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Phytophthora infestans secretes extracellular proteases with necrosis inducing activity on potato

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Abstract

The proteolytic activity present in the extracellular preparation (ECP) from suspension media of infective structures of the late blight pathogen *Phytophthora infestans* was partially characterized. The proteolytic activity was analyzed in gelatin-containing SDS-PAGE. A discrete band of digested gelatin was visualized at approximately 45 kDa in ECPs from zoospores and germinating cysts media. Treatment of ECP with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) or incubation at 100 °C for 5 min completely abolished the proteolytic activity in the zymograph assay. When microinjected in potato leaves, ECP induce localized necrosis within 24 h post inoculation. This necrosis appeared in potato and was not visible in two non-host plants. Moreover, the necrosis seems to be dependent on active host metabolism. Treatments of ECP with Proteinase K, PMSF and boiling inhibited their ability to induce the necrotic response. These results suggest a correlation between, proteolytic and necrosis-inducing activities in ECP. A preliminary characterization with protease inhibitors suggests that the ECPs contain serine protease(s).

Abbreviations: ECP – extracellular preparation; PMSF – phenylmethylsulfonyl fluoride; DFP – diisopropyl fluorophosphate; HR – hypersensitive reaction.

Introduction

A large and growing body of evidence suggests that matrix proteases play an important role in cell-to-cell interactions, tumor invasion and pathological processes in animal cells (Stetler-Stevenson et al., 1993; Birkedal-Hansen, 1995). Thus, proteolytic enzyme components of the excretory/secretory products have been implicated as mediators of relevant processes in host–parasite interactions such as: (1) invasion and dissemination through host tissues (Hoetz et al., 1990); (2) conversion of host tissues into nutrients essential for parasite metabolism (Hill et al., 1993) and (3) suppression and modulation of host immune mechanisms (Verwaerde et al., 1988). Recently, a parallel has been described for the mechanism of pathogenesis between plant-pathogenic fungi and fungal pathogenesis in insects (Clarkson and Charnley, 1996). Nevertheless, knowledge about the role of extracellular proteases in plant diseases, especially during invasion events, is just emerging. At least two hypotheses, which do not exclude each other, can be proposed about the role in pathogenesis of extracellular proteases produced by plant pathogens. On the one hand, the pathogen-secreted proteases (psp) can be considered as cell-wall-degrading (cwd) or disorganizing enzymes like e.g. hydrolases (cutinases, cellulases, pectinases and glucanases), that play a role in the invasion of the host

tissue (Knogge, 1996). On the other hand, the psp can be considered as components involved in the interactions with host cells and as such participating in the establishment of the host recognition and specificity. Although molecular and genetic studies have been performed on extracellular proteases in bacterial plant pathogens (Down et al., 1990; Murphy and Walton, 1996), in moulds, and in mutualistic and entomopathogenic fungi (Leger et al., 1992; Jarai et al., 1994; Reddy et al., 1996), no such studies have been described with plant-pathogenic fungi.

Fungi constitute the most diverse and damaging group of plant pathogens. There is an enormous diversity among plant-pathogenic fungi in their life styles, the symptoms they cause and the specificity for hosts, and this is thought to depend on differences in the functions of pathogenicity factors. We focused our studies on the patho-system potato–*Phytophthora infestans*. *P. infestans* belongs to the Oomycetes, a class of organisms that have a fungal-like growth morphology, but whose biochemical and genetic characteristics differ from true fungi. The molecular basis of pathogenicity and host specificity of *P. infestans* is poorly understood (Judelson, 1996, 1997; Govers et al., 1997). In an attempt to identify pathogenicity factors of the potato late blight agent *P. infestans*, we performed the partial characterization of an extracellular proteolytic activity secreted by this pathogen. In this paper, we focus on proteolytic activity detectable in extracellular proteins secreted by infective structures of the pathogen. Furthermore, the extracellular preparations (ECPs) were assayed in microinjection experiments for their ability to generate a hypersensitive-like response on potato leaves.

Materials and methods

Growth of P. infestans *and plant material*

The isolate used in this study was *P. infestans* 'INTA' (races 1, 4, 7, 8, 10, 11, mating type A2) provided by EEA, INTA-Balcarce, Argentina. Mycelia of *P. infestans* were maintained and propagated in Petri dishes containing V8 or rye agar media and on potato tuber slices. Tubers were thoroughly washed with 2% sodium hypochlorite, rinsed with 70% ethanol and flamed. Tuber slices were inoculated with *P. infestans* mycelium and put at 18 °C in the dark and 100% relative humidity. Mycelium from potato tuber slices or Petri dishes was harvested 5 and 15 days after inoculation, respectively, into sterile water at 4 °C and sporangia concentration was adjusted to 10^4 sporangia per ml. After 3 h of incubation, the zoospores released into the water were collected. Cyst formation was initiated by vortexing the zoospore suspension for 2 min. Germinating cysts were obtained by incubating the cysts in the dark for 2 h at 18 °C.

Potato tubers (*Solanum tuberosum* L cv. Pampeana) were planted in flower pots containing a sterile soil/vermiculite mixture (3 : 1), and plants were grown at 25 °C, with a 14 h photoperiod for 15 days, and then for 15 days at 18 °C with the same photoperiod. Leaves from the 3rd and 4th internodes of plants of approximately 30 days old were used for experiments.

Extracellular preparations from zoospores and germinating cyst suspension media

Zoospore and germinating cyst suspensions were centrifuged at $5000 \times g$ for 15 min. The supernatants were filtrated through a 0.22 μm membrane (Millipore) and one volume of 100% ethanol was added (typically 80–100 ml). Solutions were kept at 4 °C overnight. Then, they were centrifuged at $5000 \times g$ for 10 min at 4 °C; the pellets were dried and resuspended in a minimal volume (typically 200–300 μl) of 10 mM Tris-HCl (pH 7.5). They were named ECPs and stored at 4 °C until use.

Gelatin zymography

Samples were electrophoresed in 10% polyacrylamide gels containing 0.1% SDS and 0.5 mg ml^{-1} of copolymerized gelatin. Before electrophoresis, samples were mixed with 5× Laemmli sample buffer (Laemmli, 1970) without dithiotreitol and electrophoresed without boiling. After electrophoresis, SDS was removed from the gel by washing in 2.5% Triton X-100, 50 mM Tris-HCl (pH 8), 5 mM $CaCl_2$ for 30 min and incubated in the same buffer without Triton X-100 at 37 °C overnight. The gel was stained with Coomassie Blue R-250 and the presence of gelatinolytic activity was detected by the appearance of clear zones.

SDS-PAGE analysis and gelatin blot

Protein composition of ECPs was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis

(SDS-PAGE) (Maniatis et al., 1982) with a 5% stacking gel and 12% resolving gel. Prior to electrophoresis, ECP samples were suspended in 5× Laemmli sample buffer (Laemmli, 1970) and electrophoresed without boiling. After electrophoresis, gels were silver stained as described by Maniatis et al. (1982). Molecular weights were estimated by comparison with known molecular weight standards (New England Biolabs). Proteins separated on a non-stained SDS-PAGE were transferred into 0.2 mm 10% polyacrylamide gels containing 0.5 mg ml^{-1} of copolymerized gelatin by capillary transfer procedure. These gels were treated as described above in gelatin zymography.

Determination of proteolytic activity

Azocasein assay: Azocasein prepared in our laboratory (Kirtley and Koshland, 1972) was used as substrate. The reaction mixture contained 0.5% azocasein, 50 mM Tris-HCl (pH 8), 5 mM $CaCl_2$ and sample to a final volume of 0.5 ml. Incubations were performed at 37 °C for 12 h and stopped by adding one volume of ice-cold 10% trichloroacetic acid (TCA). After 30 min at 4 °C, samples were centrifuged at 3000 × *g* for 10 min and A_{335} of TCA-soluble products was measured, as described by Herrera Seitz et al. (1997). ECPs were preincubated with proteinase inhibitors at 4 °C for 30 min and then incubated as described above. Protease inhibitors were purchased from Sigma.

Plate assay: A Petri dish was covered with a mix containing 1.4% agarose and 1% gelatin in 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8). Samples were loaded in 2-mm-diameter wells and incubated overnight at 37 °C. The plate was stained as described above in gelatin zymography.

Leaf microinjection

Microinjection assays were done in leaflets of fully expanded detached potato leaves (see 'Growth of *P. infestans* and plant material' above). ECP samples were microinjected with a hypodermic syringe, with a 28-gauge needle in the central vein of leaflets. Each leaflet was microinjected with 20 µl of ECP containing 400 ng of protein. Protein content was determined by the micro-biuret assay (Goa, 1953) using BSA as standard. After infiltration, treated leaves were kept at 18 °C, 100% RH and 14 h photoperiod. Necrosis appearance was monitored visually 16–48 h after microinjection. For different treatments, aliquots of ECP were preincubated with Proteinase K (GibcoBRL), DNAse I (Sigma), RNAse A (Sigma) or PMSF (Sigma) at 37 °C for 30 min.

Partial characterization of the potato necrotic response, using metabolic inhibitors

Immediately prior to infiltration, cycloheximide (Sigma), α-amanitin (Sigma) or sodium vanadate (Sigma) were mixed with ECP to give final inhibitor concentrations of 5×10^{-4}, 2×10^{-4} and 5×10^{-5} M, respectively (He et al., 1993). As a negative control, the inhibitors were mixed with distilled water to the same final concentration. α-Amanitin was infiltrated into leaves 30 min before infiltration with ECP. As a positive control, ECP was infiltrated at the same final concentration without any added inhibitor.

Results and discussion

Secretion of proteins with proteolytic activity from infective structures of Phytophthora infestans

The first approach consisted of looking for secreted proteinase activity in zoospore or cyst suspensions of the oomycete *P. infestans*. ECPs were analyzed in polyacrylamide gels in the absence or presence of copolymerized gelatin (Figure 1). As shown in lane 1, a very reduced number of protein bands was evidenced after $AgNO_3$ staining. At the same time, a non-stained lane with separated proteins was transferred to a second gelatin-containing gel and developed for gelatinolytic activity. A gelatinolytic band of digested gelatin was visualized at approximately 45 kDa, coincident with the most abundant protein band (Figure 1a, lane 2). Other, much less intense proteolytic bands with higher molecular weights were also detected. When ECP was loaded onto a gelatin-containing gel, a different mobility was observed. In Figure 1b, lanes 3 and 4 show that a gelatinolytic band with identical electrophoretical mobility was detected in ECPs from both zoospores and germinating cysts of *P. infestans.* Figure 1b also shows that (i) the irreversible inhibitor PMSF completely abolished the proteolytic activities present in ECPs from both zoospores and germinating cysts (Figure 1b, lanes 5 and 6) and (ii) incubation of ECPs at 100 °C also results in the loss of the proteolytic activity (Figure 1b, lanes 7 and 8). The same proteolytic activity was present in ECPs from *P. infestans* growing on V8, rye

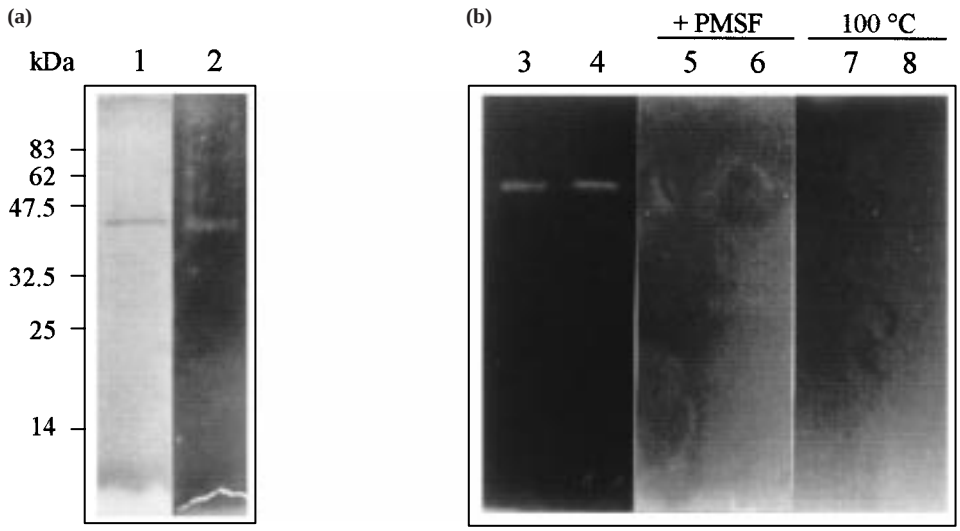

Figure 1. Proteolytic activity in ECPs of *P. infestans.* (a) SDS-PAGE analysis of ECPs from zoospores by silver staining (lane 1), and blotting to a gel containing 0.5% gelatin and Coomassie Blue staining (lane 2). Molecular weight markers (kDa) are indicated on the left. (b) Gelatin zymography in polyacrylamide gels containing 0.5% copolymerized gelatin. ECPs from *P. infestans* zoospores (lanes 3, 5 and 7) and from germinating cysts (lanes 4, 6 and 8) were treated with 1 mM PMSF (lanes 5 and 6), or boiled (lanes 7 and 8).

medium or potato tuber slices (data not shown). Therefore, it was excluded that the proteolytic activity was of potato origin.

ECPs induce necrosis in potato leaves

To determine whether the factor(s) present in ECPs from *P. infestans* were biologically active as inducers that can trigger necrosis on potato, the ECPs from zoospores were microinjected into leaves of cultivar Pampeana. Although this cultivar is susceptible to *P. infestans* infection (Laxalt et al., 1996), it has a high level of horizontal resistance to late blight (Ing. Agr. M. Huarte, INTA Balcarce Argentina, pers. comm.). ECPs were microinjected in detached potato leaves. Leaf tissue in the infiltrated area collapsed within 24 h post inoculation (hpi) and became clearly necrotic at 24–48 hpi, as visualized by a dry and papery collapsed area (Figure 2a). No necrosis was observed beyond the infiltrated area. Figure 2 also shows that no visible reaction was observed when the samples were boiled (Figure 2b) or when buffer alone was infiltrated (Figure 2c). We infiltrated the ECPs in two non-host species, a solanaceous (*Salpichroa organifolia*) and a monocotyledonous species (*Tradescantia* sp.). None of them showed any visible reaction to the infiltration of ECP (Figure 2d and e, respectively).

To elucidate if the necrosis induced by ECP was due to an active metabolic plant response, we microinjected samples of ECP with eukaryotic metabolic inhibitors. The treated ECPs did not trigger any response, whereas the usual necrosis was elicited by untreated ECP (data not shown). This result suggests that active host cell metabolism participates in the necrotic reaction. Thus, the timing, localization and appearance of the necrotic lesions and the requirement of an active host metabolism are reminiscent of a hypersensitive response (HR) as has been found in many incompatible plant–pathogen interactions. In the potato–*P. infestans* pathosystem, it was reported that HR occurs in both compatible and incompatible interactions (Cuypers et al., 1988; Freytag et al., 1994). However, to be able to prove that an HR is occurring upon injection of ECPs more experiments have to be performed: for example, to monitor expression of PR proteins, production of active oxygen species (AOS) and/or synthesis of antimicrobial compounds (phytoalexins). Consequently, we decided to use the term necrosis to describe the observed response. So far, we do not know if other potato genotypes share the same response upon infiltration of ECPs from *P. infestans* 'INTA' or if other *P. infestans* isolates secrete the same proteolytic activity. These experiments are currently in progress.

Recently, evidence for the presence of caspase-like protease(s) in plants that show HR-cell death was

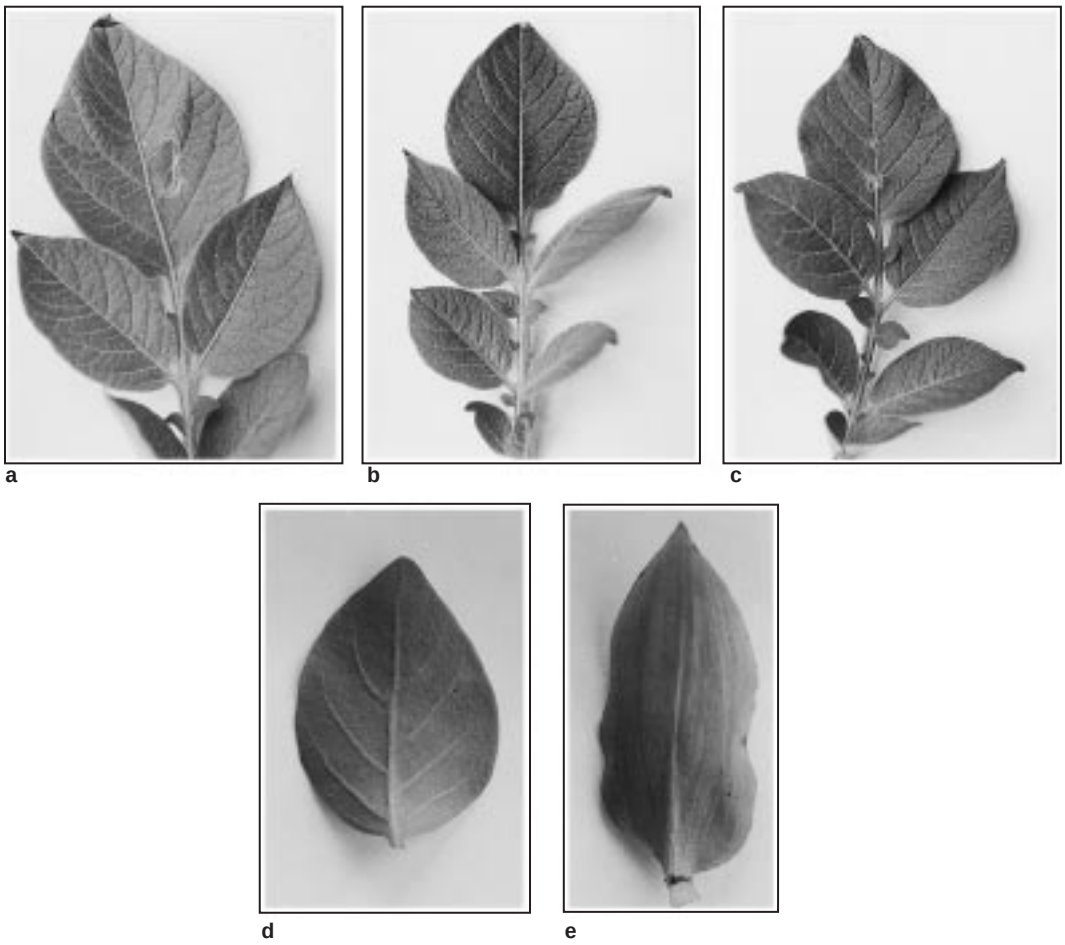

Figure 2. ECPs induce necrosis in potato leaves. ECPs from *P. infestans* zoospores were microinjected into leaflets of detached potato leaves (a–c) and into the non-host (d and e). ECP without treatment (a), ECP incubated at 100 °C for 5 min (b), buffer Tris-HCl 10 mM (pH 7.5) (c). Microinjections of ECPs into the non-host leaves: a dicotyledonous *Salpichroa organifolia* (*Solanaceae* family) (d) and a monocotyledonous *Tradescantia* sp. (*Commelinaceae* family) (e). Pictures were taken at 48 h post infiltration. Each microinjection consisted of 20 µl of ECP containing 400 ng of protein.

presented (Del Pozo and Lam, 1998), therby increasing the parallelism between components of host–pathogen interactions and cell death in animal and plant-systems (Withman et al., 1994; Keller et al., 1998; Van der Biezen and Jones, 1998). It is known that synthetic caspase inhibitors can abolish HR-cell death. However, Del Pozo and Lam (1998) showed that antipain (a non-specific serine/cysteine protease inhibitor) was able to abolish HR, without inhibiting the caspase-like activity, suggesting the involvement of other antipain-sensitive protease(s) in HR-cell death induction. Since *P. infestans* is a hemibiotrophic pathogen, it would be interesting to test if the proteases secreted by the pathogen could be involved in triggering the host tissue HR-cell death as part of the invading strategy of the pathogen.

A family of small, 10 kDa extracellular elicitors proteins, termed elicitins, is produced by several species of *Phytophthora* (Ricci et al., 1989). Several lines of evidence argue against a putative elicitin-like activity in our preparations. INF1 produced by expressing *inf1* in *E. coli* does not induce an HR on potato, but is an active elicitor on tobacco (Kamoun et al., 1997). Moreover, *inf1* is not expressed in zoospores (Kamoun et al., 1997), which also argues against an INF1-mediated response in our assays and fits with the fact that no

band of 10 kDa is present in the ECPs in our study. Additionally, elicitins are soluble in 60% ethanol and in contrast to our results its HR-inducing activity persists after boiling (S. Kamoun, pers. comm.).

Partial characterization of the necrosis inducing factor(s)

To investigate the nature of the necrosis inducing factor(s) and their relationship with the proteolytic activity, ECP samples were treated with Proteinase K (50 $\mu g\,ml^{-1}$), DNAse I (30 U μl^{-1}), RNAse A (50 $\mu g\,ml^{-1}$) and PMSF (1 mM). The remaining proteolytic activity after treatments was evaluated in plates containing 1% gelatin. An aliquot of the treated-ECPs was infiltrated in potato leaflets and the development of necrosis was observed. Figure 3a(1) shows the necrosis-inducing activity present in H_2O-treated ECP. Figure 3a(2) shows that treatment with Proteinase K completely abolished the necrotic response. In the plate assay (Figure 3b(2)), the high proteolytic activity was due to the presence of Proteinase K. In contrast, treatment of ECPs with DNAse I or RNAse A did not modify either the necrotic response in infiltrated potato leaves (Figure 3a(3) and (4)) or the proteolytic activity as visualized in plates (Figure 3b(3) and (4)). Figure 3a and b(5) shows that when PMSF, an irreversible inhibitor of serine proteases, was included in ECP samples, a weakly necrotic spot can be seen in Figure 3a(5) (ECP+PMSF), which correlates with the weak proteolytic activity observed in the plate assay (Figure 3b(5)). PMSF alone did not display any visible signal (Figure 3a and b(6)). Together these results indicate that both induction of the necrotic reaction by ECP infiltrated into potato leaflets and the proteolytic activities appear to depend on heat-unstable proteinaceous factor(s). However, since no purified secreted proteases were assayed, we cannot assume that the necrosis factor(s) described here rely exclusively on the proteolytic activity present in ECP. In addition, it cannot be ruled out that a product of proteolysis of some host cell component(s) could act as the signaling molecule to trigger the necrosis. The finding that a necrotic response could be elicited by an extracellular proteinaceous fraction from *P. infestans* sharing proteolytic activity provides a way to dissect the role of secreted proteases during pathogenic processes. Nevertheless, further investigations are needed to support the hypothesis that secreted proteases could function as avirulence proteins in some plant–pathogen interactions.

Preliminary characterization of the proteolytic activity of ECPs

Several protease inhibitors were assayed in order to characterize the proteolytic activity of ECPs. Figure 4 shows that the proteolytic activity present in ECP samples was strongly inhibited by serine protease inhibitors like PMSF, DFP and chymostatin (100%, 100% and 95%, respectively) and pepstatin (80%). Leupeptin, an inhibitor of serine-type trypsine activity, inhibited the activity by 30%, while E-64, an inhibitor of cysteine proteolytic activity, had no effect. These results suggest that at least part of the proteolytic activity present in

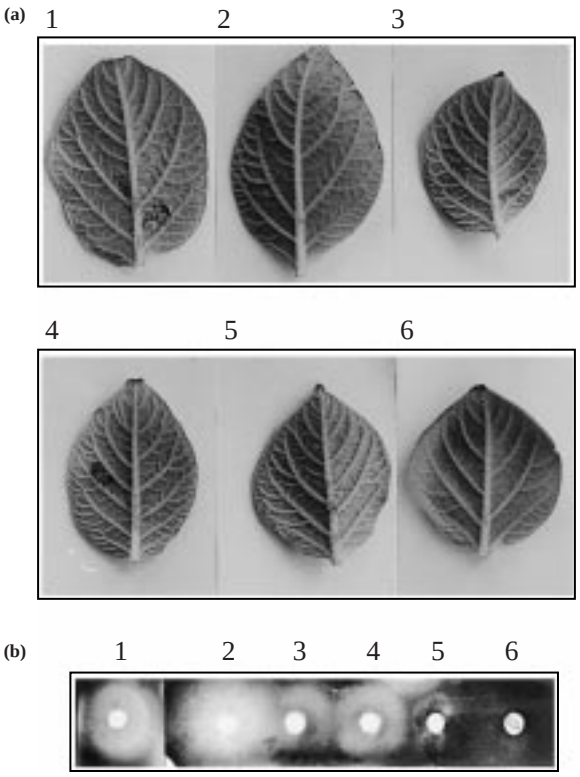

Figure 3. Partial characterization of the HR-inducing factor(s). (a) ECPs from *P. infestans* zoospores were microinjected into leaflets of detached potato leaves. For treatments ECPs were preincubated at 37 °C for 30 min before microinjections, with water (1), Proteinase K (50 $\mu g\,ml^{-1}$) (2), DNAse I (30 U μl^{-1}) (3), RNAse A (50 $\mu g\,ml^{-1}$) (4), PMSF (1 mM) (5) or PMSF (1 mM) alone (6). Pictures were taken at 24 hpi; 20 μl of treated ECPs was microinjected per assay. (b) Proteolytic activity present in treated ECPs were assayed in plates containing 1% gelatin and detected by Coomassie Blue staining; 10 μl of treated ECPs was used per assay.

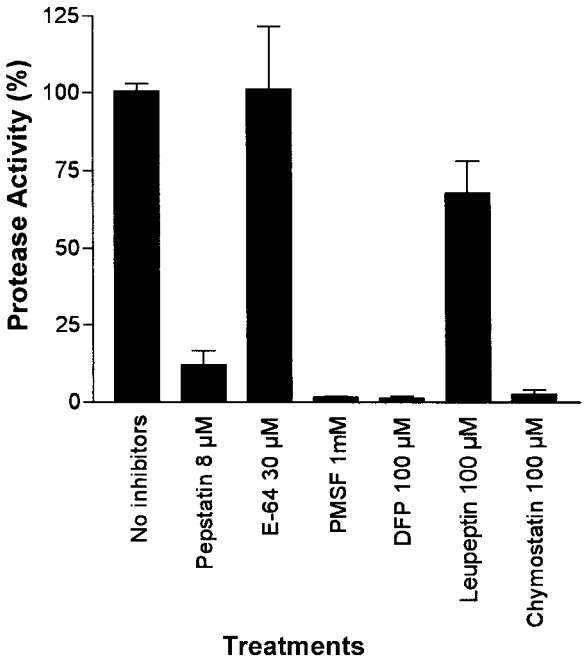

Figure 4. Effect of proteinase inhibitors on proteolytic activity present in ECPs. ECPs from *P. infestans* zoospores were preincubated with proteinase inhibitors. Bars represent the mean ± SE of three independent experiments. Protease activities are expressed as a percentage of activity obtained in the control (ECPs preincubated with water).

ECPs is of the serine type. We cannot explain the inhibition by pepstatin, since it is an inhibitor of aspartil proteases, and at this moment there is no report on the inhibition of serine proteases by pepstatin. We cannot rule out the possibility that more than one protease may be present in ECPs.

Protease inhibitors in plants have been postulated to be involved in the complex mechanism of defense against insects and pathogens (Ryan, 1990). During pathogen attack, production of these inhibitors is highly regulated by a signal transduction pathway that is transduced as a wound response involving jasmonic acid (Koiwa et al., 1997). In potato, two families of serine protease inhibitors (I and II) have been described to be induced after wounding (Ryan, 1990). In addition, two serine protease inhibitors (PSPI-21 and PSPI-22) accumulate in potato tubers infected with *P. infestans* zoospores (Valueva et al, 1998). Altogether, these facts make pathogen proteases and plant protease inhibitors and their regulation a very interesting aspect of plant–pathogen interactions. Probably, a very sensitive equilibrium, in timing and concentration, between the secretion of pathogen proteases and the induction of plant protease inhibitors constitutes an important chapter (which includes invasion and recognition) in the plant–pathogen encyclopedia.

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