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Roles of glycosylation on the antifungal activity and apoplast accumulation of *St*APs (*Solanum tuberosum* aspartic proteases)

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Abstract

Specific roles of glycosylation appear to be protein-dependent. Plant aspartic proteases (APs) contain two or more consensus *N*-glycosylation sites; however, the importance of them is not well understood. *St*APs (*Solanum tuberosum* aspartic proteases) are bifunctional proteins with both proteolytic and antimicrobial activities. These proteins are accumulated into the intercellular washing fluid of potato tubers and leaves after wounding or infection. In this paper we investigated the importance of glycosylation on the *St*APs apoplast accumulation, biochemical parameters, and fungicidal activity. Assays to evaluate the importance of *St*APs glycosylation groups by using glycosylation inhibitors demonstrate that carbohydrate portions are essential to *St*APs accumulation into the apoplast of tubers and leaves after wounding or detachment, respectively. Bifunctional activity of *St*APs is differentially affected by this post-translational modification. Results obtained show that not significant changes were produced in the physicochemical properties after *St*APs deglycosylation (pH and thermal-optimum activity and index of protein surface hydrophobicity). Otherwise, *St*APs antifungal activity is affected by deglycosylation. Deglycosylated *St*APs (dg*St*APs) fungicidal activity is lower than native *St*APs at all concentrations and times assayed. In summary, glycosylation has not a significant role on the *St*APs conformational structure. However, it is involved in the *St*APs subcellular accumulation and antifungal activity suggesting that it could be necessary for *St*APs membrane and/or protein interactions and subsequently its biological function(s).
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1. Introduction

Glycosylation is a post-translational process in which carbohydrate residues are covalently attached to the protein. These glycosyl residues have been found to facilitate protein folding, to prevent protein aggregation, to protect against proteolytic attack and to act as surface cell receptors and targeting signals [1–4]. However, specific roles seem to be protein-dependent. For some glycoproteins, N-linked oligosaccharides are necessary for overall stability [5–7] whereas for others, the presence of carbohydrate chains is only required during the folding process [8] or during secretion [9].

Aspartic proteases (EC 3.4.23) (AP) constitute one of the four super-families of proteolytic enzymes. They are present in a wide variety of organisms, such as viruses, fungi, animals and plants [10–13]. Previously, we have identified three potato aspartic proteases, one from tubers (*St*AP1) [14] and two from leaves (*St*AP2 and *St*AP3) [15]. Two of these isoforms, *St*AP1 and *St*AP3, have been purified and characterized. In agreement with previous reports showing that many plant APs contain one or more N-linked glycans, *St*APs are glycosylated [14,15]. On the other hand, *St*APs are induced by both abiotic and biotic stress [15,16], have extracellular localization and have antimicrobial activity *in vitro* towards *Phytophthora infestans* and *Fusarium solani*, two potato pathogens [16–18].

Unlike the case of mammalian glycoproteins, in plant glycoproteins, the role of glycans in the folding and stability depends

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on the characteristics of individual protein [19–22]. *In vitro* studies have shown that once the protein is folded, glycosylation is often important for its stability [23–25]. On the other hand, the remotion of N-linked sites has occasionally little effect in the protein structure and function [26].

Different glycosylation inhibitors have been used to show the importance of glycosylation. In plant proteins, Brefeldin A (BFA) inhibits secretion and vacuolar protein transport in plant cells [27,28]. Most of extracellular *N*-glycosylated glycoproteins are not secreted in the presence of the inhibitor tunicamycin [29–32].

The aim of this work was to analyze the importance of glycosylation in the *St*APs apoplast accumulation, activities (antifungal and proteolytic), and biochemical properties.

2. Material and methods

2.1. Plant and fungal material

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

Tubers were washed and sterilized by immersion in 5% (w/v) sodium hypochlorite for 20 min. Tuber wounding was carried out cutting disks of parenchyma (4–6 mm diameter, 10 mm thick) in sterile conditions and incubating them for 0 h, 24 h and 48 h after wounding at 18 °C in the dark in a moist chamber.

Potato plants (*S. tuberosum* L. cv. Pampeana INTA) were grown in pots containing a sterile mixture of soil: vermiculite (2:1 v/v) and maintained at 25 °C for 6 weeks with a 14 h photoperiod. Light was supplied by Osram L36W/20 cool white fluorescent tubes, which supplied 120 μmol m^{-2} s^{-1} PAR measured 30 cm from the source. Wounding of leaves was performed by detaching leaves from plants and placing them at 18 °C in a moist chamber. Leaves were harvested at different times after detachment (wounding: 0 h, 24 h and 48 h in moist chamber).

Intercellular washing fluids (IWFs) of potato tubers and leaves were obtained as described by Guevara et al. [16,17]. Tuber disks and leaves 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. Tuber and leaves 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.

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

2.2. Purification of potato aspartic proteases

Potato tubers (*S. tuberosum*) were washed and sterilized by immersion in 5% (w/v) sodium hypochlorite for 20 min. Sterile disks of tuber parenchyma (10 mm diameter, 2 mm thick) were prepared, washed extensively, suspended in sterile water (15 disks/23 ml water) and aerated for 24 h at 25 °C in an orbital shaker at 60 cycles min^{-1}. Then the tuber disks were homogenized in 100 mM sodium acetate pH 5.2, 0.5% (w/v) sodium metabisulfite by applying four pulses of 10 s separated by 30 s intervals using a VirTis 45 homogenizer (The Virtis Co., Gardiner, New York, NY) set at speed 10. Homogenates were filtered through cheesecloth, centrifuged at 12,000 × *g* for 20 min; the resulting supernatant was stored at −20 °C. *St*AP1 was purified using the protocol described by Guevara et al. [14].

Leaves from 6-week-old potato plants were detached and placed at 18 °C in a moist chamber. Leaves were harvested at 24 h after detachment and homogenized in two 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) 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. *St*AP3 was purified according to Guevara et al. [15].

The purity of proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide gels [33]. Gels were stained with silver nitrate [34].

2.3. Detection of StAPs apoplast accumulation assays

After wounding, potato tuber disks and leaves were inoculated with a 100 μl of 100 mM sodium acetate buffer, pH 5.2 containing or not 0.5 mg ml^{-1} of tunicamycin (Sigma–Aldrich) [35] or 100 μg ml^{-1} of brefeldin A (BFA) (Sigma–Aldrich) and incubated during 6 h [36]. Subsequently, tuber disks and leaves were incubated for 0 h, 24 h and 48 h at 18 °C in the dark in a moist chamber.

*St*APs apoplast accumulation was monitored in intercellular washing fluid (IWF), obtained as indicated above, from treated or untreated tubers and leaves.

2.4. Gel electrophoresis and immunoblot analysis

Tuber and leaf IWFs corresponding to 10 mg of fresh tissue were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gel [33]. Gels were stained with silver nitrate [34].

For inmunoblotting, IWFs were electrophoresed and transferred to nitrocellulose using a semi dry electrophoretic transfer cell (trans Blot, BioRad) at 12 V for 10 min. The membrane was blocked in Tris buffered saline containing 5% (w/v) dried low fat milk for 45 min. The antiserum (anti-*St*AP1 [14] and the second antibody (alkaline phosphatase-coupled goat–antirabbit IgG) were used at 1/10,000 and 1/5000, respectively, in block-

ing solution containing 1% dried low fat milk. Bound antibody was detected using BCIP/NBT according to procedures recommended by the manufacturer (Sigma, St. Louis, USA).

For immunoblotting analysis of leaves IWFs, carbohydrate epitopes were destroyed by periodate oxidation, as described in Heimgartner et al. [37]. IWF were electrophoresed and transferred onto nitrocellulose. The membranes were oxidized with 10 mM periodic acid in 100 mM sodium acetate, pH 5, at room temperature for 30 min in the dark and subsequently quenched with 25 mM NaOH. The oxidized membranes were further processed in the same way as untreated membranes.

The intensity of the immuno-positive bands on the western blots was quantified by image analysis (GelPro Analyzer 4.0, Gel-Pro Software, Media Cybernetics, LP, USA).

2.5. Chemical deglycosylation of StAPs

Carbohydrate epitopes were destroyed by periodate oxidation, according to Heimgartner et al. [37]. Aliquots of *St*AP1 or *St*AP3 were oxidized with 10 mM periodic acid (Sigma–Aldrich) at room temperature for 30 min in the dark and subsequently neutralized with 25 mM NaOH.

The efficacy of this protocol in the destruction of *St*APs carbohydrate portion was evaluated as follows:

- To analyze the extent of *St*AP1 deglycosylation, we took in account that previous results reported the capacity of *St*AP1 to bind to concanavalin-A [14]. According to this fact, dg*St*AP1capacity of binding to a column of matrix-linked concanavalin-A was evaluated as described by Guevara et al. [14].
- On the basis of previous results that showed that contrary to native *St*AP3, dg*St*AP3 is able to immuno cross-react with anti*St*AP1-IgG [15]; dg*St*AP3 capacity to immuno cross-react with anti*St*AP1-IgG was evaluated as described by Guevara et al. [15].

2.6. Analytical procedures and measurement of enzymatic activities

Protein concentration was measured by the bicinchoninic acid method [38], using bovine serum albumin (BSA) as standard.

Proteolytic activity was determined using bovine haemoglobin as substrate according to Sorgine et al. [39] with some modifications. Substrate (100 μg) was incubated in 400 μl of 0.15 M acetate buffer, pH 3.5 at 37 °C. The reaction was started by the addition of 100 μl of *St*APs or dg*St*APs (0.5 mg/ml). After 6 h, 500 μl of cold 10% trichloroacetic acid (TCA) was added; samples were placed in ice for 10 min and centrifuged for 5 min at 5000 × *g*. Absorbance at 280 nm was measured in the supernatant. One unit (U) is defined as the amount of the enzyme producing an increase in absorbance of 0.1 units at 280 nm, in 1 h, at 37 °C. The effect of pepstatin A (a specific aspartic protease inhibitor) was tested in these assays as described by Guevara et al. [14].

2.7. Temperature-activity and pH-activity optimum assays.

Temperature-activity and pH-activity optimum of purified *St*APs and deglycosylated *St*APs (dg*St*APs) were determined. The *St*APs pH-activity optimum was determined incubating 0.05 mg of *St*APs or dg*St*APs with haemoglobin as substrate according to described above and using a set of buffers with different pH ranges: 0.2 M citrate–phosphate buffer in the pH range 3–7 and 0.2 M carbonate–bicarbonate buffer in the pH range 8–10.

Temperature-activity optimum determination assay was performed by incubation of 0.05 mg *St*APs or dg*St*APs with haemoglobin as substrate at 35 °C, 45 °C, 55 °C, 65 °C and 75 °C for 3 h. All measurements were performed in triplicate and comparisons were made to the control conditions (pH 5.2 and 37 °C, respectively).

2.8. Surface hydrophobicity

Surface hydrophobicity of soluble *St*APs was determined using hydrophobic fluorescence probe ANS (1-anilino-8-naphtalene-sulfonate) [40]. *St*APs or dg*St*APs (1 mg ml^{-1}) were serially diluted with its own buffering solution to a final volume of 2 ml. The range of enzyme concentrations analyzed was 0.01–1% of initial protein concentration. After ANS stabilizing at 20 °C, 10 μl of this probe (8 mM) were added to *St*APs or dg*St*APs solutions. The relative fluorescence intensity (RFI) of ANS-protein was measured with a Shimadzu RF-5301PC spectrophotofluorometer at wavelength (λ_{ex}, λ_{em}) 370–470 nm. The RFI was standardized adjusting the reading to 80% full scale for ANS in methanol. The net RFI was obtained by subtracting the RFI of each sample measured without a probe from that with a probe. The initial slope (S_0) of the RFI versus protein concentration was calculated by linear regression analysis, and these values were used as an index of protein surface hydrophobicity.

2.9. Antifungal activity of dgStAPs

2.9.1. Determination of dgStAPs fungistatic activity

To assay the effects of dg*St*APs on the germination of spores of *F. solani*, *in vitro* bioassays were performed as described by Guevara et al. [16]. To quantify the effects of dg*St*APs or native *St*APs spores germination, these bioassays were examined by observation of four fields in Neubauer camera, with a bright-field microscope.

2.9.2. Determination of dgStAPs fungicidal activity

To check if dg*St*APs as well as *St*APs have fungicidal activity based in its capacity to destabilize membranes, assays involving the uptake of the fluorogenic dye SYTOX Green were performed [41]. This reagent can only penetrate cells that have compromised plasma membranes, and it fluoresces upon binding to DNA of dead cells.

F. solani spores were incubated 3 h or overnight at 25 °C with water (control experiments) or exposed to different amounts of *St*AP1, *St*AP3 or dg*St*APs as described in Mendieta et al. [18].

SYTOX Green probe (molecular probes) was added at a final concentration of 1 μM and qualitative detection of SYTOX Green uptake was made after 30 min with a Nikon Eclipse E200 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a B-2A Fluorescein filter set. Positive controls included spores treated with 0.5% (w/w) Triton X-100. Fluorescence was measured using a FluoresKan Ascent (Thermo Electron Corporation, Finland) 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.

2.9.3. Protease labeling

*St*AP1, *St*AP3, dg*St*AP1 or dg*St*AP3 were labeled with fluorescein isothiocyanate (FITC), Isomer I using FluoroTag™ FITC Conjugation Kit (Sigma). Labeled proteases were isolated from non-labeled protease and free label according to the procedure recommended by the manufacturer.

2.9.4. Binding of StAPs to spores and hyphae surface

F. solani spores (2.7×10^6 spores ml^{-1}) were incubated overnight with different concentrations of *St*APs or labeled-dg*St*APs (0.3 μM, 1.2 μM, 1.9 μM, 6.5 μM and 10 μM) at 25 °C and 100% relative humidity. After incubation, the number of fluorescent spores was evaluated. Controls were performed replacing the protease solutions with 10 μM of pepsin or water. The binding of *St*APs or dg*St*APs to surface of spores was examined by observation with a Nikon Eclipse E200 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a B-2 filter set (Nikon) for fluorescein detection by counting the fluorescent and non-fluorescent spores on a Neubauer camera. The results from three independent experiments were analyzed to calculate the percentage of fluorescence.

2.10. Statistical analysis

To determine a possible correlation between dg*St*APs and *St*APs proteolytic activity, FITC-dg*St*AP binding, antifungal activity and membrane permeabilization effect, two-way analysis of Variance followed by Tukey's test (SigmaStat 3.0) were assayed. Differences between values were considered statistically significant if $p < 0.05$.

3. Results

3.1. StAPs accumulation in the apoplast of potato tubers and leaves

In order to elucidate if *St*APs glycosylation process has importance on the accumulation enzymes in the apoplast after wounding, assays using tunicamycin and BFA were performed. Sterile water, BFA, or tunicamycin were applied before the initiation of abiotic stress conditions, and samples were incubated at different times of wounding or detachment.

Analysis by SDS-PAGE shows that no differences were observed regardless whether or not BFA or tunicamycin were

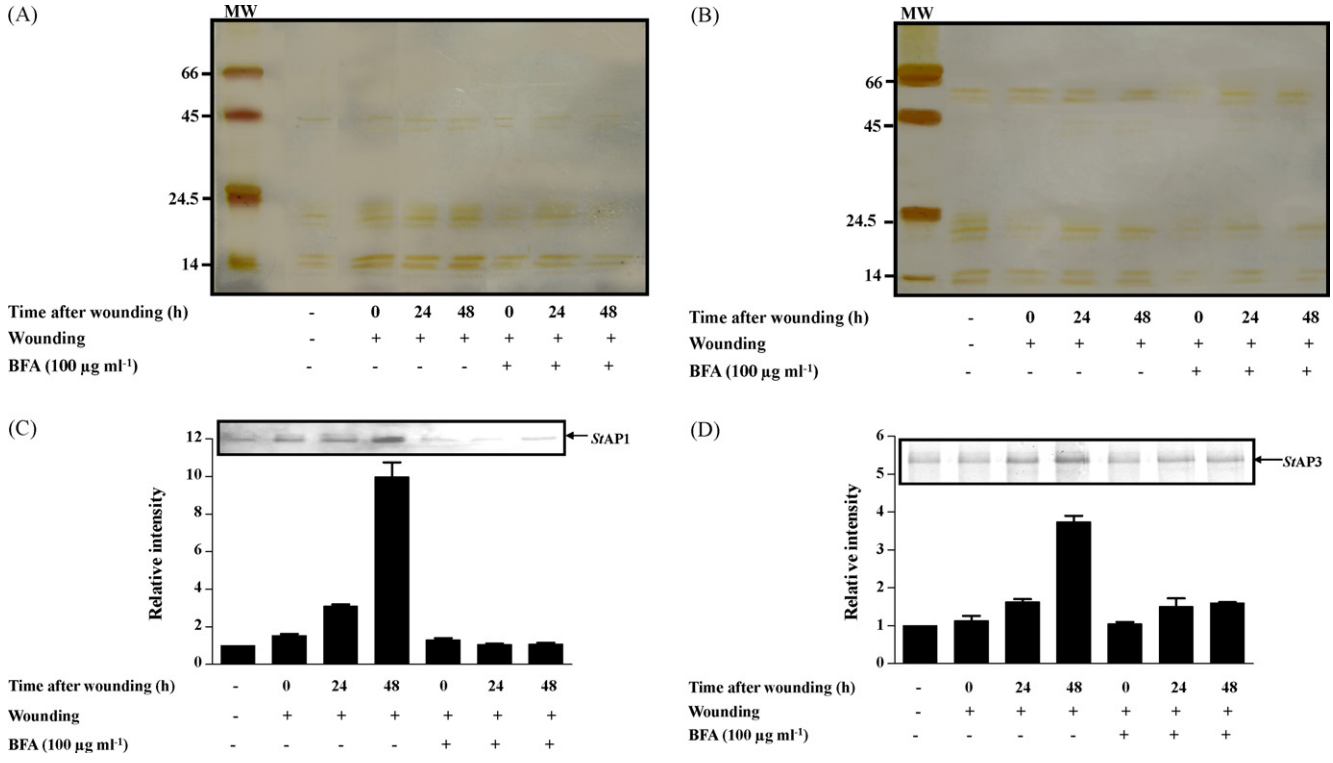

Fig. 1. *St*APs accumulation into potato tubers and leaves apoplast after wounding and/or BFA treatment. SDS-PAGE analysis of intercellular washing fluid from tubers (A) and leaves (B) after wounding and/or BFA treatment. Western blot and densitometry analysis of *St*AP1 (C) and *St*AP3 (D) accumulation into tubers and leaves apoplast, respectively, after wounding and/or BFA treatment. Anti-*St*AP1 was used as primary antibody as is described in Section 2. The samples corresponded to 10 mg of fresh weight. Each point represents the mean ± S.D. ($n = 3$).

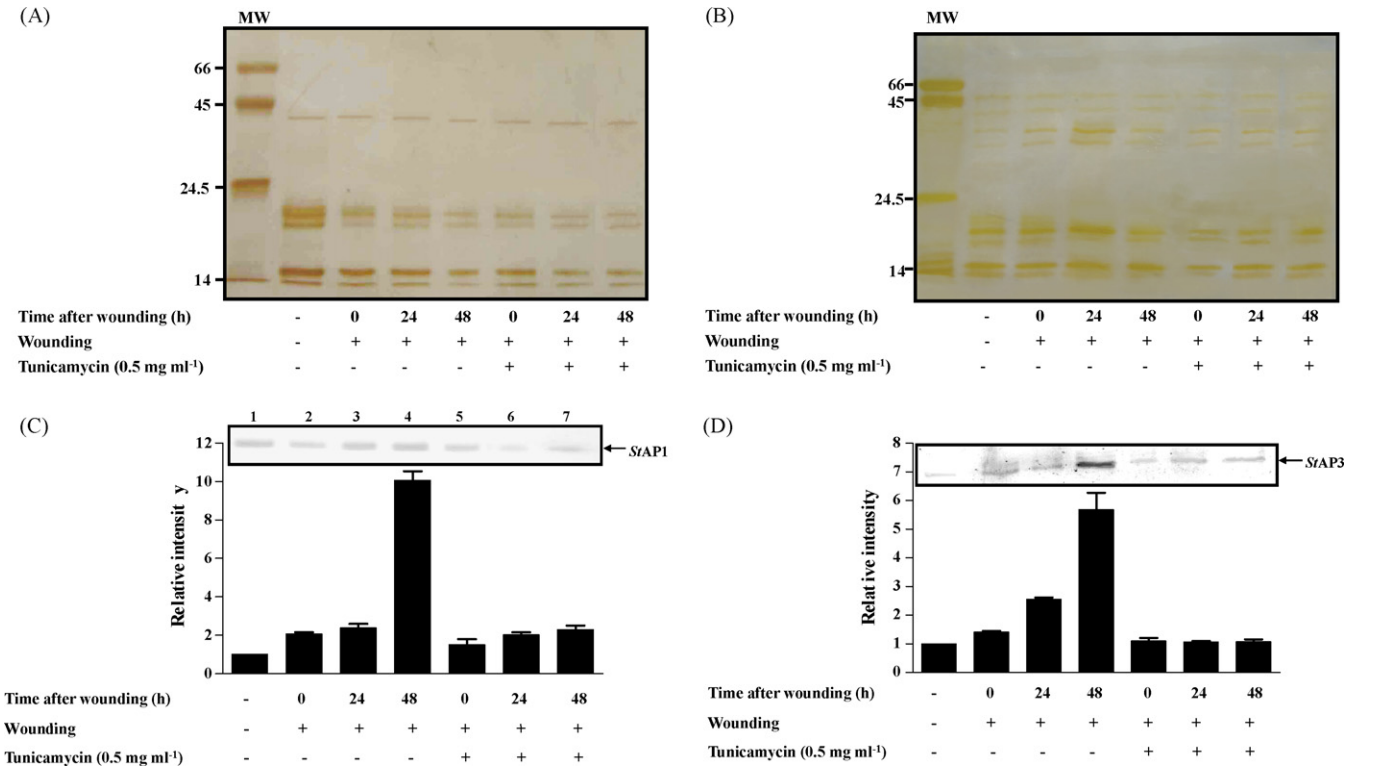

Fig. 2. *St*APs accumulation into potato tubers and leaves apoplast after wounding and/or tunicamycin treatment. SDS-PAGE analysis of intercellular washing fluid from tubers (A) and leaves (B) after wounding and/or tunicamycin treatment. Western blot and densitometry analysis of *St*AP1 (C) and *St*AP3 (D) accumulation into tubers and leaves apoplast, respectively, after wounding and/or tunicamycin treatment. Anti-*St*AP1 was used as primary antibody as is described in Section 2. The samples corresponded to 10 mg of fresh weight. Each point represents the mean ± S.D. ($n = 3$).

added (Figs. 1 and 2). SDS-PAGE analysis show the same protein patterns in IWFs of control (healthy) and IWFs of tuber disks and leaves after different wounding or detachment times. Jointly, and according to our previous reports [14], western blot analysis using anti*St*AP1-IgG showed that the amount of *St*AP1 and *St*AP3 increase in IWFs of potato tuber disks and leaves after 48 h of wounding or detachment, respectively. These increases were six and 2.5-fold higher, respectively, than their controls (Fig. 1C and D). However, when BFA (Fig. 1C and D) or tunicamycin (Fig. 2C and D) were previously applied to potato tuber disks and leaves, accumulation of *St*AP1 and *St*AP3 IWFs was not observed.

3.2. Physicochemical properties of StAPs

Efficacy of deglycosylation protocol was tested for *St*AP1 and *St*AP3 as described in Section 2. Contrary to results described for native *St*AP1 [14], dg*St*AP1 was unable to bind to concanavalin-A matrix column (data not shown) indicating that the deglycosylation protocol was successful. Likewise, dg*St*AP3 was able to immuno cross-react with anti*St*AP1-IgG in dose-dependent form (data not shown).

The effects of pH and temperature on the proteolytic activity of dg*St*APs were studied and compared with the values previously reported for native *St*APs [14,15]. Similar values were determined in curves of *St*AP1 and dg*St*AP1 proteolytic activity versus pH ($p < 0.05$). Both proteins presented the highest activity at pH 5, and then the activity dropped as the pH was increased (Fig. 3A and B). Significant differences ($p < 0.05$) in the pH optimum activity were detected between *St*AP3 and *dg*S*t*AP3 (Fig. 3B). While native *St*AP3 has the optimum pH activity at pH 3, dg*St*AP3 has the optimum pH activity at pH 5.

Native and deglycosylated *St*APs showed similar profiles of proteolytic activity versus temperatures ($p < 0.05$). (Fig. 4A and B). Temperature optimum activity of these proteins was detected at 37 °C. However, as temperature increased, a steady decline of activity was observed. At 85 °C a small amount of activity was detected for either form of *St*APs.

No significative changes ($p < 0.05$) were detected in the values of surface hydrophobicity exposure between native *St*APs and dg*St*APs (Table 1).

Table 1
Effect of deglycosylation on exposure surface hydrophobicity of *St*APs

	ANS So	
	*St*AP1	*St*AP3
Control	8.26 ± 1.9 a	7.14 ± 1.5 a
Deglycosylated	9.52 ± 0.5 a	8.35 ± 2.3 a

ANS So: initial slope of relative fluorescence intensity vs. protein concentration plot in the presence of ANS (1-anilino-8-naphtalene-sulfonate). Different letters mean values significantly different ($p < 0.05$; two-way ANOVA following by Tukey's test, SigmaStat).

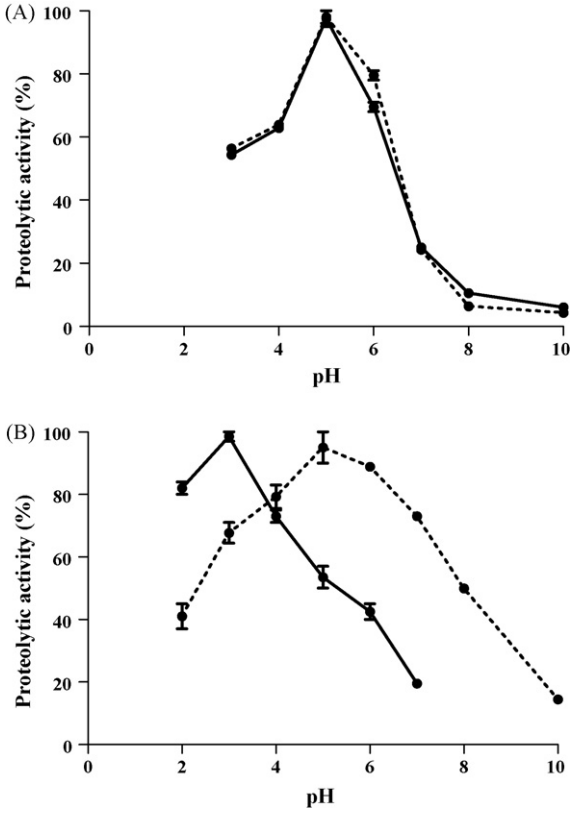

Fig. 3. Effect of deglycosylation on the *St*APs pH optimum proteolytic activity. pH optimum proteolytic activity values were determined for *St*AP1 (A) and *St*AP3 (B) using haemoglobin as substrate, as described in Section 2. The percentages of proteolytic activity were calculated as the percentage of *St*APs activity at pH 5 for *St*AP1 and pH 3 for *St*AP3. The amount of *St*APs or dg*St*APs used to perform these assays was 0.05 mg ml^{-1}. Each point represents the mean ± S.D. ($n = 3$), native form (—) and deglycosylated form (· · ·).

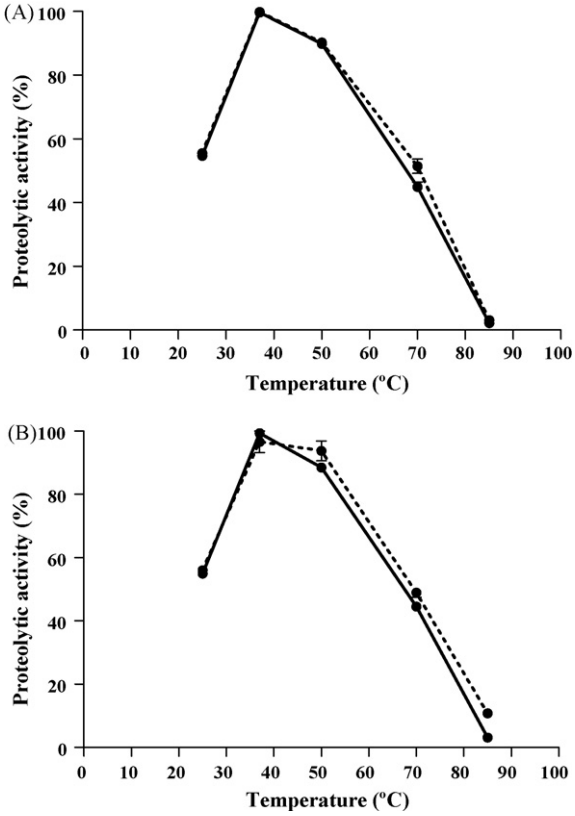

Fig. 4. Effect of deglycosylation on *St*APs temperature optimum proteolytic activity. Temperature optimum proteolytic activity values were determined for *St*AP1 (A) and *St*AP3 (B) using haemoglobin as substrate, according to described in Section 2. The percentages of proteolytic activity were calculated as the percentage of *St*APs activity at 37 °C. The amount of *St*APs or dg*St*APs used to perform these assays was 0.05 mg ml^{-1}. Each point represents the mean ± S.D. ($n = 3$), native form (—) and deglycosylated form (· · ·).

3.3. dgStAPs in vitro antifungal activity

Previously reported assays show that *St*APs have fungicidal activity. This antifungal effect against *F. solani* is caused by a direct interaction with the spores surfaces and, subsequently, by membrane permeabilization [18].

In order to analyze if glycosylation could have an effect on *St*APs antifungal activity dose–response experiments were performed. *F. solani* spores were incubated with different amounts of *St*APs or dg*St*APs and percentage inhibition of spore germination were determined. Results show that both dg*St*APs are able to inhibit spore germination in the same dose-dependent form ($p < 0.05$) as native *St*APs (Fig. 5A and B).

To determine if dg*St*APs have fungicidal activity and fungal membrane permeabilization capacity, as well as the native forms, quantitative assays of SYTOX green uptake were performed. Fig. 5C and D shows that at both, short and long times of incubation (3 h and overnight), dg*St*APs have a similar dose–effect patterns than native *St*APs. However, a lower effect ($p < 0.05$) was detected on spore viability and membrane destabilization when *St*APs are deglycosylated.

Binding capacity to the surface of fungal spores was evaluated for dg*St*AP and compared with the values previously determined for native *St*APs [18]. Deglycosylated *St*APs were able to bind to spores surface at all concentrations assayed (Fig. 6) and in the same dose-binding pattern than native *St*APs (data not shown).

4. Discussion

Oligosaccharide portions of glycoproteins are involved in numerous biological processes, including protein folding, secretion, cell–cell interaction, conformational stability and proteolytic susceptibility [1–4]. Plant APs contain conserved, occupied *N*-glycosylation sites; however, the importance of this post-translational modification on the activity and subcellular localization of each plant AP, is different [26,45,46]. Specifically, *St*APs are glycosylated, are bifunctional enzymes, presenting proteolytic and antimicrobial activities and their accumulation into the apoplast of tubers and leaves is induced after wounding and infection [17].

In this paper we show that the inhibition of protein *N*-glycosylation by tunicamycin abolish the accumulation of *St*AP1 and *St*AP3 into the IWFs of potato tubers and leaves after wounding. This result suggests that the glycans are essential for the final *St*APs apoplast accumulation induced by wounding in potato tubers and leaves. This effect could have several explana-

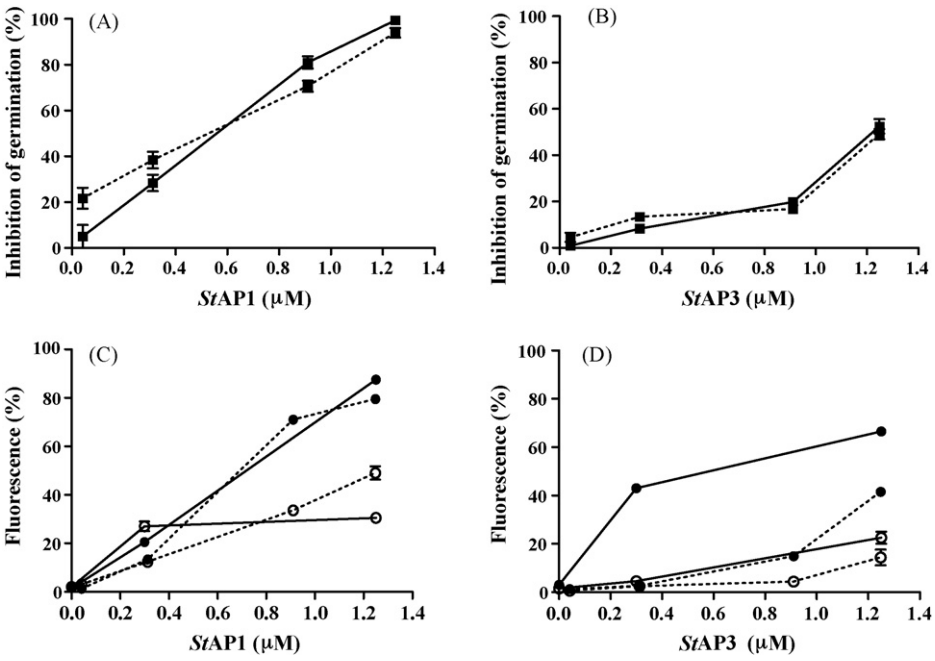

Fig. 5. Effect of deglycosylation on the *St*APs antifungal activity. The capacity of *St*APs and dg*St*APs to inhibit *Fusarium solani* germination was evaluated after incubation of spores with different amounts of *St*AP1 or dg*St*AP1 (A) and *St*AP3 or dg*St*AP3 (B) as is described in Section 2. Native form (–■–) and deglycosylated form (· · ·■· · ·). Fungicidal activity of *St*APs and dg*St*APs was evaluated by SYTOX Green uptake on *F. solani* spores. The spores were incubated for 3 h (—) or overnight (· · ·) with increased amounts of *St*AP1/dg*St*AP1 (C) or *St*AP3/dg*St*AP3 (D); after that, 1 μM SYTOX Green was added as described in Section 2. The number of the fluorescent spores treated with 0.5% (w/w) of Triton was taken as 100%. Data are averages of triplicate measurements. Native form (●) and deglycosylated form (◯).

tions. Likewise to phytepsin, another plant aspartic proteinase, glycosylation might have a role in the protection of the enzyme against premature proteolytic cleavage and, consequently, on the intracellular stability, by preventing its degradation [50]. Another explanation could be the breaking of the *St*APs interaction with other(s) protein(s) involved in the protein transport to the apoplast. The results obtained are in accordance with several reports that show that tunicamycin inhibits protein secretion in cultured cells [42,31,43] and has a dramatic effect on the intracellular transport of storage glycoproteins [37]. On the

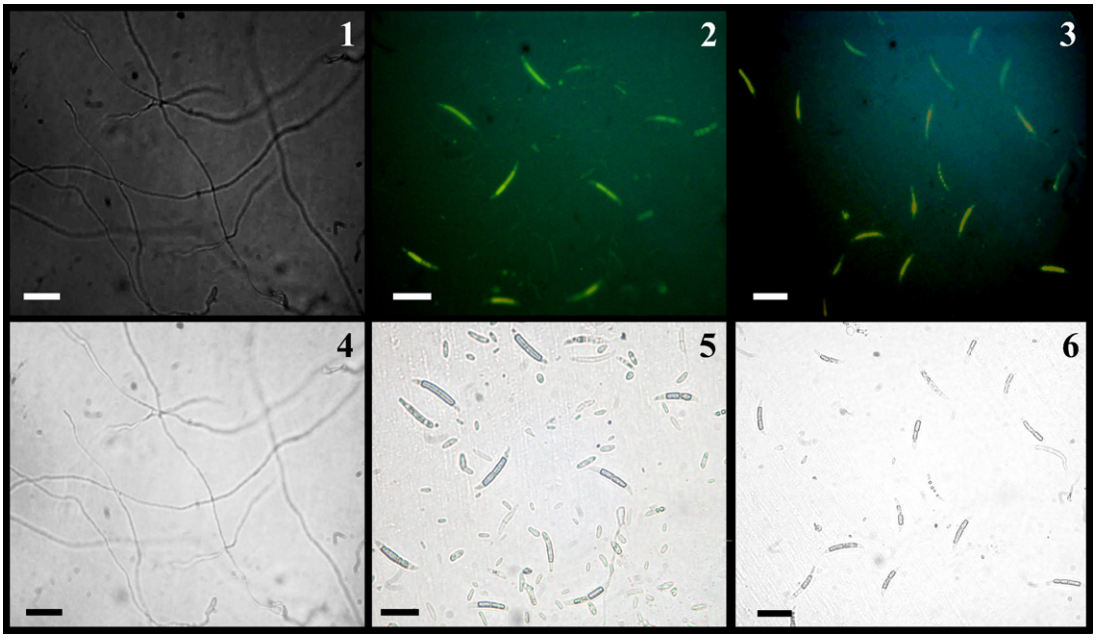

Fig. 6. Effect of *St*APs deglycosylation on *St*APs binding capacity to fungal spores surface. Spores of *F. solani* were incubated with 3.75 μM of FITC-labeled *St*APs. After 16 h of incubation, the labeled protein was detected by fluorescence microscopy. Panels 1, 2 and 3: fluorescence microscopy. Panels 4–6: light-field microscopy. Panels 1 and 4: controls. Panels 2 and 5: 3.75 μM of FITC-labeled *St*AP1. Panels 3 and 6: 3.75 μM of FITC-labeled *St*AP3, bars: 15 μm.

other hand, *St*APs apoplast accumulation induced by wounding was inhibited by BFA. Brefeldin A is known to inhibit Golgi-mediated vesicular traffic by disrupting the Golgi apparatus. Additionally, several reports demonstrate that BFA is able to inhibit protein secretion at an early step of protein transport in animal cells [44] and to block protein secretion in plant cells [47–50]. Based in these antecedents and in the results obtained here, *St*APs Golgi-mediated vesicular traffic is necessary to *St*APs apoplast accumulation induced by wounding in potato tubers and leaves.

In this work, we have also analyzed the importance of glycosylation on two previously reported *St*APs activities, proteolytic and antimicrobial [16–18]. Initially, we checked the efficacy of the deglycosylation protocol used. Results obtained show that this protocol was efficient for both *St*APs and that reactive free aldehyde groups highly released by periodic acid oxidation did not affect the physicochemical properties of *St*APs. In a second step, we analyzed the effect of deglycosylation on the pH and temperature optimum activities, and we found that there are not significant differences in these parameters between deglycosylated *St*AP1 and its native form. However, dg*St*AP3 optimum pH (pH 3) is significatively different to *St*AP3 optimum pH (pH 5). This result suggests that deglycosylation process could change *St*AP3 conformation, in an ANS undetectable form. As a final result, a change in the optimum pH of *St*AP3 is detected.

Results obtained using aromatic hydrophobicity assays (ANS), showed that *St*APs folding does not suffer significant changes when the glycosydic portion is removed from these proteins.

Analysis of antifungal activity of dg*St*APs shows that fungicidal activity was affected by deglycosylation in both *St*APs, since values obtained were ever lower than those obtained for native forms, at all concentrations and times assayed.

Previous reports indicated that, for some plant APs, hyperglycosylation improves either secretion or stability and consequently, in some cases, it produces a decrease of enzyme proteolytic activity [45]. In addition, Costa et al. [46], suggested that oligosaccharides will play a dramatic role in the conformation stability, activity and specificity of *Cynara cardunculus* aspartic proteases. Here, we present evidences that differ from the above described. The results obtained show that glycosylation is involved in *St*APs subcellular accumulation induced by wounding and only in one of two *St*APs activities, i.e. the antifungal activity, but it does not have any effect on *St*APs proteolytic activity and conformational structure *in vitro*.

Future efforts would be focused to perform assays to determine if glycosylation has any role on the *St*APs membrane and/or protein interactions and subsequently in *St*APs biological function(s).

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