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**Antimicrobial activity of an aspartic protease from *Salpichroa organifolia* fruits**

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Accepted Article

**Significance and Impact of the Study:** This study provides insights on the antimicrobial activity of an aspartic protease isolated from *Salpichroa oranifolia* fruits on plant and human pathogens. The proteinase inhibited *Fusarium solani* and *Staphylococcus aureus* in a dose-dependent manner due to the alteration of the cell plasma membrane barrier but not due to its proteolytic activity. Antimicrobial activity of salpichroin suggests its potential applications as an important tool for the control of pathogenic microorganisms affecting humans and crops of economic interest. Therefore, it would represent a new alternative to avoid the problems of environmental pollution and antimicrobial resistance.

### **Abstract**

Plant proteases play a fundamental role in several processes like growth, development and in response to biotic and abiotic stress. In particular, aspartic proteases are expressed in different plant organs and have antimicrobial activity. Previously, we purified an aspartic protease from *Salpichroa oranifolia* fruits called salpichroin. The aim of this work was to determine the cytotoxic activity of this enzyme on selected plant and human pathogens. For this purpose, the growth of the selected pathogens was analysed after exposure to different concentrations of salpichroin. The results showed that the enzyme was capable of inhibiting *Fusarium solani* and *Staphylococcus aureus* in a dose-dependent manner. It was determined that 1.2  $\mu\text{M}$  of salpichroin was necessary to inhibit 50% of conidial germination, and the minimal bactericidal concentration was between 1.9- 2.5  $\mu\text{M}$ . Using SYTOX Green dye we were able to demonstrate that salpichroin cause membrane permeabilization. Moreover, the enzyme treated with its specific inhibitor pepstatin A did not lose its antibacterial activity. This finding demonstrates that the cytotoxic activity of salpichroin is due to the alteration of the cell plasma membrane barrier but not due to its proteolytic activity. Antimicrobial activity of the aspartic protease could represent a potential alternative for the control of pathogens that affect humans or crops of economic interest.

### **Keywords**

Antimicrobials, cytotoxicity, proteinase, fungi, Staphylococci

## Introduction

Proteases are an important group of enzymes capable of hydrolysing peptide bonds with key roles in physiology and metabolic regulation. They are classified according to their mechanism of catalysis. The MEROPS database (MEROPS: the database of proteolytic enzymes, their substrates and inhibitors, <http://merops.sanger.ac.uk>) considers seven families of proteases: aspartic, cysteine, glutamic, metallo, asparagine, serine and threonine (Rawlings *et al.*, 2018). In plants, five classes of endoproteases have been described: serine, cysteine, aspartic, metallo and threonine proteases (Ako and Nip, 2006; Rawlings *et al.* 2016). These enzymes are ubiquitously distributed in all living organisms and play a fundamental role during reproduction, nutrition, growth, defense and senescence. They are also involved in degradation of misfolded proteins, protein turnover, defense mechanisms and programmed cell death (González- Rábade *et al.*, 2011). In plants, proteases play a fundamental role in development, growth and in response to biotic and abiotic stress. Aspartic proteases (Enzyme Commission number, EC 3.4.23) have two aspartic residues responsible for the catalytic activity, are most active at acidic pH and are specifically inhibited by pepstatin A (McDonald *et al.*, 2009). They are expressed in different plant organs such as seed, tuber, leaf, flower, and root (Simoes *et al.*, 2004). The majority of plant aspartic proteases are synthesized as single-chain preproenzymes and the mature enzyme can be either single or two-chain protein. The precursor is characterized by a hydrophobic N-terminal signal sequence, responsible for translocation into the endoplasmic reticulum, and a C-terminal domain with an insertion of approximately 100 amino acids, named as plant-specific insert (PSI). The PSI is an independent domain-exclusively found in plant aspartic proteases, which is closely related to saposin-like proteins (SAPLIPS) (Simoes *et al.* 2004; González-Rábade *et al.*, 2011; Bryksa *et al.*, 2017). It was shown that this insert can integrate into membranes causing leakage, and this process depends on pH and lipid composition (Egas *et al.*, 2000; Bryksa *et al.*, 2011; Muñoz *et al.*, 2011; Muñoz *et al.*, 2014; Bryksa *et al.*, 2017). The expression of these proteases is related to a plant defense response. It was described that plants proteases are induced after being challenged by pathogens, suggesting that their activity affects the growth of the pathogen directly or indirectly. In addition, if the genes that encode these enzymes are deleted or silenced, the susceptibility of plants to pathogens increases (Guevara *et al.*, 2002; Jashni *et al.*, 2015).

In the last years, the interest in proteolytic enzymes from plants has grown due to its potential application in the field of medicine, biotechnology, agronomic and pharmaceutical (González- Rábade *et al.*, 2011). In particular, plant proteases are interesting as an alternative to conventional drugs due to their antimicrobial activity against bacteria and fungi. Currently, chemical treatments are extensively used in crops in order to avoid fungal pathogen infection (Salas *et al.*, 2015). However, these methods cause environmental pollution, especially by increasing the pesticide residues that are harmful to human health (Aktar *et al.*, 2009; Rizzati *et al.*, 2016). Therefore, the search for alternative antimicrobial treatments for humans and crops is growing due to the increasing spread of antibiotic-resistant pathogens (Pei *et al.*, 2017; Strachan and Davies, 2017).

*Salpichroa organifolia* (Lam.) is a perennial herb found in the north and centre of Argentina that belongs to the Solanaceae family. Previously, we reported the isolation of a novel aspartic protease from *Salpichroa organifolia* ripe fruits (Rocha *et al.*, 2015) capable to inhibit *Phytophthora capsici* growth (Rocha *et al.*, 2016). The relationship between the purified enzyme, called salpichroin, and other plant aspartic proteases containing the PSI domain was evaluated by phylogenetic analysis using the Neighbor-Joining method of the MEGA 5 program. Salpichroin showed high similarity to an aspartic protease from *Solanum lycopersicum* (SIAP1-like), both belonging to the A1 family of aspartic proteases and a fragment of salpichroin matched with the C-terminal domain of SIAP1-like where the PSI is located (Rocha *et al.*, 2015). The aim of this work was to determine the cytotoxic activity of salpichroin on selected plant and human pathogens, and to evaluate if the effect was mediated by membrane disruption. Besides, this work would allow us to evaluate the capacity of this new enzyme to be used as a potential tool of biotechnological interest in medicine and agronomic fields.

## Results & Discussions

In this study, we analysed the antimicrobial activity of salpichroin on both plant and human pathogens.

### *Antifungal activity of salpichroin*

We have previously reported the cytotoxic effect of salpichroin against *Phytophthora capsici* (Rocha *et al.*, 2016), a phytopathogen that causes significant losses in crops of economic interest. In order to analyse antifungal activity of the aspartic protease over *F. solani* different amounts of salpichroin (0.3, 0.75, 1.2, 1.9, 2.5, 3.75  $\mu$ M) were incubated with conidia of the pathogen. The results showed that the enzyme was capable of inhibiting conidia germination of *F. solani* in a dose dependent manner (Fig 1a). Besides, a concentration of 1.2  $\mu$ M of salpichroin was necessary to inhibit 50% of conidial germination, the same amount reported as the minimal concentration for inhibiting strains of *P. capsici* (Rocha *et al.*, 2016). These results are similar to those obtained by Mendieta *et al.* (2006), who described the antifungal activity of two aspartic proteases of *Solanum tuberosum*, StAP1 and StAP3. This activity was attributed to the ability of these enzymes to bind to the surface and to penetrate cells with compromised plasma membranes. Furthermore, Muñoz *et al.* (2010) proposed that the presence of the PSI domain could be correlated with the antimicrobial activity.

To determine whether the antifungal activity of salpichroin was associated to the damage on fungal membranes, we performed an assay based on the uptake of the fluorescent dye SYTOX Green (Thevissen *et al.*, 1999). This reagent can only penetrate cells with damaged plasma membrane and fluoresces upon binding to DNA. *F. solani* conidia were incubated with different amounts of salpichroin (0.3, 0.75, 1.2, 1.9  $\mu$ M). After 20 h of incubation, SYTOX Green was added to evaluate membrane integrity. The results did not show fluorescent conidia's in absence of the aspartic protease. In contrast, in the presence of salpichroin, the SYTOX Green probe was incorporated in a dose dependent manner (Fig 1b). These results indicate that salpichroin, the purified protease from *S. originifolia* was able to induce membrane permeabilization of *F. solani* conidia (Fig 1c). Moreover, the pattern of dose-response curves for membrane damage and conidia viability were similar, showing a correlation between them. This result could be explained by the presence of a PSI domain in salpichroin sequence. Tryptic digestion products of the aspartic protease salpichroin were sequenced and one of the fragments matched with the C-terminal domain of StAP1-like, where the PSI is located (Rocha *et al.*, 2015).

### *Bactericidal activity of salpichroin*

Antibacterial activity was also described as a characteristic of several plant proteases (Xia et al., 2004; Siritapetawee et al., 2012; Alam et al., 2014; Raskovic et al., 2015; dos Anjos et al., 2016; Bryksa et al., 2017; Muthu et al., 2017). With the purpose of corroborating if salpichroin was also capable of inhibiting bacterial growth, we ascertained its activity against two human pathogens, *E. coli* and *S. aureus*. To find out if salpichroin had a cytotoxic effect on bacteria, different amounts of enzyme (0.3, 0.75, 1.2, 1.9, 2.5  $\mu\text{M}$ ) were incubated with  $10^4$  c.f.u./ml of *E. coli* or *S. aureus*. After 5 h, bacteria were plated in TSA plates to obtain colony counts. Our results demonstrated that salpichroin had no effect on *E. coli* as shown in Fig 2a. None of the amounts of the aspartic protease provoke a cytotoxic effect. In contrast, the aspartic protease was able to kill bacterial cells of *S. aureus* in a dose dependent manner (Fig 2b). The MBC value determined in this assay was between 1.9- 2.5  $\mu\text{M}$ . The minimal bactericidal concentration of salpichroin was at least 2.5 times lower than the reported for other plant aspartic proteases (Muñoz et al., 2010). Thus, the enzyme of *S. origanifolia* had a higher antibacterial capacity against *S. aureus* than the proteases of *S. tuberosum*.

In order to elucidate if the cytotoxic effect observed was due to the proteolytic activity of the enzyme, salpichroin was treated with pepstatin A, a specific inhibitor of APs. When the AP was incubated with pepstatin A prior to incubation with the bacteria, no changes were observed in the cytotoxic activity (Fig 3). The effect caused by salpichroin treated with pepstatin A on the growth of *S. aureus* was similar to that produced in the absence of the inhibitor (Fig 2b). The MBC value of treated salpichroin was also between 1.9-2.5  $\mu\text{M}$ . These results suggest that bactericidal effect observed was not due to the proteolytic activity of the enzyme. These findings demonstrated that the cytotoxic capacity of salpichroin on pathogenic microorganisms was not due to its proteolytic activity, as with other plant proteases (Xia et al., 2004; López-García et al., 2012; dos Anjos et al., 2016). This result would also support the hypothesis proposed which states that the killing mechanism of the aspartic protease involves the permeabilization of cell membranes due to the presence of the PSI domain.

### *Haemolytic activity*

The effect of salpichroin was also tested in human red blood cells. After 2 h (data not shown) or 5 h of incubation with different amounts of the enzyme (0.3, 0.75, 1.2, 1.9, 2.5  $\mu$ M), the release of haemoglobin was measured by spectrophotometry. As it is shown in Table 1, salpichroin did not produce haemolysis in hRBC at any concentration evaluated and incubation time assayed, in accordance with that observed by Muñoz *et al.* (2010).

Our results show that salpichroin is able to kill some plant and human pathogens but have no effect on human red blood cells or *E. coli*. The selective toxicity was also observed by other authors, the aspartic proteases of *Solanum tuberosum* are cytotoxic for plant and human pathogens, bovine and human spermatozoa and human leukaemia cells, but do not exert toxic effect on plant cells, human T lymphocytes or erythrocytes (Mendieta *et al.*, 2006; Cesari *et al.*, 2007; Mendieta *et al.*, 2010; Muñoz *et al.*, 2010). This outcome is due to the fact that the interaction of PSI domain with the membrane varies with lipid composition (Muñoz *et al.*, 2011, 2014; Bryksa *et al.*, 2011, 2017). Therefore, the selective cytotoxicity of salpichroin could be attributed to a possible mechanism of action involving the PSI domain. However, the results of our experiments only showed that the cytotoxic activity of the enzyme is due to its capability to provoke membrane permeabilization and is not related to its proteolytic activity, but we do not have direct evidence to demonstrate that the PSI is involved. Future assays should be conducted to corroborate this hypothesis. These could include the recombinant expression of the protein containing or not the PSI domain and verifying its activity.

Antimicrobial activity of salpichroin suggests its potential applications as an important tool for the control of pathogenic microorganisms affecting humans and crops of economic interest. Therefore, it would represent a new alternative to avoid the problems of environmental pollution and antimicrobial resistance.

## Materials and methods

### *Plant and microbial material*

Ripe fruits from *S. origanifolia* were collected from healthy plants grown near Luján city (Buenos Aires, Argentina). The mature fruits are about 2 cm long and look like small white eggs (Fig. S1 and S2)

After collection, fresh fruits were washed thoroughly with distilled water and then stored at 4°C until they were used.

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

*S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were grown in triptein soy broth (TSB) at 37 °C to exponential phase. The bacteria were harvested from broth by centrifugation at 800 x g for 10 min, washed and resuspended in sterile PBS at a concentration of 10<sup>4</sup> c.f.u./ml.

### *Aspartic protease purification*

Fresh and mature fruits were ground with cold ethanol at -20 °C (0.5% p/v) and kept for 2 h for complete protein precipitation with gentle stirring, before vacuum filtration. The precipitate was dissolved in a 50 mM potassium phosphate buffer (pH 7.0) and then centrifuged at 26000 x g for 20 min at 4°C. The supernatant called crude extract was stored at -20°C. Purification of the aspartic protease (AP) was performed as described by Rocha *et al.* (2015). Salpichroin, the purified AP showing proteolytic activity, was stored at -20°C. The purity of salpichroin was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 14% acrylamide gels (Laemmli, 1970) and-mass spectrometry (Rocha *et al.*, 2015).



#### *Analytical procedures and measurement of enzymatic activity*

Protein concentration was measured by the Bradford's method (1976) using bovine serum albumin (BSA) as standard. Proteolytic activity was measured with  $\alpha$ -casein as described previously (Parisi *et al.* 2008). One unit of caseinolytic activity (Ucas) was defined as the amount of enzyme required to cause a unit increase in absorbance per minute at 280 nm across a 1 cm path length, under the assay conditions. Salpichroin, the purified enzyme used for antimicrobial assays, presented an average caseinolytic activity of 160 Ucas.

#### *Assay for antifungal activity*

To assay the salpichroin effect on conidia germination of *F. solani*, 10  $\mu$ l of a suspension of  $5 \times 10^5$  conidia  $\text{ml}^{-1}$  in 50 mM sodium acetate buffer (pH 5.2) containing 30% sucrose were incubated with different amounts of salpichroin (0.3, 0.75, 1.2, 1.9, 2.5, 3.75  $\mu\text{M}$ ). After 20 h incubation at 25 °C the slides were evaluated under a light microscope by counting on a Neubauer camera.

#### *Assay for fungal membrane permeabilization*

*F. solani* conidia were incubated overnight at 25 °C with water (controls) or exposed to different amounts of salpichroin (0.3, 0.75, 1.2 or 1.9  $\mu\text{M}$ ) as described by Guevara *et al.* (2002).

SYTOX Green probe (Molecular Probes) was added at a final concentration of 1  $\mu\text{M}$  and qualitative detection of SYTOX Green uptake was done after 30 min incubation with a Nikon Eclipse E200 fluorescence microscope equipped with a B-2A Fluorescein filter set. 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.

### *In vitro bactericidal assays*

Potential antibacterial activity was tested against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) strains. The bacteria were grown in triptein soy broth (TSB) at 37 °C to exponential phase. Then, they were harvested from broth by centrifugation at 800 x g for 10 min, washed and resuspended in sterile PBS at a concentration of 10<sup>4</sup> c.f.u./ml. One hundred microliters of bacterial suspension were added to 96-well polystyrene microtiter plate containing dilutions of salpichroin untreated or treated with 1 µM of pepstatin A (Sigma Chemical Company, St. Louis, MO, USA) during 30 min at 37 °C. After 5 h incubation at 37°C, bacteria were dispersed and aliquots were plated on triptein soy agar (TSA) plates to obtain colony counts. Pathogen viability after protein treatment was determined from the number of colonies obtained on buffer-treated control plates compared with the number of colonies from protein-treated samples. Minimal bactericidal concentration (MBC) values were calculated as the lowest concentration of salpichroin (µM) that results in more than 99.9% death of the bacteria being tested.

### *Haemolysis assay*

Fresh human red blood cells (hRBC) were rinsed in PBS and centrifuged three times for 10 min at 800 x g. Then they were resuspended in PBS to a final erythrocyte concentration of 4% (v/v). The hRBC suspensions (100 µl) were incubated with different concentrations of salpichroin with agitation for 2 or 5 h at 37°C. Samples were then centrifuged at 800 x g for 10 min, and the release of haemoglobin was monitored by measuring the absorbance of the supernatant at 550 nm. Controls of zero and 100% haemolysis consisted of hRBC suspended in PBS and 1% Triton X-100, respectively (Muñoz *et al.*, 2011)

### *Data analysis*

All data are expressed as mean ± SD of at least two independent experiments carried out in triplicate. Statistical analyses were performed by ANOVA followed by the Bonferroni Test using the GraphPad Prism 4 statistical program by Graph Pad Software, Inc. (San Diego, CA, USA). Data were considered significantly different if p < 0.05.

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## Conflict of interest

No conflict of interest to declare

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Treatment	Concentration ( $\mu\text{M}$ )	Haemolysis of hRBC (%)
Triton X-100		100
PBS Buffer		$0.19 \pm 0.18$
Salpichroin	0.3	$0.27 \pm 0.07$
	0.75	$0.23 \pm 0.11$
	1.2	$0.69 \pm 0.41$
	1.9	$0.96 \pm 0.24$
	2.5	$1.34 \pm 0.18$

**Table 1.** Effect of salpichroin on human red blood cells

Fresh human blood cells (hRBC) were incubated with different amounts of salpichroin for 5 h. Controls of zero and 100% haemolysis consisted of hRBC suspended in PBS and 1% Triton X-100, respectively. Values represent the media  $\pm$  SD of three independent experiments. Statistical analysis was performed by ANOVA followed by the Bonferroni test.

\*SD: significant difference compared to PBS buffer treatment.

## Legends of figures

**Fig. 1.** Effect of salpichroin on *F. solani* spores. (a) Inhibition of germination was evaluated after incubation spores with different amounts of salpichroin (0.3, 0.75, 1.2, 1.9, 2.5 or 3.75  $\mu\text{M}$ ) or buffer as a control (0  $\mu\text{M}$  of salpichroin). (b) Membrane permeabilization was detected by salpichroin induced SYTOX Green uptake. Data are means  $\pm$  SD of triplicate measurements. (c) Membrane permeabilization detected by fluorescence microscopy. Panels 1, 3 and 5, light-field microscopy; panels 2, 4 and 6, fluorescence microscopy. Panels 1 and 2, control (without salpichroin); panels 3 and 4, 0.3  $\mu\text{M}$  of salpichroin; panels 5 and 6, 1.9  $\mu\text{M}$  of salpichroin. Magnification: 10X.

**Fig. 2.** Bactericidal effect of salpichroin on human pathogens. *E. coli* (a) or *S. aureus* (b) were incubated with different amounts of salpichroin or buffer as a control (0  $\mu\text{M}$  of salpichroin). After incubation, c.f.u. were counted and compared with control. Data are means  $\pm$  SD of triplicate measurements.

**Fig. 3.** Effect of pepstatin A on salpichroin cytotoxic activity. *S. aureus* were incubated with different amounts of salpichroin treated with pepstatin A or only with pepstatin A as a control (0  $\mu\text{M}$  of salpichroin). After incubation, c.f.u. were counted and compared with control. Data are means  $\pm$  SD of triplicate measurements.

## Supporting Information legends

**Fig. S1.** Ripe fruits from *Salpichroa organifolia*

**Fig. S2.** Unripe fruits from *Salpichroa organifolia*







