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An aspartic protease with antimicrobial activity is induced after infection and wounding in intercellular fluids of potato tubers

M.G. Guevara[1], C.R. Oliva[1], M. Huarte[2] and G.R. Daleo[1]
[1]*Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CC 1245, 7600 Mar del Plata, Argentina (Fax: +54 223 4753150; E-mail: gguevara@mdp.edu.ar);*
[2]*Unidad Integrada Facultad de Ciencias Agrarias INTA, Balcarce, Argentina*

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Abstract

Aspartic proteases (APs) one of the main proteinase classes, have different physiological functions in animals, fungi and viruses. In plants, knowledge of the biological roles of APs is less well developed. An AP has been purified from potato tuber and leaves (Guevara et al., 1999, 2001). In this paper, the changes in the level of AP in response to infection by *Phytophthora infestans* (*P. infestans*) and wounding were studied in intercellular washing fluids (IWFs) from tuber disks of two potato cultivars differing in their susceptibility to *P. infestans*. A differential induction was observed between both cultivars: in the resistant cultivar, induction was higher and faster in infected tissues than in wounded ones. In the susceptible cultivar, a lower and later accumulation was observed than in the resistant cultivar. In addition, AP had a direct inhibitory effect on the germination of cysts of *P. infestans* and conidia of *Fusarium solani*. The pattern of accumulation and *in vitro* activity of AP suggest that this enzyme may have a role in the defense response of potato.

Abbreviations: AP – aspartic protease; BCIP – 5-bromo-4-chloro-3-indolyl phosphate; BSA – bovine serum albumin; FPLC – fast protein liquid chromatography; NBT – nitroblue tetrazolium; PR proteins – pathogenesis-related proteins; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Introduction

Proteolysis plays an important role in many biological processes, including protein degradation and turnover, protein processing and pathogen attack. Among the main proteinase classes, aspartic proteases (APs) are a class of endopeptidases that are active at acidic pH, inhibited by pepstatin A and show a conserved three-dimensional structure. This group of proteinases is generally part of the secretory system and is activated either outside the cell or inside the lysosome/vacuole (Davies, 1990; Tang and Wong, 1987).

APs have been purified from monocotyledoneous (Belozersky et al., 1989; Doi et al., 1980; Sarkkinen et al., 1992) and dicotyledoneous species (Guevara et al., 1999, 2001; Heimgartner et al., 1990; Polanowski et al., 1985; Rodrigo et al., 1989, 1991), as well as from gymnosperms (Bourgeois and Malek, 1991). The hydrolytic specificities of some plant APs have been determined (Faro et al., 1992; Kervinen et al., 1993), but little is known about their biological function. Rodrigo et al. (1991) have reported, in the intercellular fluid of tobacco and tomato plants, the constitutive expression of APs that degrade pathogenesis-related proteins (PR proteins) and suggest that these proteinases may be involved in the turnover of PR proteins as well as in the pathogenesis process itself.

A few studies have shown that proteases are important in plant defense. For example, one study suggested

that a Cys endoprotease confers resistance to maize against fall armyworm (Jiang et al., 1995). It is possible that exopeptidases, such as leucine aminopeptidase-A or the tomato wound-induced carboxypeptidases, have a role in plant defense (Chao et al., 1999; Metha et al., 1996; Pautot et al., 1993; Walling and Gu, 1996) by inactivating proteins essential for pathogen or insect growth and pathogen spread.

We have previously reported the purification of an AP from potato tuber disks. This AP has a molecular mass of 40 kDa, is monomeric and has partial homology with other plant APs (Guevara et al., 1999). In this paper, we show a differential pattern of accumulation of potato tuber AP in two potato cultivars with different degrees of field resistance to *Phytophthora infestans* and the inhibitory effect of purified AP towards *P. infestans* and *Fusarium solani*. Our results suggest that AP may constitute a component of the potato-defense response arsenal.

Materials and methods

Plant and fungal material

Two potato cultivars with different degrees of field resistance against late blight disease were used. Whereas cv. Bintje is susceptible to *P. infestans* (Wastie, 1991), cv. Pampeana INTA has a high level of field resistance, free of the known *Solanum demissum* R genes (Huarte et al., 1997). Potato tubers (*Solanun tuberosum* cv. Bintje and cv. Pampeana) were provided by the Balcarce Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina; cv. Pampeana INTA (MP1 59.789/12 × Huinkul MAG) the Argentine Breeding Program (INTA-Balcarce).

F. solani f. sp. *eumartii*, isolate 3122 and *P. infestans* race 1, 4, 7, 8, 10, 11, mating type A2, were obtained from the INTA Collection, Balcarce (Argentina). *P. infestans* was grown on potato tuber slices. Mycelia were harvested in sterile water 5 days post-inoculation and the sporangial concentration was adjusted to 10^5 sporangia ml^{-1}. Zoospores and cysts were produced as described by Pieterse et al. (1994).

Tubers were washed and sterilized by immersion in 5% (w/v) sodium hypochlorite for 20 min. Sterile disks of parenchyma (4–6 mm diameter, 10 mm thick) were prepared and inoculated with droplets containing 1.25×10^3 zoospores of *P. infestans* or sterile water and were incubated for 0, 14, 24 and 38 h at 18 °C in the dark in a moist chamber.

Isolation of intercellular washing fluid

Intercellular washing fluids (IWFs) of potato tubers were obtained from tissue surrounding the inoculation site (a band of approximately 8 mm around the site). The tissue was cut and the pieces were washed four to five times with distilled water under gentle agitation. After washing, the tissue was immersed in a large excess of buffer containing 50 mM HCl–Tris, pH 7.5, 0.6 M NaCl and 0.1% (v/v) 2-mercaptoethanol and submitted to vacuum during three 10-s periods separated by 30-s intervals, as described by Pinedo et al. (1993). The pieces of tuber tissue were dried on filter paper, placed in a fritted glass filter inserted in a centrifuge tube and centrifuged for 20 min at 400*g*. The recovered extract was used immediately or conserved at −20 °C.

Preparation of total tuber soluble extract

Potato tuber disks were homogenized in 100 mM sodium acetate pH 5.2, 0.5% (w/v) sodium metabisulfite, 1 mM PMSF, 1 mM benzamidine, 10 mM EGTA, 0.2 mM leupeptin, 1.5 mM pepstatin A and 0.5 M NaCl by applying four pulses of 10 s with 30 s intervals using a VirTis 45 homogenizer (The Virtis Co., Gardiner, New York, NY) set at 20% full speed. Homogenates were filtered through cheesecloth, centrifuged at 12 000*g* for 20 min. The resulting supernatant represented the total tuber soluble extract (TTSE). After extraction of IWF as indicated above, the same procedure was applied to obtain total soluble extract minus IWF.

Determination of fresh weight/dry weight relationship

Potato tuber disks were individually weighed and dried at 50 °C until the weight was constant. The dry weight of each disk was recorded and the ratio fresh weight/dry weight calculated.

Glucose-6-phosphate dehydrogenase and α-mannosidase activities

Glucose-6-phosphate dehydrogenase activity (EC 1.1.1.49), as a marker enzyme for the cytosolic fraction, was measured for 15 min at 18 °C in 1.1 ml of a solution containing 50 µl extract, 65 mM $MgCl_2$, 3 mM glucose-6-phosphate, 1.2 mM $NADP^+$ and 85 mM triethanolamine, pH 7.6. The absorbance at 340 nm was continuously recorded.

α-Mannosidase (EC 3.2.1.24), as a marker for the vacuolar fraction, was measured using a standard assay (Boller and Kende, 1979). The assay mixture contained in 0.5 ml, 0.05 ml enzyme, 50 µmol succinic acid adjusted to pH 5.0 with NaOH, and 0.3 µmol *p*-nitrophenol-α-mannopyranoside as substrate. The enzyme was incubated 5 min in buffer at 37 °C and the reaction started by the addition of substrate. It was stopped after 2 h by adding 0.8 ml of 1 M Na_2CO_3, after which the absorbance was measured at 405 nm.

Protein concentration

Protein concentration was measured by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin (BSA) as standard.

Proteolytic activity

Proteolytic activity was measured with hemoglobin as substrate (Orlowsky et al., 1984). One proteolytic unit (U) is defined as the amount of enzyme producing an increase in absorbance of 0.1 at 750 nm, in 1 h, at 37 °C.

Gel electrophoresis

IWFs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide gels (Laemmli, 1970). Gels were stained with silver nitrate (Oakley et al., 1980).

Production of serum and immunoblotting

The AP was purified (Guevara et al., 1999) and the antigen (250 µl of purified AP solution 1 mg ml^{-1}) was emulsified with Freund's complete adjuvant (Sigma, St. Louis, MO, USA) for the first injection and incomplete adjuvant (Sigma, St. Louis, MO, USA) for all the following boost injections. Serum was collected 8 days after each boost.

For immunoblotting, IWFs were electrophoresed and transferred to nitrocellulose in 25 mM Tris–HCl containing 192 mM glycine and 20% (v/v) methanol, pH 9.2, using a semi-dry electrophoretic transfer cell (Trans-Blot, Bio Rad, Hercules, USA) at 12 V for 20 min. The nitrocellulose sheet was soaked for 2 h with a solution containing 100 mM Tris–HCl, pH 8.0 and 1% (w/v) BSA. The membrane was washed four times with 100 mM Tris–HCl, pH 8.0 containing 0.3% (v/v) Tween 20 (TBST) and incubated overnight with the serum diluted 10 000-fold with 100 mM Tris–HCl, pH 8.0, and 1% BSA. After four washes with TBST solution, the blot was allowed to react for 2 h with goat anti-rabbit antibody (1 : 10 000 v/v) labeled with alkaline phosphatase (Sigma Chemical Co, St. Louis, MO, USA). Bound antibody was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) according to procedures recommended by the manufacturer (Sigma). The intensity of the immuno-positive bands on the Western blots were quantified by image analysis (TN-Image, Image Analysis Software, Compuserve, IBMAPP, Rockville, USA).

Assay for antifungal activity

To assay the effects of AP on the germination of cysts of *P. infestans*, a method was designed using microtiter dishes, incubating 50 µl of a suspension of 2×10^5 cysts ml^{-1} in 50 mM sodium acetate, pH 5.2, containing 0.04 mM 2-mercaptoethanol with different amounts of purified AP, with or without 0.08 mM pepstatin A. Inhibition of germination was determined after 20 h incubation at 18 °C. The assay with pepsin and trypsin was done in the same conditions.

The bioassay of inhibition of conidial germination of *F. solani* in microtitre dishes, involved incubating 10 µl of a suspension of 5×10^5 conidia ml^{-1} in 50 mM sodium acetate buffer, pH 5.2, containing 30% sucrose, 0.04 mM 2-mercaptoethanol with different amounts of purified AP, with or without 0.08 mM pepstatin A. Inhibition of germination was determined after 20 h incubation at 25 °C. The assays with pepsin and trypsin were made in the same conditions.

To quantify the effects of purified AP on the germination of cysts of *P. infestans* and conidial germination of *F. solani*, these bioassays were examined, by observation of four fields in Neubauer camera, with a bright-field microscope.

Results

Immunological analysis of IWFs from tuber disks treated with water (wounding) or inoculated with *P. infestans* showed that AP was induced in both cultivars, either by wounding or by infection, although to a different extent and at different times. In cv. Pampeana, the amount of AP began to increase 14 h after wounding or infection (Figure 1A). When the intensity of the immunological reaction was estimated by densitometry, the levels of AP were 12- and 3.5-fold higher in infected disks as compared to wounded ones,

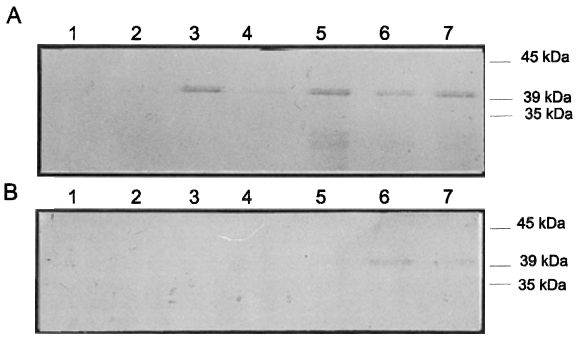

Figure 1. Western blot analysis of the temporal induction of 40 kDa AP in intercellular fluids of potato tubers disks. The samples corresponded to 2 mg of fresh weight. Lane 1: healthy; lanes 2, 4 and 6: 14, 24 and 38 h after mechanical wounding and inoculated with sterile water infection; lanes 3, 5 and 7: 14, 24 and 38 h after mechanical wounding and inoculated with zoospores of *P. infestans*. (A) cv. Pampeana INTA and (B) cv. Bintje.

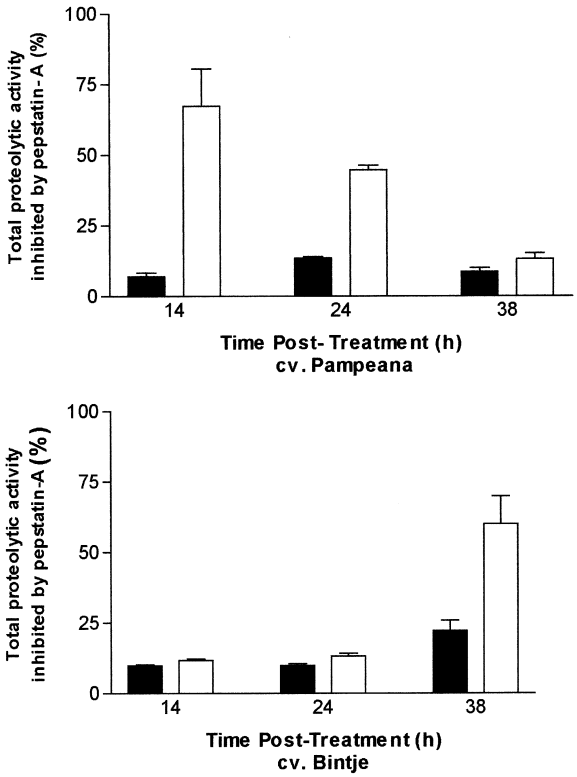

Figure 2. Percentage of total proteolytic activity inhibited by pepstatin A to intercellular fluids of potato tubers disks. The samples corresponded to 1 g of fresh weight. Black bars: after mechanical wounding and inoculated with sterile water. White bars: after mechanical wounding and inoculated with infection with zoospores of *P. infestans*. Values are normalized to proteolytic activity inhibited by pepstatin A present in intercellular fluids of healthy potato tubers disks.

after 14 and 24 h, respectively (results not shown). In cv. Bintje, AP was induced only 38 h after wounding and/or infection, and at lower levels to those obtained with cv. Pampeana (Figure 1B). No signals were detected in the preimmune control, indicating the specificity of the antibody (results not shown).

The possible contamination of IWFs with intracellular components was tested by measuring activities of α-mannosidase and glucose-6-P-dehydrogenase, as markers for vacuoles and cytoplasm, respectively. Less than 1% of total marker enzyme activity was found in the IWFs of healthy or infected tubers.

Total proteolytic activity increased after mechanical wounding and/or infection in both cultivars. The percentage of total proteolytic activity in IWFs from cv. Pampeana inhibited by pepstatin A, calculated as a percentage of total proteolytic activity from cv. Pampeana potato tuber disks after 14 h infection, was higher in infected disks than in wounded ones, after 14, 24 and 38 h wounding respectively (Figure 2). The maximum percentage of proteolytic activity inhibited by pepstatin A was detected after 14 h in infected disks and it decreased after 24 and 38 h (Figure 2). In infected disks of cv. Bintje, only after 38 h was the percentage of proteolytic activity inhibited by pepstatin A activity comparable to that obtained in Pampeana cv. after 14 h in infected disks (Figure 2).

Purified potato AP was assayed for antifungal activity against *P. infestans* and *F. solani* (Table 1). The degree of inhibition of cyst and conidia germination was measured *in vitro*. For *P. infestans*, the germination of cysts was inhibited by as little as 0.33 $\mu g\,ml^{-1}$ of AP. When pepstatin A was added, the inhibitory activity was not detected. The specificity of this effect was studied by incubation of cysts with trypsin or pepsin. No inhibition of cysts germination was observed with these proteases even at a concentration of 48 $\mu g\,ml^{-1}$. AP also inhibited completely the germination of conidia of *F. solani* at the highest concentration assayed (48 $\mu g\,ml^{-1}$). At lower concentrations (24 and 12 $\mu g\,ml^{-1}$), germination and the growth of hyphae were inhibited in a dose-dependent manner as compared to the growth in non-treated conidia. The calculated IC_{50} (the concentration at which 50% of inhibition was observed) for *F. solani* was 32.16 $\mu g\,ml^{-1}$. When pepstatin A was added to the assay, neither the germination nor the growth of hyphae were inhibited. Commercial proteases did not inhibit

Table 1. In vitro inhibitory activity of potato AP toward *P. infestans* and *F. solani*[a]

Treatment	% Inhibition	
	P. infestans	*F. solani*
50 mM sodium acetate pH 5.2+ 0.04 mM β-mercaptoethanol	0	0
0.08 mM pepstatin A	0	0
AP 0.16 μg/ml	0	0
AP 0.33 μg/ml	100	0
AP 1.6 μg/ml	100	0
AP 3.3 μg/ml	100	0
AP 12 μg/ml	100	12 ± 1
AP 30 μg/ml	100	29.5 ± 2.3
AP 35 μg/ml	100	70.8 ± 5.5
AP 40 μg/ml	100	95.8 ± 2
AP 48 μg/ml	100	100
Boiled AP 0.33 μg/ml	0	0
Boiled AP 48 μg/ml	0	0
AP 0.33 μg/ml+ 0.08 mM pepstatin A	0	0
AP 48 μg/ml+ 0.08 mM pepstatin A	0	0
IWF 1 μl	80 ± 0.8	91 ± 10.5
IWF 1 μl+0.08 mM pepstatin A	45.3 ± 2.7	75 ± 6
Pepsin 48 μg/ml	0	0
Trypsin 48 μg/ml	0	0

[a]Specific activity: pepsin 68 U/mg; trypsin 10 U/mg; AP 0.66 U/mg. One proteolytic unit (U) was defined as the amount of enzyme producing an increase in absorbance of 0.1 at 750 nm, in 1 h, at 37 °C, using hemoglobin as substrate. IWF was obtained from potato tubers after 14 h of inoculation. The results are means (±SD) of at least three independent experiments.

Table 2. AP activity in fractions from 14 h infected tubers[a]

Fraction	Activity (U/g FW)	Specific activity (U/mg proteins)
TTSE	14.56 ± 0.21	0.33 ± 0.01
IWF	13.00 ± 0.02	1.29 ± 0.17
TTSE after IWF extraction	0.70 ± 0.07	0.026 ± 0.003

[a]Proteolytic activity was determined as indicated in Materials and methods. One proteolytic unit (U) was defined as the amount of enzyme producing an increase in absorbance of 0.1 at 750 nm, in 1 h, at 37 °C, using hemoglobin as substrate. Results are the means (±SD) of three independent experiments. Potato tubers of cv. Pampeana were inoculated with *P. infestans* race 1, 4, 7, 8, 10, 11, mating type A2 as described in Materials and methods.

conidial germination at these concentrations (results not shown).

Table 2 also indicates that IWFs contain most of the AP proteolytic activity (total and specific activities) present in the total tuber extracts. According to our previous results, the calculated concentration of purified AP *in vivo* is 27 μg g^{-1} fresh weight (Guevara et al., 1999). Hence, since 1 g of fresh weight contains 0.85 ± 0.03 ml of liquid, the concentrations that are likely to occur *in vivo*, especially in the intercellular spaces, are the same or higher than those that were shown to be effective in this report.

Discussion

In this study, the evidence presented shows that there is a different pattern of accumulation of potato AP, in response to wounding and infection with *P. infestans*, in IWFs from two potato cultivars differing in their susceptibility to *P. infestans*. The results show that in a susceptible cultivar, the level of accumulation of this enzyme was lower and occurred later than in a resistant cultivar. In both cultivars, AP accumulation was higher and occurred earlier in infected tissues than in wounded ones. There are only a few examples about the induction of plant APs. An AP message was induced when tomato leaves were wounded (Schaller and Ryan, 1996) and when cauliflower seeds were treated with polyethylene glycol (Fujikara and Karssen, 1995). On the other hand, the mRNA levels of an AP from *Arabidopsis* were not significantly affected by wounding (Mutlu and Gal, 1999) and tobacco and tomato APs were constitutive, being present in healthy and infected leaves at similar levels (Rodrigo et al., 1989, 1991).

Our results indicate that the *in vitro* antimicrobial activity of AP varied between pathogens. The AP concentrations needed to completely inhibit the germination of cysts of *P. infestans* were significantly lower than those previously reported for potato proteins active against *P. infestans* (Niderman et al., 1995; Liu et al., 1994; Woloshuk et al., 1991). We excluded the possibility that the microbial inhibitory effect was due to contaminants in the AP preparation for the following reasons: (1) the AP used was purified to homogeneity, giving a single band in SDS-PAGE after silver staining (Guevara et al., 1999); (2) in the sequence analysis of the samples no contaminations were detected (Guevara et al., 1999) and (3) the antimicrobial effect was specifically reversed by pepstatin A (Guevara et al., 1999). How AP inhibits the growth of *P. infestans* and *F. solani* is not apparent from the data presented, although it is clear that the overall inhibition observed is dependent of AP proteolytic activity.

Table 1 shows the antimicrobial activity of IWF from potato tuber disks after 14 h inoculation. This

activity was reduced by the addition of pepstatin A. This result is a strong evidence that the AP acted within the IWF. The antimicrobial activity that was not inhibited by pepstatin A could be attributed to other protein/s with antimicrobial activity. Several proteins with inhibitory activity toward *P. infestans* have been previously reported (Niderman et al., 1995; Liu et al., 1994; Woloshuk et al., 1991).

The facts that AP accumulates in the induced plants, was found in IWF and inhibits the germination of *P. infestans* and *F. solani*, suggest that potato AP may constitute a component of the potato-defense response against pathogens.

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