Antimicrobial activity of potato aspartic proteases (StAPs) involves membrane permeabilization

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Solanum tuberosum aspartic proteases (StAPs) with antimicrobial activity are induced after abiotic and biotic stress. In this study the ability of StAPs to produce a direct antimicrobial effect was investigated. Viability assays demonstrated that StAPs are able to kill spores of *Fusarium solani* and *Phytophthora infestans* in a dose-dependent manner. Localization experiments with FITC-labelled StAPs proved that the proteins interact directly with the surface of spores and hyphae of *F. solani* and *P. infestans*. Moreover, incubation of spores and hyphae with StAPs resulted in membrane permeabilization, as shown by the uptake of the fluorescent dye SYTOX Green. It is concluded that the antimicrobial effect of StAPs against *F. solani* and *P. infestans* is caused by a direct interaction with the microbial surfaces followed by membrane permeabilization.

INTRODUCTION

Plants, as well as animals, can defend themselves against pathogenic microbes via cationic antimicrobial proteins and peptides, which can kill micro-organisms without any harmful effect on the host (Hancock & Lehrer 1998; Levy, 2004; Brogden, 2005).

Several plant proteins capable of inhibiting fungal growth *in vitro* have been isolated and characterized. Among these proteins, called antifungal proteins (AFPs), glucanases and chitinases (Mauch *et al.*, 1988), thaumatin-like proteins (Hejgaard *et al.*, 1992; Vigers *et al.*, 1991; Woloshuk *et al.*, 1991), several families of basic-cysteine-rich peptides (Broekaert *et al.*, 1997), chitin-binding proteins (Raikhel *et al.*, 1993), ribosome-inactivating proteins (Leah *et al.*, 1991), aspartic proteinases (Guevara *et al.*, 2002, 2004) and proteinase inhibitors (Terras *et al.*, 1993) have been found. AFPs may be part of the preformed defence barriers or may be induced upon perception of a micro-organism. There is evidence that these proteins may have a direct antimicrobial activity *in vivo*. In particular, previous studies have shown enhanced resistance to microbial pathogens by transgenic plants overexpressing some of these antimicrobial proteins (Dixon *et al.*, 1996).

Aspartic proteases (EC 3.4.23) (AP) are a class of widely distributed proteases present in animals, microbes, viruses and plants (Davies, 1990; Rawlings & Barrett, 1995). Biological functions of plant APs have not been characterized as well as those of their mammalian, microbial or viral

counterparts (Davies, 1990; Rawlings & Barrett, 1995; Mutlu & Gal, 1999).

Previously, we reported the first evidence of the bifunctional activity – proteolytic and antimicrobial – of plant APs (Guevara *et al.*, 2002, 2004). We have identified three potato (*Solanum tuberosum*) aspartic proteases, one from tubers (StAP1) (Guevara *et al.*, 1999) and two from leaves (StAP2 and StAP3) (Guevara *et al.*, 2001). Two of these isoforms, StAP1 and StAP3, have been purified and characterized. Both proteins are induced by both abiotic and biotic stress (Guevara *et al.*, 2001, 2002), have an extracellular localization and produce a direct inhibitory effect *in vitro* on the germination of cysts of *Phytophthora infestans* and conidia of *Fusarium solani* (Guevara *et al.*, 2002, 2004).

The aim of this work was to determine whether the StAPs produce a cytotoxic effect on fungal cells mediated by a direct interaction with fungal surface structures. The results presented here provide insight into the antimicrobial mode of action of StAPs.

METHODS

Biological materials. Potato tubers (*Solanum tuberosum* cv. Pampeana) were provided by the Balcarce Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. *S. tuberosum* cv. Pampeana INTA (MPI 59.789/12 × Huinkul MAG) is a cultivar from the Argentine Breeding Programme (INTA-Balcarce).

Fusarium solani f. sp. *eumartii*, isolate 3122 (EEA-INTA, Balcarce, Argentina) was grown at 25 °C on potato dextrose (glucose) agar (PDA) plates supplemented with 100 µg ampicillin ml⁻¹, and spores were collected from 8-day-old cultures by suspension in sterile water.

Abbreviations: AP, aspartic protease; StAP, *Solanum tuberosum* aspartic protease; DAB, 3,3'-diaminobenzidine.

Phytophthora infestans, mating type A2, was grown on V₈-agar medium and on potato tuber slices. Mycelia were harvested in sterile water and stimulated to release zoospores by incubation for 2–3 h at 4 °C. After filtration through muslin, the resultant suspension was observed by light microscopy for quantification of zoospores.

Protein purification. Sterile disks (10 mm diameter, 2 mm thick) of potato tuber were prepared, washed extensively and suspended in sterile water (15 disks in 23 ml water) and aerated for 24 h at 25 °C in an orbital shaker at 60 cycles min⁻¹. StAP1 was purified from tubers using the protocol previously described by Guevara *et al.* (1999).

Potato leaves were detached and placed at 18 °C in a moist chamber. StAP3 was purified from leaves using the protocol previously described by Guevara *et al.* (2001).

The purity of proteins was checked by SDS-PAGE using 15% acrylamide gels (Laemmli, 1970). Gels were stained with silver nitrate (Oakley *et al.*, 1980).

Analytical procedures and measurement of enzymic activities. Protein concentration was measured by the bicinchoninic acid method (Smith *et al.*, 1985), using BSA as standard.

Proteolytic activity was measured with haemoglobin as substrate according to the method described by Anson (1979). One unit (U) is defined as the activity required to produce an increase in absorbance of 0.1 at 750 nm, in 1 h at 37 °C.

Determination of the fungicidal properties of StAPs. Test samples containing 30 µl of the spore suspension (2×10^4 spores ml⁻¹) were incubated with different amounts (0.3, 0.75, 1.2, 1.9, 2.5, 3.75, 6.25 or 10 µM) of either StAP1, StAP3 or 10 µM pepsin, a mammalian aspartic protease (EC 3.4.23.1) (Sigma) in a final volume of 50 µl. Samples were mixed, incubated for 15, 30 and 60 min at 25 °C and then spread on PDA plates. After 3 days at 25 °C, colonies were counted. Bioassays were performed three times and the mean number of c.f.u. was calculated for each treatment (De Luca *et al.*, 1999). The IC₅₀ (concentration needed to reduce spore viability by 50%) was determined.

Detection of H₂O₂. Detection of H₂O₂ *in situ* was performed by an endogenous peroxidase-dependent histochemical staining procedure using 3,3'-diaminobenzidine (DAB) (Sigma), as described by Thordal-Christensen *et al.* (1997) with minor modifications. Briefly, the spores (final concentration 2.7×10^6 spores ml⁻¹) were incubated with water (control) or different amounts of StAP1, StAP3 or pepsin (1.2, 1.85 or 3.75 µM) in the presence of 0.5 mg DAB ml⁻¹ for 1 h and observed microscopically after previous rinsing. This time was necessary to ensure DAB uptake by the fungal cells. DAB polymerizes instantly and locally as soon as it comes into contact with H₂O₂ in the presence of peroxidase.

Protease labelling. Purified StAP1, StAP3 and pepsin (1 mg) were labelled with FITC, Isomer I, using the FluoroTag FITC Conjugation kit (Sigma). Labelled proteases were separated from unlabelled protease and free label according to the procedure recommended by the manufacturer. Assays using haemoglobin as substrate were performed in order to check the proteolytic activity of labelled StAPs. No changes were observed in the specific activity of FITC-labelled StAPs as compared to unlabelled StAPs.

Binding of StAPs to the surface of spores and hyphae. *F. solani* spores (2.7×10^6 spores ml⁻¹) were incubated with different concentrations of labelled-StAPs (0.3, 0.75, 1.2, 1.9, 2.5 and 3.75 µM) for three different lengths of time (15 min, 30 min and 2 h). After 16 h incubation of spores at 25 °C and 100% relative humidity, growing hyphae were treated with FITC-labelled StAPs.

Similarly, *P. infestans* cysts (2×10^5 cysts ml⁻¹) were incubated with different amounts of FITC-labelled-StAPs (from 0.1 nM to 2 µM) for 16 h. After incubation, the number of fluorescent spores or hyphae was evaluated. Controls were performed replacing the protease solutions with 10 µM of pepsin or water. The binding of StAPs to the surface of spores and hyphae was examined with a Nikon Eclipse E200 fluorescence microscope equipped with a B-2 filter set (Nikon) for fluorescein detection. A quantitative test was performed by counting the fluorescent and non-fluorescent spores on a Neubauer camera. The results from three independent experiments were analysed to calculate the percentage fluorescence.

Assay for antimicrobial activity. To assay the effect of StAPs on the germination of cysts of *P. infestans* and spores of *F. solani*, *in vitro* bioassays were performed as described by Guevara *et al.* (2002). The buffer used for the incubation of *F. solani* spores was supplemented with the amount of NaCl needed to reach the final concentrations indicated (75, 150 or 300 mM). After incubation in the same conditions of time and dose as described above, the slides were evaluated for inhibition of germination under a light microscope by counting on a Neubauer camera. Three independent experiments were performed.

Assay for fungal membrane permeabilization. *F. solani* spores and mycelia were incubated overnight at 25 °C with water (controls) or exposed to different amounts of StAP1, StAP3 or pepsin (0.3, 0.75, 1.2, 1.9, 2.5, 3.75, 6.25 or 10 µM) as described by Guevara *et al.* (2002).

P. infestans cysts were incubated for 16 h at 18 °C with water (controls) or exposed to different amounts of StAP1 and StAP3 (from 0.1 nM to 2 µM) or pepsin, according to Guevara *et al.* (2002).

SYTOX Green probe (Molecular Probes) was added at a final concentration of 1 µM and qualitative detection of SYTOX Green uptake was done, after 30, 60 and 90 min incubation for *F. solani* or 30 min for *P. infestans*, with a Nikon Eclipse E200 fluorescence microscope equipped with a B-2A Fluorescein filter set. Positive controls included spores and cysts treated with 0.5% (w/w) Triton X-100. Fluorescence was measured using a FluoresKan Ascent (Thermo Electron Corp.) fluorescence measurement system, at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence values were corrected by subtracting the fluorescence value of a buffer incubated with SYTOX Green.

Statistical analysis. To determine a possible correlation between FITC-StAP binding, antifungal activity and membrane permeabilization effect, *P* values of the corresponding data were calculated by analysis of variance using Microsoft Excel.

RESULTS

Fungicidal effect of StAPs

In initial dose-response experiments, *F. solani* spores were incubated with different amounts of StAPs or pepsin for 15, 30 and 60 min at 25 °C. After this treatment the spore solutions were plated on PDA medium and incubated for 3 days to verify the presence of fungal colonies. As shown in Fig. 1, 15 min incubation with either StAP was sufficient to reduce the viability of spores in a dose-dependent manner. StAP3 had an IC₅₀ almost twofold lower (0.54 µM) than that of StAP1 (0.93 µM) after 15 min incubation (Fig. 1) as well as after 30 and 60 min (results not shown).

Fig. 1. Lethal effect of StAPs on *F. solani* spores. The spores were incubated for 15 min at 25 °C with different amounts (0.3, 0.75, 1.2, 1.9, 2.5, 3.75, 6.25 or 10 μM) of (a) StAP1 or (b) StAP3 or with 10 μM pepsin and then spread on PDA plates. Colonies were counted after 3 days. The number of colonies from control spores treated with water was taken as 100 %. Means of three experiments; error bars represent SD.

The viability of spores incubated in the presence of different amounts of pepsin was not affected.

StAPs induce ROS production

The capacity of StAPs to generate oxygen reactive species (ROS) in spores was analysed. Fungal spores were treated with different concentrations of StAP1 and StAP3 in the presence of DAB, a reagent that can enter into cells and produce an insoluble coloured complex in contact with H_2O_2 (Thordal-Christensen *et al.*, 1997). The micrographs in Fig. 2 show that in the presence of StAPs, spores developed a strong reddish-brown pellet (Fig. 2a), clearly indicating the existence of endogenous ROS in StAP-treated fungal cells. No ROS generation was detected when pepsin was used in the assays (Fig. 2a).

The quantification of this effect revealed that the generation of H_2O_2 increased as the StAP1 and StAP3 dose rose (Fig. 2b).

Interaction of StAPs with the surface of spores and hyphae of *F. solani*

To determine whether the fungicidal effect of StAPs is related to direct or indirect interaction with fungal cells, different amounts of FITC-StAPs were incubated with spores and hyphae of *F. solani*. The results showed that StAP1 and StAP3 were able to bind to the spore surface at all concentrations assayed in a dose-dependent manner (Fig. 3c, d). For both StAPs, the percentage of fluorescent spores increased in parallel with the percentage inhibition of germination. An amount 1.5-fold lower of StAP3, as compared to StAP1, was needed to obtain 50 % of fluorescent spores. Fluorescent hyphae were observed at all concentrations of StAPs assayed (Fig. 3b). The specificity of the binding was studied by incubating spores with FITC-labelled pepsin. No fluorescent spores or hyphae were observed with this enzyme even at a concentration of 3.75 μM (Fig. 3a, b).

The capacity of StAPs to bind to spores of *F. solani* was independent of the time of incubation at all StAP concentrations assayed: the percentage of fluorescent spores was the same at all times of incubation (15, 30 or 120 min) (results not shown). These results suggest that the interaction between StAPs and fungal structures is direct and stable.

StAPs induce membrane permeabilization in *F. solani*

To check whether StAPs have an effect on fungal membranes, we used an assay based on the uptake of the fluorogenic dye SYTOX Green (Thevissen *et al.*, 1999). This reagent only penetrates cells that have damaged plasma membranes and fluoresces upon binding to DNA. *F. solani* spores and hyphae were incubated with 0.3, 0.75, 1.2, 1.9, 2.5, 3.75, 6.25 or 10 μM StAP1 or StAP3 or 10 μM pepsin. These assays were performed in the same conditions as reported for antifungal activity (Guevara *et al.*, 2002). SYTOX Green was then added to evaluate membrane integrity.

Fig. 4(a) and (b) show the effect of StAPs on SYTOX Green uptake by *F. solani* spores and hyphae respectively, observed by fluorescence microscopy. No fluorescent spores or hyphae were observed in the presence of pepsin or buffer. The fluorescent probe was incorporated into the fungal spores and hyphae in a dose-dependent manner in the presence of either StAP (Fig. 4c, d). These results indicate that StAP1 and StAP3 are able to induce membrane permeabilization in spores and hyphae of *F. solani*. The percentage fluorescence values increased between 30 and 60 min after incubation of spores with different amounts (0–6.25 μM) of StAP1 (Fig. 4c). However, no significant increases in the percentage fluorescence with time were observed when different amounts of StAP3 were used (Fig. 4d). No SYTOX Green uptake was detected at

Fig. 2. Detection of H_2O_2 in spores of *F. solani*. (a) Spores stained with DAB in the presence of water (a_1), $3.75 \mu\text{M}$ of pepsin (a_2), $3.75 \mu\text{M}$ of StAP1 (a_3) or $3.75 \mu\text{M}$ of StAP3 (a_4). Uptake of DAB is confirmed by the dark staining (reddish-brown) reaction in the spores. Bars, $15 \mu\text{m}$. (b) Percentage of DAB-stained spores as a function of StAP1 (white bars) or StAP3 (black bars) concentration. Means of three experiments; error bars represent SD.

concentrations below $0.3 \mu\text{M}$ of StAP1 and $0.25 \mu\text{M}$ of StAP3.

Ionic strength sensitivity of the antimicrobial activity of StAPs

Ionic strength sensitivity of antimicrobial activity has been reported for antimicrobial proteins and peptides (Bruhn *et al.*, 2003; Theis *et al.*, 2003). Table 1 shows the effect of increasing the ionic strength on the antimicrobial activity of StAPs. The cytotoxic activity of both StAPs was unaffected by the addition of 75 mM NaCl. At higher NaCl concentrations, the cytotoxic activity of both StAPs decreased.

Effect of StAPs on *P. infestans*

To study the antimicrobial activity of StAPs on another potato pathogen, *P. infestans*, we analysed the inhibition of germination, surface binding and membrane permeabilization of *P. infestans* cysts in the presence of different amounts of StAPs and pepsin. As shown in Fig. 5, the concentration of StAP1 (5 nM) needed to inhibit germination by 50 %

was almost 70-fold lower than that of StAP3 (370 nM) (Fig. 5a, b). Also, both StAPs were able to bind to the fungal surface (Fig. 5c, d) and to permeabilize cell membranes (Fig. 5e, f). When pepsin was incubated with cysts of *P. infestans*, inhibition of germination, protein binding to surfaces and membrane permeabilization were not observed.

DISCUSSION

Antimicrobial proteins and peptides are an important component of the natural defences of most living organisms against invading pathogens and are found in a wide range of eukaryotic organisms, from humans to plants (Ganz, 2003; Lehrer, 2004; Zasloff, 2002; Brogden *et al.*, 2003; Vizioli & Salzet, 2002). Plant APs have been implicated in protein processing and/or degradation in different plant organs, as well as in plant senescence, stress responses, programmed cell death and reproduction (Mutlu & Gal, 1999; Davies, 1990; Rawlings & Barrett, 1995; Simões & Faro, 2004; Ge *et al.*, 2005). StAP1 and StAP3 have been associated with the plant defence mechanism in *S. tuberosum*, and their

Fig. 3. Ability of StAPs to bind to fungal surface structures. (a, b) Spores (a) and hyphae (b) of *F. solani* were incubated with 3.75 μM of FITC-labelled StAPs. After 16 h incubation, the labelled protein was detected by fluorescence microscopy. Panels 1, 2, 3 and 4, fluorescence microscopy; panels 5, 6, 7 and 8, light-field microscopy. Panels 1 and 5, control; panels 2 and 6, 3.75 μM of FITC-labelled StAP1; panels 3 and 7, 3.75 μM of FITC-labelled StAP3; panels 4 and 8, 3.75 μM of FITC-labelled pepsin. Bars, 15 μm. (c, d) Percentage of fluorescent spores (■) and percentage inhibition of spore germination (□) after incubation with StAP1 (c) or StAP3 (d). The number of spores treated with 3.75 μM of FITC-labelled-StAP was taken as 100%. Means of three experiments; error bars represent SD.

antimicrobial activity, *in vitro*, against the potato pathogens *F. solani* and *P. infestans* has been reported (Guevara *et al.*, 2002, 2004). However the mode of action of these enzymes is unknown.

The first point analysed in this work was whether the antimicrobial activity of StAPs involved just the arrest of mycelial growth or the killing of the microbial spores. The results obtained show that the viability of the spores of *F.*

Fig. 4. Membrane permeabilization induced in spores and hyphae of *F. solani* after incubating with StAPs, detected by StAP-induced SYTOX Green uptake. (a, b) Spores (a) and hyphae (b) of *F. solani* were incubated with 3.75 μM of StAPs in the presence of 1 μM SYTOX Green. After 30 min incubation, the fluorescence was detected by fluorescence microscopy. Panels 1, 2, 3 and 4, fluorescence microscopy; panels 5, 6, 7 and 8, light-field microscopy; panels 1 and 5: control. Panels 2 and 6, 3.75 μM of StAP1; panels 3 and 7, 3.75 μM of StAP3; panels 4 and 8, 3.75 μM of pepsin. Bars, 15 μm . (c, d) Spores of *F. solani* were incubated with different amounts of StAP1 (c) or StAP3 (d) for 30 (●), 60 (■) or 90 (□) min in the presence of 1 μM SYTOX Green. Data are means $\pm$ SD from triplicate measurements. The number of fluorescent spores treated with 0.5% (w/w) of Triton X-100 was taken as 100%.

solani was significantly reduced by both StAPs in a dose-dependent manner, clearly indicating that these proteases possess fungicidal (not fungistatic) properties. The lethal doses necessary to kill 50 % of the spores of *F. solani* (2.5 μ M StAP1 and 7.75 μ M StAP3) are in the same magnitude order as values reported for this parameter for snakain-1 and 2, two potato antimicrobial peptides (Berrocal-Lobo *et al.*, 2002;

Segura *et al.*, 1999). On the other hand, the concentrations of StAPs needed to kill 50 % of cysts of *P. infestans* (5 nM StAP1 and 370 nM StAP3) 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). All these assays showed that the antimicrobial effect of StAPs is higher towards *P. infestans*

Fig. 5. Effect of StAPs on cysts of *P. infestans*. (a, b) Inhibition of germination was evaluated after incubating cysts with different amounts of StAP1 (a) or StAP3 (b). Capacity of StAPs to bind to the fungal surface was evaluated after incubating cysts with different amounts of FITC-labelled StAP1 (c) or FITC-labelled StAP3 (d). The number of cysts treated with 20 nM of FITC-labelled StAP1 and 3 μ M of FITC-labelled StAP3 was taken as 100 %. (e, f) Ability of StAPs to permeabilize cyst membranes was analysed by incubating cysts with different amounts of StAP1 (e) or StAP3 (f) for 30 min in the presence of 1 μ M SYTOX Green. Data are means $\pm$ SD of triplicate measurements. The number of fluorescent spores treated with 0.5 % (w/w) of Triton was taken as 100 %.

Table 1. Influence of NaCl concentration on the fungicidal activity of StAPs towards *F. solani*

The fungicidal activity according to the standard assay is normalized to 100%. The calculation of the residual activity (%) is based on the IC₅₀ values (concentration needed to reduce spore viability by 50%), because IC₁₀₀ values were not reached upon addition of higher concentrations of NaCl. The results are means $\pm$ SD ($n=3$).

NaCl (mM)	Relative fungicidal activity (%)	
	StAP1	StAP3
25	100	100
75	98.8 $\pm$ 1.2	100
150	71.7 $\pm$ 2.2	85.5 $\pm$ 1.5
300	60.0 $\pm$ 1.1	62.0 $\pm$ 2.3

than *F. solani* and that StAP1 is a more potent antimicrobial protein than StAP3 for these potato pathogens.

The mechanism of action of antifungal proteins includes fungal cell wall polymer degradation, membrane channel and pore formation, damage to ribosomes, inhibition of DNA synthesis, and inhibition of the cell cycle (Selitrennikoff, 2001; Brogden, 2005). To obtain more information about the mode of action of StAPs as antifungal proteins, we investigated whether StAP1 and StAP3 exert a direct effect on spores and mycelia of *F. solani* and *P. infestans*. Binding experiments performed with FITC-labelled StAPs confirmed that both proteins are able to bind to spores and mycelia of *F. solani* and cysts of *P. infestans* in a dose-dependent manner. Using the SYTOX Green assay we were able to show that StAPs cause membrane permeabilization of spores and hyphae of *F. solani* and cysts of *P. infestans*. The patterns of binding and permeabilization observed correlated with the lethal effect of these StAPs on *F. solani* and *P. infestans* spores and cysts, since similar dose-response curves were observed. The membrane damage curves also showed the same shape as those previously obtained for the growth inhibition of *F. solani* in the presence of StAP1 and StAP3 (Guevara *et al.*, 2002, 2004).

Cation sensitivity has been described for basic antimicrobial proteins, which are thought to act via electrostatic interactions. Moreover, the results obtained here show that antimicrobial activity of StAPs decreases with increasing NaCl concentration. However, the percentage inhibition by NaCl is lower than that observed for other antimicrobial proteins and peptides in the same conditions (Bruhn *et al.*, 2003; Theis *et al.*, 2003). This result suggests that the electrostatic interaction between StAPs and negatively charged membrane components and/or specific receptors, described for other antimicrobial proteins (Thevissen *et al.*, 1997; Lehrer *et al.*, 1989), could be the initial step of StAPs' antimicrobial activity mechanism.

Reactive oxygen species (ROS) are known mediators of intracellular signalling cascades. Excessive production of

ROS may, however, lead to oxidative stress, loss of cell function, and ultimately apoptosis or necrosis (Nordberg & Arner, 2001). Here we have shown that StAPs induce the generation of H₂O₂ in fungal spores in a dose-dependent manner; this agrees with the results previously reported for histatin, a cationic peptide from man (Kavanagh & Dowd, 2004; Patrzykat *et al.*, 2002).

Future characterization of the physico-chemical properties and/or putative receptor or target site will facilitate the understanding of the mechanism of antifungal activity of StAPs.

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