porcelain mortar) in 50 mM-ammonium acetate buffer (pH 6.0) containing 1 mM-PMSF and 1 mM-DTE at 4°C . The homogenate was centrifuged (24000 g, 20 min) to remove cell wall fragments. The supernatant was frozen in liquid nitrogen and lyophilized.

Preparation of washing fluid of infection structures. To obtain extracellular proteins, polyethylene membranes with adhering differentiated structures were inverted and floated on 400 ml 50 mM-ammonium acetate buffer (pH 6.0) for 10 min. The washing fluid from 10 sheets was tested for activity of the cytoplasmic marker enzyme malate dehydrogenase to estimate the fraction of damaged structures. The washing fluid was frozen in liquid nitrogen and lyophilized.

Further treatment of extracts. The lyophilized material of the different extracts was resuspended in buffers as required for subsequent analyses and dialysed overnight. The dialysed material was centrifuged (20000 g, 30 min) to remove precipitates and either used immediately or stored in liquid nitrogen.

Enzyme assays. Pectinesterase was measured as described by Förster & Rasched (1985) with minor modifications. One millilitre of a 2% (w/v) aqueous solution of apple pectin type B (pH 7.0) (Roth, Karlsruhe, FRG) and 1 ml 10 mM-PIPES (Roth) buffer (pH 7.0) containing 300 mM-NaCl were mixed. After adding 0.5 ml enzyme extract (in 5 mM-PIPES, 150 mM-NaCl; pH 7.0) the pH decrease at 30°C was followed until a difference of at least 0.1 and maximally 0.2 pH units had been detected. In controls the enzyme solution was substituted by buffer. Another control contained distilled water rather than pectin. One unit of pectinesterase activity was defined as 1 nmol of protons liberated per minute.

The pH optimum of pectinesterase was determined by adding 0.5 ml of an aqueous enzyme solution or, in parallel controls, 0.5 ml distilled water to 2 ml of a non-buffered solution of 1% (w/v) pectin at the

respective pH. The pH was continuously measured, and kept constant by titrating with 20 mM-NaOH, for 1 h. Both samples and controls were adjusted to pH 7.0 with NaOH at the end of the assay. The difference in the required moles of NaOH corresponded to the moles of carboxyl groups formed as a result of pectinesterase activity.

Malate dehydrogenase (EC 1.1.1.37) activity was measured according to Sigma Technical Bulletin no. 340-UV. The assay was performed at room temperature by adding 100 μl sample to 2.9 ml 100 mM-potassium phosphate buffer containing $136\ \mu\text{M}$ -NADH and $150\ \mu\text{M}$ -*cis*-oxaloacetate (pH 7.5). The decrease in A_{340} per minute was used as a measure of malate dehydrogenase activity.

Protein determination. Protein concentration was determined by using a commercially available protein assay (BioRad) based on the method by Bradford (1976), with γ -globulin as standard.

Column chromatography. *Chromatofocusing.* Chromatofocusing was performed on a DEAE Si = 500 (Serva) column (13 ml gel bed, 16.8 cm long) in the pH range from 6.2 to 3.5. The column was equilibrated with 25 mM-histidine (pH 6.2). Up to 4 ml of sample in the same buffer was applied to the column and eluted with 0.2% (w/v) Servalyt 3-8 (Serva) adjusted to pH 5.0, followed by 0.2% (w/v) Servalyt 3-8 adjusted to pH 3.5. The column was then washed with 1 M-NaCl. Fractions of 1.75 ml were collected. The flow rate was $0.25\ \text{ml min}^{-1}$.

A PBE 94 (Pharmacia) column (9 ml gel bed, 17.8 cm long) equilibrated with 25 mM-ethanolamine (pH 9.0) was used for chromatofocusing in the pH range from 9.0 to 6.0. In this case up to 3 ml of sample in 25 mM-ethanolamine (pH 9.0) was applied to the column and eluted with 10% (v/v) Polybuffer 96 (Pharmacia), adjusted to pH 6.0, followed by washing with 1 M-NaCl. Flow rate and fraction size were set as above.

The pH gradient was monitored using a pH microelectrode (type U-402-M3, Ingold, Steinbach, FRG) and the protein content of the fractions was estimated by determining the absorbance at 280 nm.

Each fraction was dialysed against 5 mM-PIPES containing 150 mM-NaCl (pH 7.0) overnight (4°C) and prior to pectinesterase activity determination. Active fractions were pooled for further use.

Gel permeation chromatography of crude extracts. A Sephadex G75-SF (Pharmacia) column (378 ml, 84 cm long) equilibrated with 150 mM-NaCl in 5 mM-PIPES buffer (pH 7.0) was used for M_r determination of pectinesterase in crude extracts. A 2 ml sample of crude extract was applied to the column and separated at a flow rate of $0.25\ \text{ml min}^{-1}$. Fractions of 3.75 ml were collected.

Gel permeation chromatography of separated pectinesterase isoforms. M_r determination of pectinesterase isoforms separated by chromatofocusing was performed by HPLC on a Polyol Si = 100 column (4.6×250 mm, $5\ \mu\text{m}$ pore size; Serva) equipped with a guard column (4.6×50 mm, Polyol Si = 100, $10\ \mu\text{m}$ pore size; Serva). The columns were equilibrated with 5 mM-PIPES buffer containing 150 mM-NaCl (pH 7.0). Samples of 20 μl were injected and fractions of 100 μl ($0.2\ \text{ml min}^{-1}$ flow rate) were collected. Identical fractions of six runs were pooled to assay pectinesterase activity and to measure the protein content. Bovine serum albumin (BSA, M_r 67000), egg albumin (45000), carbonic anhydrase (29000), and cytochrome *c* (12300) served as M_r markers.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed according to Laemmli (1970) using slab gels containing 12% (running gel) and 4% (stacking gel) polyacrylamide. Gels were run at a constant current of 15 mA (stacking gel) and 30 mA (running gel). Silver staining as described by Jungblut & Seifert (1990) was used to visualize separated proteins.

Reproducibility of results. Within a series of experiments, portions of spores collected at the same time were used. Each set of experiments was performed at least twice using spores harvested at different times. All tables and figures show representative values.

Results

Differentiation specificity of pectinesterase

In crude extracts of *U. viciae-fabae*, pectinesterase activity was first detected when uredosporelings began to differentiate substomatal vesicles, and activity increased during the formation of infection hyphae and haustorial mother cells. In extracts of germ tubes grown on glass plates for 24 h (non-differentiated controls), pectinesterase activity was not found. Spores treated with cycloheximide produced only short irregularly shaped germ tubes and did not express any pectinesterase activity (Fig. 1).

Separation and quantification of pectinesterase isoforms from crude extracts by chromatofocusing

Chromatofocusing of crude extracts of 24-h-old infection structures in the range from pH 6.4 to 3.5 revealed major pectinesterase activity in fractions that did not bind to the column. Two minor isoforms bound to the column and eluted at pH 5.7 (isoform B) and 4.7 (isoform C). Enzyme peaks co-eluted with major protein peaks. The addition of NaCl did not elute any further pectinesterase activity (Fig. 2). The unbound fractions contained only one pectinesterase isoform as judged by subsequent chromatofocusing on PBE 94. The pI of this isoform (A) was 8.4 (Fig. 3).

In crude extracts of 24-h-old infection structures, 78% of the total pectinesterase activity consisted of isoform A, 20% of isoform B and 2% of isoform C.

Separation and quantification of pectinesterase isoforms from washing fluid by chromatofocusing

Washing fluid prepared from infection structures that had differentiated for 24 h contained 50% of the total pectinesterase activity, but only a minor percentage of the total amount of protein and negligible amounts of malate dehydrogenase activity (Table 1). In washing fluids of 24-h-old infection structures the ratios of the three isoforms were identical to those reported for crude extracts (data not shown).

Characterization of pectinesterase isoforms

Chromatography of crude extracts on Sephadex G75-SF showed a pectinesterase activity peak corresponding to an M_r of 35000, but the different isoforms could not be separated (data not shown). On Polyol Si=100 the pectinesterase isoforms separated by chromatofocusing exhibited M_r values of 33500 (isoform A) and 40000 (isoform B) (data not shown). Isoform C was not further characterized.

Fig. 1. Expression of pectinesterase (PE) activity in crude extracts of differentiated (●), non-differentiated (■), and cycloheximide-treated (○) uredosporelings of *U. viciae-fabae* at different times after inoculation. Occurrence of germ tubes (gt), appressoria (ap), substomatal vesicles (sv), infection hyphae (ih) and haustorial mother cells (hmc) is indicated by arrows.

Fig. 2. Chromatofocusing of a crude extract prepared from 24-h-old differentiated uredosporelings of *U. viciae-fabae* on DEAE Si=500. Pectinesterase (PE) activity (—●—), absorbance at 280 nm (—) and the pH gradient (---) are shown. Activity peaks of isoforms A, B, and C are marked.

Fig. 3. Chromatofocusing of pectinesterase peak A from Fig. 2 on PBE 94. Pectinesterase (PE) activity (—●—), absorbance at 280 nm (—), and the pH gradient (---) are shown.

Table 1. Secretion of pectinesterase activity by *U. viciae-fabae*

Enzyme activities (PE, pectinesterase; MDH, malate dehydrogenase) or the protein yield in extracts and washing fluid were added to give overall values that were taken as 100%. The values in parentheses represent percentages of this total.		
	Extract*	Washing fluid
Specific PE activity [U (mg protein) ⁻¹]	2.06	56.7
Relative PE activity (U per polyethylene membrane)	2.24 (49.7)	2.27 (50.3)
Relative MDH activity ($\Delta A_{340} \text{ min}^{-1}$ per polyethylene membrane)	41.0 (99.6)	0.17 (0.4)
Protein (mg per polyethylene membrane)	1.09 (96.5)	0.04 (3.5)

* Extract of germlings scraped from a previously washed membrane.

Isoforms A and B revealed different pH optima. While isoform A showed a distinct pH optimum at pH 6.0, isoform B was most active in the range from pH 5.5 to 7.5 (Fig. 4).

Addition of divalent cations such as Ca^{2+} , Mg^{2+} and Mn^{2+} had no effect on the activity of isoforms A and B, and the addition of 10 mM-EDTA did not reduce activity. Varying the NaCl concentration from 50 mM to 1 M had no effect on the apparent pectinesterase activity, while NaCl concentrations lower than 50 mM reduced the apparent pectinesterase activity by some 20% (data not shown).

Partial purification of pectinesterase isoform A

In washing fluids the specific activity of pectinesterase was ~25 times higher than in crude extracts. Chromatofocusing of the concentrated washing fluid on PBE 94 led to a 23.6-fold enrichment of pectinesterase isoform A on a protein basis. However, pectinesterase activity was unstable and further purification steps were not attempted. After chromatofocusing of extracellular proteins, activity yields were only 34% of the activity in the washing fluid (Table 2). This was notably different from the 80–90% recovery rates on the same column when crude extracts were used (data not shown). The

Fig. 4. pH profiles of activity of pectinesterase (PE) isoform A (■) and isoform B (●) of *U. viciae-fabae*. The maximal activity of the respective isoform was set 100%. Lower activities were expressed as percentages of maximal activity.

Fig. 5. Analysis by SDS-PAGE of crude extract (CE), washing fluid (WF), and fractions containing pectinesterase isoform A activity after chromatofocusing of washing fluid on PBE 94 (CWF). Gels were loaded with 15 µg (CE; WF) or 2 µg (CWF) of protein, respectively. M_r markers (M) were β -galactosidase (M_r 116000), phosphorylase *b* (97300), BSA (67000), egg albumin (45000), carbonic anhydrase (29000), chymotrypsinogen (25000), and horse myoglobin (17800).

Table 2. Partial purification of pectinesterase (PE) isoform A from *U. viciae-fabae*

Step	Total PE activity (U)	Yield (%)	Specific PE activity (U mg ⁻¹)	Purification factor
Washing fluid	346.0	100	18.8	1
PE isoform A after chromatofocusing (PBE 94)	64.5	18.6	443.0	23.6
PE isoforms B and C after chromatofocusing (PBE 94)	53.5	15.5	12.2	0.6
Total PE activity after chromatofocusing (PBE 94)	118.0	34.1	—	—

specific activity of isoform A after chromatofocusing of washing fluid on PBE 94 was 238 times higher than the pectinesterase activity of crude extracts of the same spore batches.

The partial purification of pectinesterase isoform A was examined on silver-stained SDS-gels (Fig. 5). Crude extracts led to an almost continuous stain in the M_r range from 70000 to 8000 and individual protein bands could not be distinguished. However, only 12 peptide bands were visible when washing fluids were separated on SDS-PAGE. The pooled fractions containing pectinesterase isoform A after chromatofocusing of the washing fluid on PBE 94 revealed two prominent protein bands (M_r 62000 and 18500) and three faint protein bands (M_r 54000, 52000 and 33000).

Discussion

A large variety of necrotrophic fungi produce pectin-degrading enzymes in order to invade plant tissue (Keon *et al.*, 1987), and in some host-parasite interactions these enzymes are thought to influence the virulence of the pathogen (Köller *et al.*, 1982; Lei *et al.*, 1985; Cleveland & Cotty, 1991). In obligate biotrophs, pectic enzymes have either not been found (Keon *et al.*, 1987), or not been demonstrated beyond doubt (Van Sumere *et al.*, 1957). In this report we have shown that in *U. viciae-fabae* pectinesterases are formed only during germling differentiation, in the absence of pectin or other potentially inductive pectin degradation products. Pectinesterases are only expressed in fungal structures that in nature directly contact the pectin layer of mesophyll cells. To our knowledge, this type of regulation has not hitherto been reported for fungal pectic enzymes. In other systems pectic enzymes were shown to be either adaptive or constitutively expressed (Cooper, 1983). It is clear from the present investigation that in *U. viciae-fabae*, pectinesterase production is regulated during morphogenic differentiation of sporelings. Thus, the apparent failure to demonstrate the production of pectic enzymes in rust fungi may be due to the fact that specific stages of germling morphogenesis were not studied.

The ability of rust sporelings to infect their hosts successfully correlates with their vitality and energy resources (Niks, 1990). Constitutive expression of pectinesterases during germ tube growth on the leaf surface would waste resources. On the other hand, a time-consuming substrate induction of pectinesterases after contact with host pectin, could delay, and thus endanger, the formation of the first haustorium in the host leaf. Differentiation-specific regulation of pectinesterase appears to avoid both disadvantages and to favour successful germling morphogenesis.

In *U. viciae-fabae*, three isoforms of pectinesterase can be distinguished by their isoelectric points. The pIs of the isoforms B and C are acidic, whereas the pI of form A is weakly alkaline. Physicochemical properties determine the electrostatic interactions between extracellular enzymes of pathogens and the negatively charged carboxyl groups of the pectin of the plant mesophyll cells (Keon *et al.*, 1987). Since the pH in the apoplast of mature leaf tissue is in the range 5.0–6.5 (Grignon & Sentenac, 1991) the acidic isoforms B and C can be expected to be uncharged and thus freely diffusible. In contrast, isoform A would be positively charged, leading to adhesion to the negatively charged cell wall. Thus, the activity of isoform A would be limited to the host-parasite interface. We speculate that pectinesterase isoform A would efficiently prepare demethylated pectin specifically at the site of infection. The polygalacturonic acid would then serve as substrate for polygalacturonate lyase (EC 4.2.2.2), an enzyme that was recently demonstrated for *U. viciae-fabae* (Deising & Mendgen, 1992). Restriction of pectin degradation to relatively small areas of the host-parasite interface would cause minimal host tissue damage, a factor presumed to be necessary for successful biotrophy (Chong *et al.*, 1981; Taylor & Mims, 1991).

The main characteristics of the pectinesterase isoforms of *U. viciae-fabae* are similar to those reported for pectinesterases of other fungi. In the hemibiotroph *Phytophthora infestans* two different forms of pectinesterase were characterized (Förster & Rasched, 1985). Pectinesterase type I of *P. infestans* exhibits a broad pH optimum like isoform B in *U. viciae-fabae*, while isoform A and pectinesterase type II of *P. infestans* show distinct pH optima. The pH optima and the M_r values of pectinesterases in *U. viciae-fabae* are in the range reported for other fungi such as *P. infestans*, *Trichoderma reesei*, *Rhizoctonia solani* and *Botrytis cinerea* (Förster & Rasched, 1985; Marcovic *et al.*, 1985; Marcus *et al.*, 1986; Schejter & Marcus, 1988). To our knowledge, no fungal pectinesterases with acidic pI values have so far been detected. In SDS-gels of fractions containing partially purified isoform A only one band, corresponding to an M_r of 33000, was in the expected molecular mass range.

We thank Dr Ralph Nicholson for critically reading the manuscript and the Deutsche Forschungsgemeinschaft for grant no. ME 523-12-11 and no. ME 523-13-11.

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