

## POTATO ASPARTIC PROTEASES: INDUCTION, ANTIMICROBIAL ACTIVITY AND SUBSTRATE SPECIFICITY

M.G. Guevara<sup>1</sup>, P. Veríssimo<sup>2</sup>, E. Pires<sup>2</sup>, C. Faro<sup>2</sup> and G.R. Daleo<sup>1</sup>

<sup>1</sup> Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CC 1245, 7600 Mar del Plata, Argentina

<sup>2</sup> Centro de Neurociencias de Coimbra and Departamento Bioquímica, Universidade de Coimbra, 3004-517 Coimbra, Portugal

### SUMMARY

Plant aspartic proteinases (EC 3. 4. 23) have been associated with abiotic stress responses, but little is known about their possible involvement in biotic stress responses. Here we report the induction of an aspartic proteinase, *StAP3* (*Solanum tuberosum* aspartic protease), in potato leaves upon infection with *Phytophthora infestans* and we compare its antimicrobial activity and substrate specificity with *StAP1*, an aspartic protease from potato tubers previously characterized. Both the aspartic proteinase content and activity were significantly increased in leaves from a resistant cultivar (cv Pampeana) as compared to a susceptible one (cv Bintje). *In vitro* analysis shows that *StAP3* has antimicrobial activity towards *P. infestans* and *Fusarium solani*, like *StAP1* from tubers. Substrate specificity of *StAP1* and *StAP3* was studied, using oxidized insulin  $\beta$ -chain as substrate. Both enzymes have a common cleavage position, like other plant aspartic proteases. Additionally, *StAP1* has other two cleavage positions and *StAP3* was able to cleave the peptide bond Phe<sup>24</sup>-Phe<sup>25</sup>, a cleavage position found in other plant APs. The induction of both *StAPs* (*StAP1* and *StAP3*) in potato tubers and leaves after wounding and infection in potato resistant cultivars and their antimicrobial activity would suggest that these *StAPs* are involved in plant defense response. In a previous paper, *StAPs* antimicrobial activity was ascribed to *StAPs* proteolytic activity. Differences in the antimicrobial activity of *StAP1* and *StAP3* may be associated with the differences found in the substrate specificity between these enzymes.

*Key words:* plant proteases, aspartic proteases, *Solanum tuberosum*, antimicrobial proteins, *Fusarium solani*, *Phytophthora infestans*, plant-pathogen interaction.

### INTRODUCTION

Aspartic proteases (EC 3.4.23) are a class of widely

distributed proteases present in animals, microbes, viruses and plants (Davies, 1990; Rawling and Barret, 1995). A few studies have shown that proteases are important in plant defense against biotic stresses. For example, a cysteine endoprotease confers resistance to maize against fall armyworm (Jiang *et al.*, 1995). Exopeptidases, such as leucine aminopeptidase-A or the tomato wound-induced carboxypeptidases, have been suggested to play important roles in plant defense (Gees and Hohl, 1988; Pautot *et al.*, 1993; Metha *et al.*, 1996; Chao *et al.*, 1999) by inactivating proteins essential for pathogen or insect growth and pathogen spread. Rodrigo *et al.* (1991) have reported the constitutive expression of APs that degrades pathogenesis-related proteins (PR proteins) in the intercellular fluid of tobacco and tomato plants. The authors suggested that these proteinases might be involved in the turnover of PR proteins as well as in the pathogenesis process itself. We have previously reported the isolation and purification of an aspartic proteinase from potato tuber *StAP1* (*Solanum tuberosum* aspartic protease) that is induced by wounding and aging (Guevara *et al.*, 1999) and the purification of one (*StAP3*) of two aspartic proteinases induced after detaching from potato leaves (*StAP2* and *StAP3*) (Guevara *et al.*, 2001). Also, we have studied the changes in the level of *StAP1* in response to infection by *Phytophthora infestans* (the causal agent of late blight disease) and wounding in intercellular washing fluids (IWFs) from tuber disks of two potato cultivars differing in their susceptibility to *P. infestans*. We have shown the differential induction of *StAP1* in both cultivars: in the resistant cultivar, induction was higher and faster in infected tissues with respect to wounded ones. In the susceptible cultivar, a lower and later accumulation with respect to the resistant cultivar was observed. In addition, *StAP1* had direct inhibitory effect on the germination of cysts of *P. infestans* and conidia of *Fusarium solani* (Guevara *et al.*, 2002).

In this work we show the differential accumulation of *StAPs* in leaves of potato cultivars with different degrees of field resistance to *P. infestans* after infection with this pathogen and the inhibitory effect of purified *StAP3* towards *P. infestans* and *F. solani*. Also, we show that differences in substrate specificity of *StAP1* and

Corresponding author: M.G. Guevara  
Fax: +54.22.34753150  
E-mail: gguevara@mdp.edu.ar

*StAP3* could be associated with differences found in antimicrobial activity of these *StAPs*.

## MATERIALS AND METHODS

**Plant and fungal material, growth conditions and experimental treatments.** *P. infestans* mating type A2, was grown on V<sub>8</sub>-agar medium and on potato tuber slices. Mycelia were harvested in sterile water and stimulated to release zoospores by incubation at 4°C for 2-3 h. After filtration through muslin, the resultant suspension was observed under light microscope for quantification of zoospores and was used for inoculation.

*Solanum tuberosum* L. cv Pampeana INTA (MPI 59.789/12 x Huinkul MAG) is a cultivar from the Argentine Breeding Program (INTA-Balcarce). Potato plants (*S. tuberosum* L. cv Pampeana INTA and cv Bintje) were grown in pots containing a sterile mixture of soil and vermiculite (2:1 v/v) and maintained at 25°C for 4 weeks with a 14-h photoperiod. Light was supplied by Osram L36W/20 cool white fluorescent tubes, which supplied 120 µmol m<sup>-2</sup> s<sup>-1</sup> PAR measured 30 cm from the source. The plants were then transferred to 18°C with the same photoperiod. Six-weeks old potato plants were used for inoculation with *P. infestans*. Potato plants were inoculated by spraying with a suspension containing 2·10<sup>4</sup> sporangia ml<sup>-1</sup> of *P. infestans* using a fine glass optimizer, while control plants were sprayed with water. Plants were placed at 18°C in a moist chamber.

**Protein extraction, concentration and proteolytic activity.** Leaves were detached and harvested from the plants at 0, 24, 36, 48 and 72 h post-inoculation, with *P. infestans* (infected) or water (control), and homogenized in 2 volumes of 100 mM sodium acetate, pH 5.2, containing 4 mM DTT and 2.5 mM sodium metabisulfite. A Virtis 45 homogenizer (The Virtis Company, Inc., New York, USA) was used, at 20% full speed, for four periods of 1 min. The homogenate was filtered through cheesecloth, centrifuged at 12,000 g for 20 min and the supernatant was stored at -20°C.

Protein concentration was measured by the bicinchoninic acid method (Smith *et al.*, 1985), using bovine serum albumin (BSA) as standard. Proteolytic activity was measured with hemoglobin as substrate according to the method described by Anson (1979) with or without DTT. 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. The effect of pepstatin A (at a final concentration in the assay mixture of 0.04 mM) was also tested in all assays.

**Gel electrophoresis and immunoblot analysis.** Samples were analyzed by SDS-PAGE using 12% (w/v)

acrylamide (Laemmli, 1970). Samples were treated in denaturing buffer with SDS, β-mercaptoethanol and heating before SDS-PAGE. Preimmune serum was extracted from a rabbit prior to inoculation of the antigen. *StAP1* was purified as previously described (Guevara *et al.*, 1999). The antigen (250 µl of a 1 mg ml<sup>-1</sup> *StAP1* solution) was emulsified with Freund's complete adjuvant (Sigma, Saint Louis, USA) for 12 V for 20 min. Carbohydrate epitopes were destroyed by periodate oxidation, according to Heimgartner *et al.* (1990). The extracts were subjected to electrophoresis, transferred onto nitrocellulose, and oxidized with 10 mM periodic acid in 100 mM acetate buffer, pH 5, at room temperature for 30 min in the dark and subsequently quenched with 5% Blotto at pH 7.5.

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 then incubated overnight with rabbit anti-*StAP1* (1:10,000 v/v) in 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, Saint Louis, USA). Bound antibody was detected using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) according to procedures recommended by the manufacturer (Sigma, Saint Louis, USA).

**Purification of potato leaf aspartic protease induced by infection (*StAP3*).** Leaves were collected 24 h after infection with *P. infestans* and homogenized in 2 volumes of 100 mM sodium acetate, pH 5.2, containing 4 mM DTT and 2.5 mM sodium metabisulfite. *StAP3* was purified using the protocol described by Guevara *et al.* (2001).

**Assay for antimicrobial activity.** To assay the effects of purified *StAP3* on the germination of cysts of *P. infestans* and conidia of *F. solani*, *in vitro* bioassays were performed as described by Guevara *et al.* (2002). The assays with pepsin and trypsin were performed in the same conditions. To quantify the effects of purified *StAP3* on the cysts and conidia germination, these bioassays were examined by observation of four fields in Neubauer camera, with a bright-field microscope.

**Proteolytic activity: specificity studies with oxidized insulin β-chain.** Oxidized β-chain (5 mg/ml) was incubated with *StAP1* or *StAP3* in 0.1 M formic acid adjusted to pH 3.1 with NaOH. After 1, 3 and 24 h at 37°C, the reaction mixtures were centrifuged and the peptide fragments were separated by reversed-phase HPLC using Vydac C18 column (Alltech Associates Inc., Deerfield, IL, USA). The chromatography was carried out at

room temperature and the column was equilibrated with 0.1% TFA. The peptides were eluted with a linear gradient of acetonitrile (0-80%) in 0.1% TFA at a flow rate of 1.5 ml/min. Amino acid composition and N-terminal amino acid sequencing were performed to characterize the isolated peptides. N-terminal amino acid sequences were determined by Edman degradation using an Applied Biosystems (Foster city, CA, USA) 473-A sequencer.

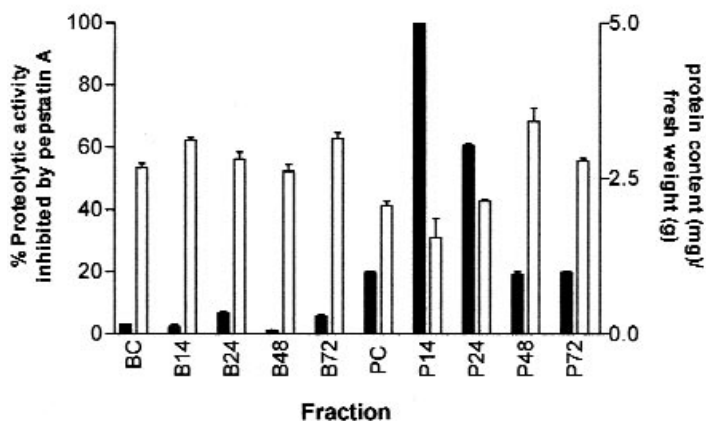
**RESULTS**

**Accumulation of aspartic proteases in potato leaves infected with zoospores of *P. infestans*.** To examine whether potato leaf APs (*StAP2* and *StAP3*) could be involved in the plant-defense response we tested the proteolytic activity inhibited by pepstatin A in leaf extracts of two potato cultivars with different degree of field resistance to *P. infestans* at different stages of infection. Fig. 1 shows that this activity was maximum in cv Pampeana INTA (resistant cultivar) 14 h after infection. In this cultivar the percentage of total proteolytic activity inhibited by pepstatin A was 5.5 and 3.5 fold higher in infected leaves as compared with the healthy ones, at 14 h and at 24 h after infection, respectively. In infected leaves of cv Bintje (susceptible cultivar), the percentage of proteolytic activity inhibited by pepstatin A did not increase upon infection with respect to healthy ones

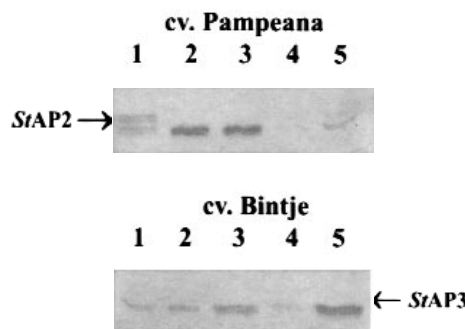
(Fig. 1). Proteolytic activity inhibited by pepstatin A was 7-fold higher in healthy leaves of cv Pampeana than in healthy leaves of cv Bintje.

Also, we analyzed these samples by western blot using polyclonal antibodies raised against *StAP1* (Fig. 2). The specificity of the antibody was tested using the homogenate of healthy leaves cv Pampeana, as antigen, incubated with different concentration of anti-AP antibodies; at all concentrations tested both *StAPs* isoforms were detected. No signal was detected in the pre-immune control. Fig. 2 shows that *StAP2* and *StAP3* were detected in healthy leaves of cv Pampeana INTA (Fig. 2, lane 1) whereas *StAP3* was detected only in cv Bintje (Fig. 2, line 5). *StAP3* was induced after infection, although to a different extent and at different times. In cv Pampeana INTA, the amount of this protease increases at 14 h after infection. *StAP2* was not detected at all times of infection tested. In cv Bintje, *StAP3* concentration increased 24 h after infection, decreased 48 h after infection and increased markedly at 72 h after infection.

**Antimicrobial activity.** To examine if *StAP3* has antimicrobial activity, cysts of *P. infestans* and conidia of *F. solani* were incubated with the purified enzyme and the degree of *in vitro* inhibition of cysts and conidia germination was measured (Table 1). For *P. infestans*, the 95% germination of cysts was inhibited by 48 mg ml<sup>-1</sup>. At lower concentrations (12 and 35 mg ml<sup>-1</sup>) the germination of cysts was inhibited in a dose-dependent manner. Only 42% of the conidia of *F. solani* were inhibited by 48 mg ml<sup>-1</sup> of this *StAP* and the calculated IC<sub>50</sub> (the concentration at which 50% of inhibition of the germination was observed) was 118 mg ml<sup>-1</sup>. When pepstatin A was added, the antimicrobial activity was not detected. The specificity of this effect was studied by incubation of both, cysts and conidia, with trypsin or pepsin. No inhibition was observed with these proteases even at a concentration of 48 mg ml<sup>-1</sup>.



**Fig. 1.** Protein content and percentage of proteolytic activity inhibited by pepstatin A in potato leaves infected with *P. infestans*. **B:** cv Bintje; **P:** cv Pampeana and **C:** control. White bars: Total protein content in leaves measured as described in Materials and methods. Black bars: Percentage of total proteolytic activity from potato leaves (corresponding to 1 g of fresh weight) inhibited by pepstatin A. Values are normalized to as a percentage of proteolytic activity inhibited by pepstatin-A from cv Pampeana potato plant leaves 14 h after infection (corresponding to 1 g of fresh weight).



**Fig. 2.** Western blot analysis of the temporal induction of *StAP* in potato leaves. Proteins were extracted from leaves (corresponding to 3 mg of fresh weight) of (1) control plants, (2) 14 h, (3) 24 h, (4) 48 h and (5) 72 h after inoculation with zoospores of *P. infestans* as described in Materials and methods.

**Table 1.** *In vitro* inhibitory activity (%) of *StAPs* toward *P. infestans* and *F. solani*<sup>a</sup>.

Treatment	<i>P. infestans</i>		<i>F. solani</i>	
	<i>StAP1</i>	<i>StAP3</i>	<i>StAP1</i>	<i>StAP3</i>
50 mM sodium acetate pH 5.2 + 0.04 mM $\beta$ -mercaptoethanol	0	0	0	0
0.08 mM pepstatin A	0	0	0	0
AP 0.16 $\mu\text{g ml}^{-1}$	0	0	0	0
AP 0.33 $\mu\text{g ml}^{-1}$	100	0	0	0
AP 1.6 $\mu\text{g ml}^{-1}$	100	50 $\pm$ 24	0	0
AP 12 $\mu\text{g ml}^{-1}$	100	55 $\pm$ 2	12 $\pm$ 1	0
AP 35 $\mu\text{g ml}^{-1}$	100	76 $\pm$ 3.2	70.8 $\pm$ 5.5	21 $\pm$ 3.5
AP 48 $\mu\text{g ml}^{-1}$	100	95 $\pm$ 1	100	42 $\pm$ 4
Boiled AP 0.33 $\mu\text{g ml}^{-1}$	0	0	0	0
Boiled AP 48 $\mu\text{g ml}^{-1}$	0	0	0	0
AP 0.33 $\mu\text{g ml}^{-1}$ +0.08 mM pepstatin A	0	0	0	0
AP 48 $\mu\text{g ml}^{-1}$ +0.08 mM pepstatin A	0	0	0	0
Pepsin 48 $\mu\text{g ml}^{-1}$	0	0	0	0
Trypsin 48 $\mu\text{g ml}^{-1}$	0	0	0	0

<sup>a</sup> Specific activity: pepsin 68 U ml<sup>-1</sup>; trypsin 10 U ml<sup>-1</sup>; *StAPs* 0.66 U ml<sup>-1</sup>. 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. The results are means ( $\pm$  SD) of at least three independent experiments.

**Digestion of oxidized insulin  $\beta$ -chain by potato leaf aspartic protease.** Since the antimicrobial activity of potato leaf aspartic proteinase was ascribed to its proteolytic activity, the question about the specificity of these proteinases was raised. As a first approach to address this question, the hydrolytic specificities of *StAP1* and *StAP3* were studied with oxidized insulin  $\beta$ -chain. The proteinases were incubated with this substrate and the insulin peptide fragments were separated by RP-HPLC (Fig. 3) and identified by N-terminal amino acid sequencing. The same insulin cleavage pattern was obtained after 1, 3 and 24 h incubation, indicating that all possible peptide bonds were already cleaved after 1 h for each protease.

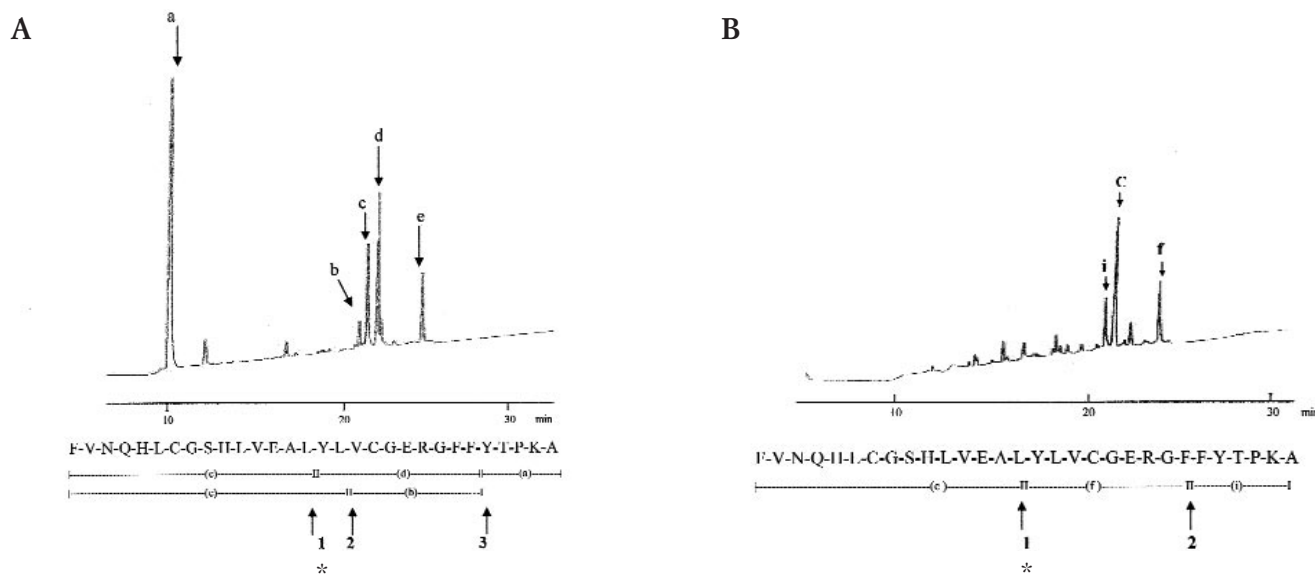
Three cleavage sites were identified for the action of the *StAP1* (Leu<sup>15</sup>-Tyr<sup>16</sup>; Leu<sup>17</sup>-Val<sup>18</sup> and Phe<sup>25</sup>-Tyr<sup>26</sup>) (Fig. 3A); whereas only two cleavage sites were identified for *StAP3* (Leu<sup>15</sup>-Tyr<sup>16</sup> and Phe<sup>24</sup>-Phe<sup>25</sup>) (Fig. 3B). Both proteases showed high specificity for peptide bonds located between amino acid residues with large hydrophobic side chains, such as Leu, Phe and Tyr.

## DISCUSSION

In this study, evidence is presented that one potato leaf aspartic protease (*StAP3*) is induced after infection with *P. infestans* (Fig. 1 and 2). These results were dif-

ferent from those obtained in potato leaves after detaching, where *StAP2* and *StAP3* are induced after this treatment (Guevara *et al.*, 2001). The induction of *StAP3* by a pathogen or by wounding can be explained since there are precedents for cross talk between both pathways (Karban *et al.*, 1987). Our results also show that in the cultivar with high level of field resistance (cv Pampeana INTA), *StAPs* activity and protein levels increase higher and faster than in the susceptible cultivar (cv Bintje). In cv Pampeana *StAPs* activity follows the same pattern of *StAPs* protein levels whereas, in cv Bintje, *StAPs* activity and protein levels follow different patterns at 24 and 72 h after infection (Fig. 1 and 2). These differences in cv Bintje suggest the presence of endogenous or exogenous aspartic protease inhibitor(s). There are only a few examples about the induction by abiotic stress of plant APs; an AP message is induced when tomato leaves are wounded (Schaller and Ryan, 1996), APs are induced when cauliflowers seeds are treated with polyethylene glycol (Fujicara and Karssen, 1995) and *StAP1*, *StAP2* and *StAP3*, all potato aspartic proteases, are induced in potato tubers and leaves by wounding (Guevara *et al.*, 1999, 2001, 2002). This is the second report about the induction of APs by biotic stress; we have previously reported the induction of *StAP1* in potato tubers of a resistant cultivar after infection with *P. infestans* (Guevara *et al.*, 2002).

The differences found in the *StAP* protein content



**Fig. 3.** RP-HPLC purification of peptide fragments from oxidised insulin  $\beta$ -chain digested by **A)** purified *StAP1* and **B)** purified *StAP3*. Digestion was carried out at pH 3.1, 37°C, for 8 h. Cleavage positions are indicated by arrows. Digested peptide fragments are numbered and areas are indicated. (\*) indicate the common *StAPs* cleavage positions.

and activity after abiotic and biotic stress in tubers and leaves of resistant potato cultivar suggest that *StAP* protein level and activity may be connected with a low or high plant defense response.

*StAP3* has antimicrobial activity, as reported for *StAP1* (Guevara *et al.*, 2002). The *StAP3* concentration 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* (Woloshuk *et al.*, 1991; Liu *et al.*, 1994; Niderman *et al.*, 1995) but 10-fold higher than the *StAP1* concentration that cause 100% inhibition of the germination of cysts of *P. infestans* (Guevara *et al.*, 2002). We previously described that with *StAP1* the calculated  $IC_{50}$  for *F. solani* was 32.16 mg ml<sup>-1</sup> (Guevara *et al.*, 2002), that for *StAP3* the concentration needed to obtain 50% inhibition for germination of conidia of *F. solani* was 118 mg ml<sup>-1</sup>. We excluded the possibility that the microbial inhibitory effect was due to contaminants in the *StAP3* preparations by the following reasons: (1) *StAP3* used was purified to homogeneity, giving a single band in SDS-PAGE after silver staining; 2) the antimicrobial effect was specifically reversed by pepstatin A. How these *StAPs* inhibit the growth of *P. infestans* is not apparent from the data presented, although it is clear that the overall inhibition observed is dependent on *StAP3* proteolytic activity.

When we analyzed the substrate specificity of purified *StAP1* and *StAP3*, using oxidized insulin  $\beta$ -chain as substrate we found that both enzymes have a common cleavage position, Leu<sup>15</sup>-Thyr<sup>16</sup> (Fig. 3A and 3B). This peptide bond is also cleaved by other described plant APs, as GIAP, HvAP, Cardosin A, Cardosin B and *Cucurbita maxima* L. AP (Polanowsky *et al.*, 1985; Faro

*et al.*, 1992, 1995; Kervinen *et al.*, 1993; Bleux *et al.*, 1998). Additionally, *StAP1* has two cleavage positions: Leu<sup>17</sup>-Val<sup>18</sup> which is common with cardosin A and cardosin B (Faro *et al.*, 1992, 1995) and Phe<sup>25</sup>-Tyr<sup>26</sup> is common with GIAP, HvAP, Cardosin A, Cardosin B and *Cucurbita maxima* L. AP (Polanowsky *et al.*, 1985; Faro *et al.*, 1992, 1995; Kervinen *et al.*, 1993; Bleux *et al.*, 1998). *StAP3* was able to cleave the peptide bond Phe<sup>24</sup>-Phe<sup>25</sup>; this cleavage position is common with other APs described, Cardosin B, AP *Cucurbita maxima* L. and HvAP (Polanowsky *et al.*, 1985; Faro *et al.*, 1992; Kervinen *et al.*, 1993). It is reported that *StAP* are unable to cleave the peptide bond Phe<sup>1</sup>-Val<sup>2</sup>, suggesting that these enzymes do not have exoproteolytic activity (Polanowsky *et al.*, 1985; Faro *et al.*, 1992).

These results would suggest that the differences in the antimicrobial activity of the *StAPs* might be associated with the different substrate specificities of these enzymes, so that the antimicrobial activity of these enzymes is completely inhibited in the presence of a specific aspartic protease inhibitor.

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