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Evaluation of *in vitro* cytotoxic activity of mono-PEGylated *St*AP3 (*Solanum tuberosum* aspartic protease 3) forms

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ABSTRACT

*St*AP3 is a plant aspartic protease with cytotoxic activity toward a broad spectrum of pathogens, including potato and human pathogen microorganisms, and cancer cells, but not against human T cells, human red blood cells or plant cells. For this reason, *St*AP3 could be a promising and potential drug candidate for future therapies. In this work, the improvement of the performance of *St*AP3 was achieved by means of a modification with PEG. The separation of a mono-PEGylated *St*AP3 fraction was easily performed by gel filtration chromatography. The mono-PEGylated *St*AP3 fraction was studied in terms of *in vitro* antimicrobial activity, exhibiting higher antimicrobial activity against *Fusarium solani* spores and *Bacillus cereus*, but slightly lower activity against *Escherichia coli* than native protein. Such increase in antifungal activity has not been reported previously for a PEGylated plant protein. In addition, PEGylation did not affect the selective cytotoxicity of *St*AP3, since no hemolytic activity was observed.

1. Introduction

Antimicrobial proteins and peptides (AMPPs) are important components of the natural defences against pathogens and are found in a wide range of eukaryotic organisms, from humans to plants [1–6]. The discovery of new groups of AMPPs as potential natural antibiotics represents a hit toward the discovery of a novel generation of drugs for the treatment of bacterial and fungal infections [7]. Moreover, the broad spectrum of antimicrobial activities reported for these molecules suggests their potential benefit in the treatment of viral or parasitic infections [8,9] and cancer [10,11]. In contrast to conventional antibiotics, they act by physical disturbance or destruction of the barrier function of the plasma membrane cell without involvement of a specific receptor [12,13].

Plants, unlike mammals, lack mobile defensive cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites [14–16]. *In vitro* antimicrobial activity has been demonstrated for the following plant peptides and proteins: (i) some of the so-called pathogenesis-related proteins, which were originally identified as pathogen-elicited proteins [17,18]; (ii) a number of plant antimicrobial protein and peptide families [19]. Furthermore, plant proteins and peptides with *in vitro* cytotoxic activity and anticancer properties on human cancer cell lines have also been reported [20–25]. We have previously reported the induction after infection and the cytotoxic activity of potato aspartic proteases (*St*APs) toward plant pathogens [26–28]. Our results show that potato aspartic proteases (*St*APs) and their swaposin domain (*St*Asp-PSI) are proteins with cytotoxic activity which involves plasma membrane destabilization. The ability of these proteins to produce cell death varies with the cellular type [28–30]. We have demonstrated that the lack of hemolytic and cytotoxic activities on human lymphocytes of *St*Asp-PSI/*St*APs is attributed to the presence of cholesterol in these cell membrane types [29,31]. These results open a new perspective to test these proteins as possible candidates to develop new drugs that would be active against microbes but not against mammalian cells and considerer these

Abbreviations: AMPPs, antimicrobial proteins and peptides; ATCC, American Type Culture Collection; BSA, bovine serum albumin; DTT, dithiothreitol; hRBC, Fresh human red blood cells; mPEG-SVA, succinimidyl valerate monomethoxy polyethylene glycol; PBS, phosphate buffered saline; PDA, potato dextrose agar; PEG, polyethylene glycol; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; *St*AP3, *Solanum tuberosum* aspartic protease 3; *St*Asp-PSI, plant-specific insert of potato aspartic protease.

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proteins as conceptually promising agent in infectious diseases and cancer therapy.

The covalent attachment of polyethylene glycol (PEG) chains (PEGylation) to therapeutic peptides and proteins has become one of the most useful pharmaceutical techniques developed thus far to provide functional bioconjugates with improved therapeutic properties over their unmodified counterparts [32,33]. PEGylation, indeed, has been proposed as a method for optimizing pharmacokinetic and pharmacodynamic properties of therapeutic small drug molecules, peptides and proteins [34]. The modification leads to an increase in molecular size and steric hindrance, changes in conformation and electrostatic binding properties. This results in the reduction of renal ultrafiltration, the masking of proteolytic and immunogenic sites and the shielding from proteolytic enzymes, antibodies or antigen processing cells [34–36]. This strategy can prolong the plasma circulating half-life, augment the *in vivo* stability [34,37–40], and diminish the phagocytosis and immunogenicity of peptides and proteins [36,41–43]. Due to these benefits, PEGylation plays an increasingly important role in the production of enhanced peptide and protein delivery systems [44].

There are few works in which PEGylation is used to improve plant proteins therapeutic potential, reducing their immunogenic behavior and extending the permanence of the injected drugs in the body. Examples of this include histaminase from *Lathyrus sativus* shoots for alternative treatment of histamine-mediated affections [45]; α-momorcharin and momordica anti-HIV protein derived from *Momordica charantia* L., for antitumor and antivirus therapies [46,47]; pokeweed mitogen, a plant lectin able to enhance the cytotoxicity of human lymphokine-activated killer cells [48]; and recombinant phenylalanine ammonia-lyase, originated from parsley (*Petroselinum crispum*) for the treatment of phenylketonuria [49], among others [50–52].

In this work, the improvement of the performance of *St*AP3 was achieved by means of a covalent modification with PEG. The separation of a mono-PEGylated *St*AP3 fraction could easily be performed by gel filtration chromatography. The mono-PEGylated *St*AP3 fraction was studied in terms of *in vitro* antimicrobial activity, exhibiting higher antimicrobial activity against *Fusarium solani* spores and *Bacillus cereus*. In addition, PEGylation did not affect the selective cytotoxicity of *St*AP3, since no hemolytic activity was observed.

2. Materials and methods

2.1. Materials and microorganisms

Succinimidyl valerate monomethoxy polyethylene glycol (mPEG-SVA, 5 kDa) was purchased from Laysan Bio Inc. (Arab, AL, USA). Sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) were supplied by Sigma (St. Louis, MO, USA). All the reagents were purchased in the highest purity and used without further purification.

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 ampicillin. Spores were collected from 8-day-old cultures by suspension in sterile water.

B. cereus and *Escherichia coli* were provided by the American Type Culture Collection (ATCC) and were grown in Luria–Bertani medium at 37 °C with continuous shaking. Bacterial growth was quantified by measuring absorbance at 600 nm.

2.2. Protein purification and preparation of PEG-StAP3 (PEGylated-StAP3)

Potato leaves were detached and placed at 18 °C in a moist chamber. *St*AP3 was purified from leaves using the protocol previously described by Guevara et al. [53]. A solution of purified *St*AP3 (5 ml, 0.6 mg/ml) in 50 mM Tris–HCl pH 8, was added to a 40-fold molar excess of mPEG-SVA. The mixture was incubated at 25 °C with stirring at 500 rpm, and the reaction was quenched after 6 h by addition of 2 ml 1 M glycine solution. The mixture was then concentrated to 230 μl using Vivaspin 15R (MW cut-off 5 kDa) (VIVASCIENCE, Germany), and 0.4% SDS (w/v) and 0.2 mM DTT were added.

2.3. Size exclusion chromatography (SEC)

PEG-*St*AP3 conjugates were analyzed by size exclusion chromatography on an equilibrated Superose 12 HR (10/30) column (Pharmacia, Uppsala, Sweden), connected to a fast-protein liquid chromatography system, at a constant flow rate of 0.4 ml/min at room temperature. The column was calibrated using a mixture of four proteins of known molecular mass, *i.e.* pyruvate kinase (230 kDa), native *St*AP3 (45 kDa), glyceraldehyde-3P-dehydrogenase (36 kDa), and lysozyme (14.3 kDa). The column was equilibrated and eluted with 20 mM Tris–HCl pH 8, 0.4% SDS (w/v), and 0.2 mM DTT. Fractions of 0.4 ml were collected and the elution was monitored at 280 nm.

2.4. Gel electrophoresis

Fractions from the size exclusion chromatography corresponding to different peaks were pooled and then analyzed by SDS-PAGE using 12% acrylamide. Gel was stained with Coomassie Brilliant Blue R250 coloidal [54]. Samples were treated in denaturing buffer with SDS, β-mercaptoethanol and heated before SDS-PAGE.

2.5. Analytical procedures for protein quantification

Protein concentration was measured by the Bradford method [55], using BSA as the standard.

2.6. Functionality assays

The fraction containing mono-PEG-*St*AP3 species was the employed for biological studies. Prior to assays, this fraction was dialyzed against 20 mM Tris–HCl pH 8, for 48 h at 4 °C, using a cellulose membrane (Sigma D9652-100) to remove DTT and SDS. The fraction was then stored at −20 °C for further analyses.

2.6.1. Cytotoxic activity assays

To evaluate the effect of mono-PEG-*St*AP3 on the germination of *F. solani* spores, *in vitro* bioassays were performed as described by Guevara et al. [26]. To quantify the effect of mono-PEG-*St*AP3 on spore germination, the bioassays were examined by observation of four fields in Neubauer camera with a bright-field microscope. The results from three independent experiments were analyzed to calculate the percentage of inhibition.

B. cereus and *E. coli* were grown in Luria–Bertani medium at 37 °C with continuous shaking to exponential phase. The bacteria were harvested from broth by centrifugation at 3500 rpm for 10 min, washed and resuspended in sterile PBS at a concentration of 10^4 c.f.u./ml. The concentration of bacteria was verified and quantified by culture on sheep blood agar plates. One hundred microliters of bacterial suspension were plated on 96-well polystyrene microtiter plates (BD Biosciences), and serial dilutions of mono-PEG-*St*AP3 were added to individual wells in triplicate and incubated for 6 h at 37 °C with rocking. Bacteria were subsequently dispersed and aliquots were plated on blood agar plates to obtain colony counts. Pathogen viability after protein treatment was determined from the number of colonies obtained on the buffer-treated control plates compared to the number of colonies

from protein-treated samples. The half maximal inhibitory concentration (IC_{50}) was calculated as the concentration of protein required to inhibit microbial growth by 50%.

2.6.2. Cell membrane permeabilization ability of mono-PEG-StAP3

F. solani spores were incubated overnight at 25 °C with water as control or exposed to different amounts of mono-PEG-*St*AP3, as described by Guevara et al. [26]. SYTOX Green probe (Molecular Probes) was added to a final concentration of 0.5 μM and qualitative detection of SYTOX Green uptake was performed. After 30 min incubation, the fluorescence of the sample was observed 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 FluorosKan 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.

2.6.3. Hemolysis assay

Fresh human red blood cells (hRBC) were rinsed in PBS, centrifuged for 10 min at 800 rpm three times, and resuspended in PBS to a final erythrocyte concentration of 4% (v/v). The hRBC suspension (100 μl) was added to a 96-well microtiter plate and incubated with different concentrations of mono-PEG-*St*AP3 in PBS. Controls of zero and 100% hemolysis consisted of hRBC suspended in PBS and 1% (w/w) Triton X-100, respectively. These suspensions were incubated with agitation for 3 h at 37 °C. The samples were centrifuged at 800 rpm for 10 min, and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at 550 nm.

3. Results and discussion

3.1. StAP3 PEGylation

Native *St*AP3 was incubated with mPEG-SVA (1:40 molar ratio) in 50 mM Tris–HCl pH 8, and the obtained conjugated species were analyzed by size exclusion chromatography after quenching the unreacted PEGylating agent with glycine (Fig. 1A). Four peaks were obtained, corresponding to molecular weights of approximately 90 kDa, 74 kDa, 60 kDa, and 45 kDa, which could be associated to the different species through gel electrophoresis assay (Fig. 1B). The analysis suggested that the pool of peak 1 is the result of a mixture of mainly tri- and di-PEGylated species to a lesser extent; peak 2 contains di-PEGylated species with a lower content of mono-PEGylated species; peak 3 consists in mono-PEGylated species; and peak 4 contains native *St*AP3 protein. The yield of purified mono-PEGylated fraction, as determined by SEC considering the ratio of the peak areas, was found to be 46.14% of the total protein, whereas a 5.06% remained as native protein. The relative abundance of di- and tri-PEGylated species could not be determined. The apparent molecular weight of the different PEGylated species obtained from size exclusion chromatography and gel electrophoresis (SDS-PAGE) is overestimated due to the retarded mobility of PEGylated proteins, which has been previously reported [56,57]. Moreover, it has also been reported that a 5 kDa-PEG-conjugated protein increases its apparent molecular weight in 15 kDa approximately [58]. This phenomenon has been attributed to the fact that the hydrodynamic volume for a PEG-conjugated protein results higher than the expected for a protein of similar molecular weight, due to the high hydrophilicity of the PEG unit [59,60].

Taking into account the results previously described we suggest that a pool of mono-PEG-*St*AP3 free of higher-degree PEGylated species and native *St*AP3 could be obtained from peak 3 as the most abundant fraction. However, given that *St*AP3 native protein contains 30 L-lysine units [27], many of which are sterically available for PEGylation, this pool is composed of different positional isomers where PEGylation occurred in different ε-amino functional groups besides α-amino terminal group. Although it has been reported that random PEGylation can lead to great loss of bioactivity [61,62], the simplicity of production of this mono-PEG-*St*AP3 pool led us to evaluate its biological properties in comparison to those of native *St*AP3.

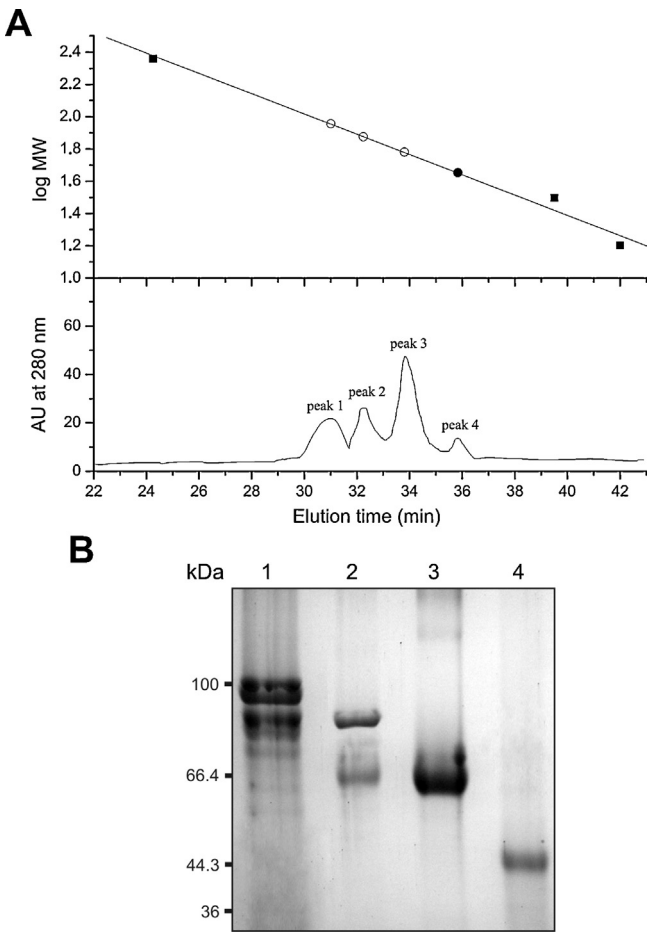

Fig. 1. Separation of PEG-*St*AP3 species. (A) Size exclusion chromatography. The upper panel shows the calibration curve as well as the elution times of the different peaks obtained after *St*AP3 PEGylation reaction. Elution times of proteins used for calibration are indicated by solid symbols, and elution times of *St*AP3-PEGylated species are indicated by open symbols. The lower panel contains the elution profile of PEG-*St*AP3 species from Superose 12 HR 10/30 column. Experiments were performed with 20 mM Tris–HCl pH 8, in the presence of 0.4% SDS (w/v) and 0.2 mM DTT. Absorption (arbitrary units, AU) was measured at 280 nm. (B) SDS-PAGE 12% analysis of fractions from the various column peaks. Each lane corresponds to a peak fraction as follows: Lane 1, peak 1; Lane 2, peak 2; Lane 3, peak 3; and Lane 4, peak 4. Gel was stained with Coomassie Brilliant Blue R250 coloidal.

3.2. Effect of mono-PEG-StAP3 on the viability of pathogen spores

In order to analyze the effect of PEGylation on the *in vitro* antimicrobial activity of *St*AP3, different amounts of mono-PEG-*St*AP3 fraction were incubated with spores of a potato pathogen, *F. solani*. Fig. 2 shows that mono-PEG-*St*AP3 was able to reduce *F. solani* spore germination in a dose-dependent manner. As shown in Table 1, the concentration of mono-PEG-*St*AP3 needed to reduce 50% spore germination (9 μg/ml) was almost 3-fold lower than the previously reported for native *St*AP3 (28 μg/ml) in the same incubation conditions [28]. These results denote that PEGylation increases

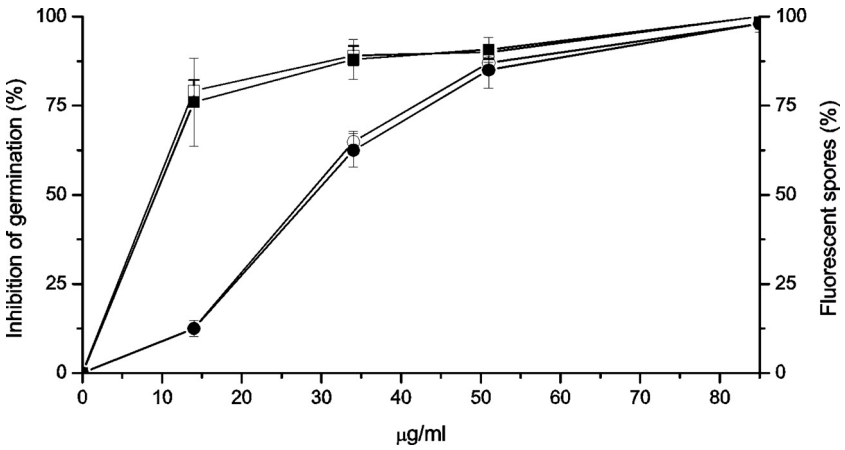

Fig. 2. Effect of mono-PEG-*St*AP3 fraction on spores of *F. solani*. *F. solani* spores (2.7×10^6 spores/ml) were incubated with different concentrations of mono-PEG-*St*AP3 (■) or native *St*AP3 (●) [28] for 16 h at 25 °C and 100% relative humidity. After incubation, the number of spores germinated was evaluated under a light microscope by counting on a Neubauer camera. Membrane permeabilization induced in spores after incubation with mono-PEG-*St*AP3 (□) or native *St*AP3 (○) [28] was detected by SYTOX Green uptake. Data reported are the means of three experiments and error bars represent the standard deviation.

cytotoxicity of *St*AP3 on spores of *F. solani*. This behavior has not been previously observed for plant proteins as far as we know, but a similar activity has also been reported by Lee et al. [38] for a recombinant antifungal insect protein. PEGylated recombinant tenecin 3 displayed a greater antifungal activity against *Candida albicans* than the native protein at the same dose, suggesting a higher interaction with fungi cell walls.

3.3. Effect of mono-PEG-StAP3 on pathogen plasma membrane integrity

We have previously reported that the antimicrobial activity of *St*APs is associated to the ability of these proteins to induce changes on the permeability of the microbial plasma membrane [28]. Based on this fact, we investigated whether PEGylation alters the capacity of *St*AP3 to permeabilize microbial plasma membranes. An assay based on the uptake of the fluorogenic dye SYTOX Green was used [63]. SYTOX Green can only penetrate cells that have compromised plasma membranes, and it fluoresces upon binding to DNA. This assay was performed incubating *F. solani* spores with different amounts of mono-PEG-*St*AP3 fraction in the same conditions reported for antifungal activity [26]. SYTOX Green was then added to evaluate membrane integrity by fluorescence quantification and microscopic examination. The fluorescent probe was incorporated into the microbial spores in the presence of different amounts of mono-PEG-*St*AP3 in a dose-dependent manner (Figs. 2 and 3). These results indicate that the PEGylated protein was able to induce membrane permeabilization in spores of *F. solani* in addition to cell death as native *St*AP3, and moreover, that PEGylation increases *St*AP3 cytotoxic activity and plasma membrane disruption ability.

Imura et al. have reported that the antimicrobial tachyplesin I peptides induce membrane disruption through the formation of toroidal pores. Moreover, it was found that PEGylation does not alter the basic mechanism of membrane permeabilization of the parent peptide [64]. On the other hand, we have previously reported that *St*Asp-PSI insertion into the membrane interface and its aggregation lead to the disruption of the membrane by a barrel-stave pore formation [31]. In addition, to determine if the mechanism of membrane permeabilization occurring for *St*AP3 is altered due to PEGylation further biophysical analyses such as differential scanning calorimetry, infrared spectroscopy, nuclear magnetic resonance and circular dichroism should be performed.

Table 1
In vitro antimicrobial activity of mono-PEG-*St*AP3 fraction.

Treatment	IC_{50} (µg/ml)[a]		
	F. solani	*B. cereus*	*E. coli*
Mono-PEG-*St*AP3	9 ± 1.2	13.2 ± 2.3	96.2 ± 5.3
Native *St*AP3 [30]	28 ± 0.7	56.2 ± 0.9	58.5 ± 1.5

[a] Means ± SD for at least three assays for each protein. The IC_{50} is the concentration of protein required to inhibit microbial growth by 50%.

3.4. Effect of mono-PEG-StAP3 on the viability of human bacterial pathogens and erythrocytes

Previously, we demonstrated that *St*APs are able to kill human pathogenic bacteria in a dose-dependent manner, but are not toxic to hRBC [30]. To determine whether PEGylation affects the bactericidal activity of *St*AP3, cultures of the Gram-positive bacterium *B. cereus* and Gram-negative *E. coli* were incubated with increasing concentrations of mono-PEG-*St*AP3 fraction for 6 h at 37 °C. Results obtained here show that mono-PEG-*St*AP3 was able to kill bacterial cells in a dose-dependent manner (Fig. 4). The antibacterial activity of mono-PEG-*St*AP3 was more effective against *B. cereus* than *E. coli*. The IC_{50} values were approximately 13.2 and 96.2 µg/ml mono-PEG-*St*AP3, respectively (Table 1). The IC_{50} values of mono-PEG-*St*AP3 were approximately 4 times lower on *B. cereus*, and approximately 1.6 times higher on *E. coli* compared to the *St*AP3 native form [30]. The greater susceptibility of mono-PEG-*St*AP3's antimicrobial effect on *B. cereus* compared to *E. coli* may be accounted for the bacterial cell membrane composition. Gram-negative bacteria have a cytoplasmatic membrane and an additional outer membrane that surrounds the cell, providing a barrier to mono-PEG-*St*AP3, whereas Gram-positive bacteria have only cytoplasmatic membrane [65,66]. In comparison, PEGylation of antimicrobial peptides tachyplesin I, nisin, α-defensin, and magainin with 5 kDa PEG chains led to a drastic decrease or even a complete loss of their antibacterial activities [64,67–69]. Nevertheless, the extent of the reduction in activity is strongly dependent on the peptide/protein evaluated. It is possible that mono-PEG-*St*AP3 decreases its ability to efficiently permeate the outer membrane due to a large steric hindrance of the PEG moiety, similar to that reported for PEGylated tachyplesin I and magainin [64,68].

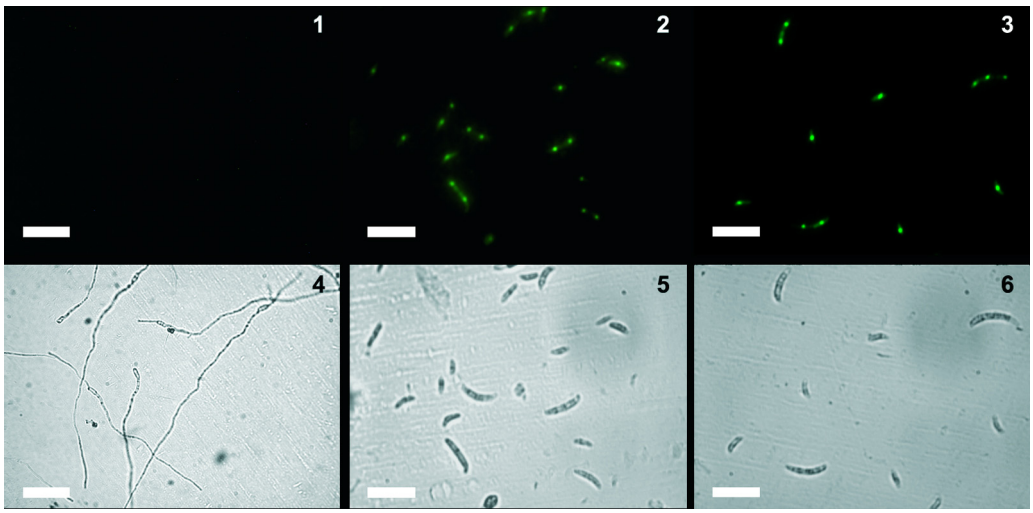

Fig. 3. Membrane permeabilization induced in spores of *F. solani* after incubating with mono-PEG-*St*AP3 fraction, detected by SYTOX Green uptake. Spores of *F. solani* were incubated with the amount of protein required to completely inhibit germination. After 30 min incubation in the presence of 0.5 μM SYTOX Green, the fluorescence was detected by fluorescence microscopy. Panels 1–3: fluorescence microscopy; panels 4–6, light-field microscopy. Panels 1 and 4, control in the presence of water; panels 2 and 5, 85 μg/ml of mono-PEG-*St*AP3; panels 3 and 6, 168 μg/ml of native *St*AP3 [28]. Bars, 15 μm.

Table 2
Effect of mono-PEG-*St*AP3 fraction on human red blood cells.

Treatment	Concentration (μg/ml)	Hemolysis of hRBC (%)
Triton X-100		100
Buffer		0.30 ± 0.28
Mono-PEG-*St*AP3	11.25	0.35 ± 0.03
	225	0.62 ± 0.20
	450	1.30 ± 0.50
	1125	2.50 ± 0.67
Native *St*AP3 [30]	11.25	0.70 ± 0.12
	225	1.20 ± 0.34
	450	2.00 ± 0.53
	1125	3.2 ± 0.33

Fresh human red blood cells (hRBC) were incubated with different concentrations of mono-PEG-*St*AP3 dissolved in PBS. Controls of zero and 100% hemolysis consisted of hRBC suspended in PBS and 1% (w/w) Triton X-100, respectively. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 550 nm. Values represent the media of three independent experiments (means ± SD).

Some antimicrobial peptides such as melittin, gramicidin S, CaLL, and surfactant protein B are also cytotoxic to mammalian cells, *e.g.* erythrocytes [70–73]. Therefore, only antimicrobial peptides/proteins and their derivatives with high antimicrobial activity and low cytotoxicity to the healthy eukaryotic cells are of practical interest. The hemolytic activity of mono-PEG-*St*AP3 fraction was tested *in vitro* on hRBC to investigate whether PEGylation affects the selective cytotoxicity of *St*AP3. As shown in Table 2, mono-PEG-*St*AP3 did not show significant hemolytic activity at all concentrations assayed.

Several reports relate the hemolytic activity of antimicrobial peptides with their capacity to strongly interact with either membranes, containing cholesterol or not [74,75]. As for the case of antimicrobial peptides unable to lyse red blood cells [76], the presence of cholesterol into the LUVs membranes strongly diminishes the capacity of *St*Asp-PSI to produce leakage at all concentration assayed [29]. The presence of cholesterol in the membranes causes a reduction in the density of hydrophilic head groups at the interfacial region of the bilayer and an increase in the packaging of the phospholipid tails in the middle of the bilayer [77]. Despite this, the high rate of hydration of phospholipid head group at the interface bilayer region found in cholesterol-rich membranes does not allow the hydrophilic mono-PEG-*St*AP3 to

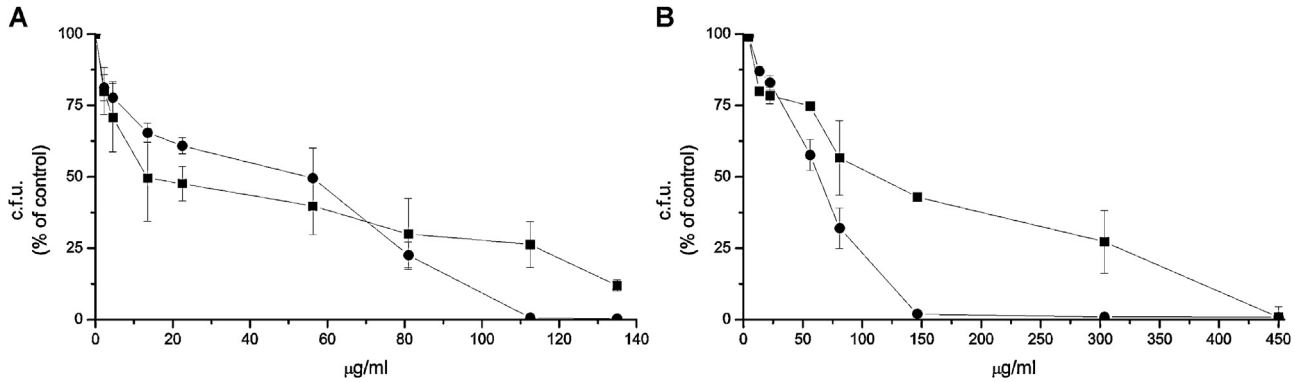

Fig. 4. Bactericidal activity of mono-PEG-*St*AP3 fraction on human pathogens. Different amounts of mono-PEG-*St*AP3 (■) or native *St*AP3 (●) [30] were incubated with (A) *B. cereus* or (B) *E. coli* cells (10^4 c.f.u./ml) in 100 μl of sterile PBS for 6 h at 37 °C with rocking. c.f.u. were counted from the number of colonies obtained on buffer-treated control plates as compared to the number of colonies from protein-treated samples. Results are representative of four separate experiments and error bars represent the standard deviation.

display any hemolytic activity, as well as the more hydrophobic native *St*AP3. This result suggests that PEGylation does not affect the selective cytotoxic activity reported for native *St*AP3 [30,78]. Future assays using calorimetry, infrared and NMR should be performed to corroborate this hypothesis.

4. Conclusions

In this work a covalent modification of *St*AP3 by PEGylation was carried out. By size exclusion chromatography it was possible to isolate a main fraction of mono-PEGylated species. The cytotoxic activity of this fraction was examined and compared to that of native protein. It is well known that the *in vitro* activity of proteins decreases with PEGylation [39]. However, the mono-PEG-*St*AP3 fraction displayed an enhanced *in vitro* antifungal activity respect native *St*AP3 toward *F. solani* spores. This is the first time that a PEGylated plant protein was found to present a higher cytotoxic activity against a pathogen than the native protein. This was ascribed to a higher interaction between fungi cell walls and the conjugated protein. On the other hand, PEGylation was found to reduce antibacterial activity toward Gram-negative bacterium, probably because outer membrane mainly acts as a mechanism of antimicrobial resistance. In addition, PEGylation did not affect the selective cytotoxicity of *St*AP3, since no hemolytic activity was observed. However, *in vivo* assays involving native *St*AP3 and PEGylated forms are being carried out to test them as new agents in therapy of infectious diseases and cancer, and will be published elsewhere.

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