Evaluation of in vitro cytotoxic activity of mono-PEGylated StAP3 (Solanum tuberosum aspartic protease 3) forms

Fernando Muñoz a,1, Pablo C. Caracciolo b,1, Gustavo Daleo a, Gustavo A. Abraham b,2, M. Gabriela Guevara a,*,2

a Plant Biochemistry Laboratory, Biological Research Institute, IIB (UNMdP-CONICET), Funes 3250, 7600, Mar del Plata, Argentina
b Instituto de Investigaciones en Ciencia y Tecnología de Materiales, INTEMA (UNMdP-CONICET), Av. Juan B. Justo 4302, 7600, Mar del Plata, Argentina

A R T I C L E   I N F O

Article history:
Available online 27 May 2014

Keywords:
PEGylation
Plant aspartic protease
Antimicrobial protein
Selective cytotoxicity

A B S T R A C T

StAP3 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, StAP3 could be a promising and potential drug candidate for future therapies. In this work, the improvement of the performance of StAP3 was achieved by means of a modification with PEG. The separation of a mono-PEGylated StAP3 fraction was easily performed by gel filtration chromatography. The mono-PEGylated StAP3 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 StAP3, since no hemolytic activity was observed.

© 2014 Published by Elsevier B.V. Open access under CC BY-NC-ND license.

1. Introduction

Antimicrobial proteins and peptides (AMPs) 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 AMPs 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 (StAPs) toward plant pathogens [26–28]. Our results show that potato aspartic proteases (StAPs) and their swaposin domain (StAsp-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 StAsp-PSI/StAPs 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: AMPs, 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 sulphate polyacrylamide gel electrophoresis; StAP3, Solanum tuberosum aspartic protease 3; StAsp-PSI, plant-specific insert of potato aspartic protease.

* Corresponding author at: 3250 Funes, 4th Floor, Mar del Plata 7600, Argentina.
Tel.: +54 223 4753030 ext. 14; fax: +54 223 4724143.
E-mail address: gguevara@mdp.edu.ar (M.G. Guevara).
1 These authors contributed equally to this paper.
2 These authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.btre.2014.05.007
2215-017X © 2014 Published by Elsevier B.V. Open access under CC BY-NC-ND license.
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 StAP3 was achieved by means of a covalent modification with PEG. The separation of a mono-PEGylated StAP3 fraction could easily be performed by gel filtration chromatography. The mono-PEGylated StAP3 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 StAP3, 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 (mPEG-SVA, 5 kDa) was purchased from Laysan Bio Inc. (Arab, AL, USA). Sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) were added. To quantify the effect of mono-PEG-StAP3 on the germination of F. solani spores, in vitro bioassays were performed as described by Guevara et al. [53]. A solution of purified StAP3 (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-StAP3 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 StAP3 (45 kDa), glyceralddehyde-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-StAP3 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-StAP3 on the germination of F. solani, in vitro bioassays were performed as described by Guevara et al. [26]. To quantify the effect of mono-PEG-StAP3 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 10^8 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-StAP3 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\textsubscript{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-StAP3, 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-StAP3 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 StAP3 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 StAP3 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-StAP3 free of higher-degree PEGylated species and native StAP3 could be obtained from peak 3 as the most abundant fraction. However, given that StAP3 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-StAP3 pool led us to evaluate its biological properties in comparison to those of native StAP3.

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 StAP3, different amounts of mono-PEG-StAP3 fraction were incubated with spores of a potato pathogen, F. solani. Fig. 2 shows that mono-PEG-StAP3 was able to reduce F. solani spore germination in a dose-dependent manner. As shown in Table 1, the concentration of mono-PEG-StAP3 needed to reduce 50% spore germination (9 μg/ml) was almost 3-fold lower than the previously reported for native StAP3 (28 μg/ml) in the same incubation conditions [28]. These results denote that PEGylation increases
cytotoxicity of STAP3 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 STAPs 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 STAP3 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-STAP3 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-STAP3 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 STAP3, and moreover, that PEGylation increases STAP3 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 STasp-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 STAP3 is altered due to PEGylation further biophysical analyses such as differential scanning calorimetry, infrared spectroscopy, nuclear magnetic resonance and circular dichroism should be performed.

### 3.4. Effect of mono-PEG-STAP3 on the viability of human bacterial pathogens and erythrocytes

Previously, we demonstrated that STAPs 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 STAP3, cultures of the Gram-positive bacterium *B. cereus* and Gram-negative *E. coli* were incubated with increasing concentrations of mono-PEG-STAP3 fraction for 6 h at 37°C. Results obtained here show that mono-PEG-STAP3 was able to kill bacterial cells in a dose-dependent manner (Fig. 4). The antibacterial activity of mono-PEG-STAP3 was more effective against *B. cereus* than *E. coli*. The IC50 values were approximately 13.2 and 96.2 µg/ml mono-PEG-STAP3, respectively (Table 1). The IC50 values of mono-PEG-STAP3 were approximately 4 times lower on *B. cereus*, and approximately 1.6 times higher on *E. coli* compared to the STAP3 native form [30]. The greater susceptibility of mono-PEG-STAP3’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-STAP3, 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-STAP3 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. 2. Effect of mono-PEG-STAP3 fraction on spores of F. solani. F. solani spores (2.7 × 10^6 spores/ml) were incubated with different concentrations of mono-PEG-STAP3 (■) or native STAP3 (●) [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-STAP3 (○) or native STAP3 (□) [28] was detected by SYTOX Green uptake. Data reported are the means of three experiments and error bars represent the standard deviation.](image)

### Table 1

*In vitro* antimicrobial activity of mono-PEG-STAP3 fraction.

| Treatment          | IC50 (µg/ml) ± SD | *F. solani* | *B. cereus* | *E. coli* |
|--------------------|------------------|-------------|-------------|-----------|
| Mono-PEG-STAP3     | 9 ± 1.2          | 13.2 ± 2.3  | 96.2 ± 5.3  |
| Native STAP3 [30]  | 28 ± 0.7         | 56.2 ± 0.9  | 58.5 ± 1.5  |

* Means ± SD for at least three assays for each protein. The IC50 is the concentration of protein required to inhibit microbial growth by 50%.
Membrane permeabilization induced in spores of *F. solani* after incubating with mono-PEG-StAP3 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-StAP3; panels 3 and 6, 168 μg/ml of native StAP3 [28]. Bars, 15 μm.

Table 2

| Treatment            | Concentration (μg/ml) | Hemolysis of hRBC (%) |
|----------------------|-----------------------|-----------------------|
| Triton X-100         | 100                   | 0.30 ± 0.28           |
| Buffer               | 11.25                 | 0.35 ± 0.03           |
| Mono-PEG-StAP3       | 225                   | 0.62 ± 0.20           |
|                      | 450                   | 1.30 ± 0.50           |
|                      | 1125                  | 2.50 ± 0.67           |
| Native StAP3 [30]    | 1125                  | 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-StAP3 dissolved in PBS. Controls of zero and 100% hemolysis consisted of hRBC suspended in PBS and 1% (w/v) 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-StAP3 fraction was tested in vitro on hRBC to investigate whether PEGylation affects the selective cytotoxicity of StAP3. As shown in Table 2, mono-PEG-StAP3 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 StAsp-PS1 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-StAP3 to

![Fig. 3](image1.png)

**Fig. 3.** Membrane permeabilization induced in spores of *F. solani* after incubating with mono-PEG-StAP3 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-StAP3; panels 3 and 6, 168 μg/ml of native StAP3 [28]. Bars, 15 μm.

![Fig. 4](image2.png)

**Fig. 4.** Bactericidal activity of mono-PEG-StAP3 fraction on human pathogens. Different amounts of mono-PEG-StAP3 (■) or native StAP3 (●) [30] were incubated with (A) *B. cereus* or (B) *E. coli* cells (10⁶ 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 StAP3. This result suggests that PEGylation does not affect the selective cytotoxic activity reported for native StAP3 [30,38]. Future assays using calorimetry, infrared and NMR should be performed to corroborate this hypothesis.

4. Conclusions

In this work a covalent modification of StAP3 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-StAP3 fraction displayed an enhanced in vitro antifungal activity respect native StAP3 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 fungus cell walls and the conjugated protein. On the other hand, PEGylation was found to reduce antibacterial activity toward Gram-negative bacteria, probably because outer membrane mainly acts as a mechanism of antimicrobial resistance. In addition, PEGylation did not affect the selective cytotoxicity of StAP3, since no hemolytic activity was observed. However, in vivo assays involving native StAP3 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.

Acknowledgements

This work was supported by National Scientific and Technical Research Council (CONICET) grant to M.G.G. and G.A.A.; Scientific Research Commission of the Province of Buenos Aires (CIC) grant to M.G.G.; University of Mar del Plata grant to M.G.G. and G.A.A.; and National Agency for Scientific and Technological Promotion grant to G.A.A. All authors are grateful for the support in microbiological assays to Dr. Abaurrea R., Dr. Scandogliero E. and Bustos E. of BAS (Laboratorio de Análisis Clínicos y Bacteriológicos, Mar del Plata, Argentina). F.M. is fellow of CONICET; G.D. is a researcher of CIC; and M.G.G., P.C.C. and G.A.A. are researchers of CONICET.

References

[1] G. Bell, P.H. Gouyon, Arming the enemy: the evolution of resistance to self-proteins, Microbiology 149 (2003) 1367–1375.
[2] M.M. Cowan, Plant products as antimicrobial agents, Clin. Microbiol. Rev. 12 (1999) 564–582.
[3] R.E. Hancock, D.S. Chapple, Peptide antibiotics, Antimicrob. Agents Chemother. 43 (1999) 1317–1323.
[4] R.E. Hancock, M.C. Scott, The role of antimicrobial peptides in animal defenses, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 8856–8861.
[5] O. Levy, Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes, J. Leukoc. Biol. 76 (2004) 909–925.
[6] K.V. Reddy, R.D. Yedery, C. Aranha, Antimicrobial peptides: premises and promises, Int. J. Antimicrob. Agents 24 (2004) 536–547.
[7] A.J. De Luca, Antifungal peptides: potential candidates for the treatment of fungal infections, Expert Opin. Investig. Drugs 9 (2000) 273–299.
[8] S. Chernyh, S.J. Kim, G. Bekker, V.A. Pleskach, V.G. Chinchar, J. Wang, L. Rollins-Smith, Inactivation of potato aspartic proteases (StAsp) involves membrane permeabilization, Microbiology 152 (2006) 2039–2047.
[9] F. Muñoz, M.F. Palomares-Jerez, G. Daleo, M.G. Guevara, Antimicrobial activity of potato aspartic proteases (StAsp) involves membrane permeabilization, Microbiology 152 (2006) 2039–2047.
[10] J.R. Mendieta, M.R. Pagano, F.F. Munoz, G.R. Daleo, Molecular cloning of a potato leaf cDNA encoding an aspartic protease (StAsp) and its expression after P. infestans infection, Plant Physiol. Biochem. 43 (2005) 882–889.
[11] M.G. Guevara, C. Almeida, J.R. Mendieta, C.J. Faro, S. Lee-Huang, Production of antiviral and antimicrobial proteins MAP30 and GAP31 in cucurbit plants using the virus plant vector ZYMV-AG1, Biochem. Biophys. Res. Commun. 292 (2002) 441–448.
[12] T. Chua, T.B. Ng, Smilaxin, a novel protein with immunostimulatory, antiprofibrin and HIV-1 reverse transcriptase inhibitory activities from fresh Smilax glabra rhizomes, Biochem. Biophys. Res. Commun. 340 (2005) 118–124.
[13] E. González De Mejía, V. Piscicu, Lectins as bioactive plant proteins: a potential in cancer treatment, Crit. Rev. Food Sci. Nutr. 45 (2005).
[14] P. Lindholm, U. Göransson, S. Johansson, P. Claeson, J. Gullbo, R. Lasron, L. Bohlin, A. Backlund, Cyclotides: a novel type of cytotoxic agents, Mol. Cancer Ther. 1 (2002) 365–369.
[15] P.H. Ngai, T.B. Ng, Phaseococcin, an antifungal protein with antiproliferative and anti-HIV-1 reverse transcriptase activities from small scarlet runner beans, Biochem. Cell. Biol. 83 (2005) 312–322.
[16] V.X. Wang, J. Jacob, P.T. Wingfield, I. Palmer, S.J. Stahl, J.D. Kaufman, P.L. Huang, P.L. Huang, S. Lee-Huang, D.A. Torchia, Anti-HIV and anti-tumor protein MAP30, a 30kDa single-strand type-I RIP, shares similar secondary structure and beta-sheet topology with the A chain of ricin, a type-II RIP, Protein Sci. 9 (2000) 138–144.
[17] M. Guevara, C. Oliva, M. Huarte, G. Daleo, An aspartic protease with antimicrobial activity is induced after infection and wounding in intercellular fluids of potato tubers, Eur. J. Plant Pathol. 108 (2002) 331–337.
[18] M.G. Guevara, C. Almeida, J.R. Mendieta, C.J. Faro, S. Lee-Huang, Molecular cloning of a potato leaf cDNA encoding an aspartic protease (StAsp) and its expression after P. infestans infection, Plant Physiol. Biochem. 43 (2005) 882–889.
[19] J.R. Mendieta, M.R. Pagano, F.F. Munoz, G.R. Daleo, M.G. Guevara, Antimicrobial activity of potato aspartic proteases (StAsp) involves membrane permeabilization, Microbiology 152 (2006) 2039–2047.
[20] F. Muñoz, M.F. Palomares-Jerez, G. Daleo, J. Villalain, M.G. Guevara, Cholesterol and membrane phospholipid compositions modulate the leakage capacity of the swaposin domain from a potato aspartic protease (StAsp-Psi), Biochem. Biophys. Acta 1811 (2011) 1038–1044.
[21] F.F. Muñoz, J.R. Mendieta, M.R. Pagano, R.A. Paggi, G.R. Daleo, M.G. Guevara, The swaposin-like domain of potato aspartic protease (StAsp-Psi) exerts antimicrobial activity on plant and human pathogens, Peptides 31 (2010) 777–785.
[22] F. Muñoz, M.F. Palomares-Jerez, G. Daleo, J. Villalain, M.G. Guevara, Possible mechanism of structural transformations induced by StAsp-Psi in lipid membranes, Biochim. Biophys. Acta 1838 (2014) 339–347.
[23] N.V. Katre, The conjugation of proteins with polyethylene glycol and other polymers: altering properties of asparaginase by conjugation to linear and branched monomethoxy poly(ethylene glycol), J. Control. Release 40 (1996) 199–209.
[24] F.M. Veronese, G. Pasut, PEGylation, successful approach to drug delivery, Drug Dev. Ind. Pharm. 28 (2002) 437–459.
[25] F. Muñoz, G. Pasut, PEGylation, successful approach to drug delivery, Drug Dev. Ind. Pharm. 30 (2004) 601–626.
[26] F.M. Veronese, G. Pasut, PEGylation, successful approach to drug delivery, Drug Dev. Ind. Pharm. 30 (2004) 1529–1551.
[27] F. Muñoz, F. Ruano-Rubio, F. Fernandez-American, N. Fernandez-American, A. Varela, A. Peralta, R.A. Gallegos, H. Díaz, R. Vázquez, M. de la Torre, P. Verissimo, E.V. Pires, G.R. Daleo, Antimicrobial activity of plant and human pathogens, Peptides 31 (2010) 777–785.
M. Li, Y. Chen, Z. Liu, F. Shen, X. Bian, Y. Meng, Anti-tumor activity and immuno-logical modification of ribosome-inactivating protein (RIP) from Momordica charantia by covalent attachment of polyethylene glycol, Acta Biochim. Biophys. Sin. 41 (2009) 792–799.

Y. Meng, S. Liu, J. Li, X. Zhao, Preparation of an antioxidant and antiviral agent: chemical modification of alpha-MMC and MAP30 from Momordica charantia L. with covalent conjugation of polyethylene glycol, Int. J. Nanomed. 7 (2012) 3133–3142.

T. Ueno, Y. Kodera, Y. Kimoto, K. Sakurai, M. Hiroto, A. Matsushima, H. Nishimura, S. Takai, Y. Inada, Polyethylene glycol-modified polyolefin monomer (PWM) as a potential non-immunogenic stimulator of lymphokine-activated killer cells, J. Biomat. Sci. Polym. Ed. 7 (1996) 753–758.

K. Ibeda, E. Schiltz, T. Fujii, M. Takahashi, K. Mitsuji, Y. Kodera, A. Matsushima, Y. Inada, G.E. Schulz, H. Nishimura, Phenylalanine ammonia-lyase modified with polyethylene glycol: potential therapeutic agent for phenylketonuria, Amino Acids 29 (2005) 283–287.

J. Matousek, J. Matousek, Plant ribonucleases and nucleases as antiproiferative agents targeting human tumors growing in mice, Recent Pat. DNA Gene Seq. 4 (2010) 29–39.

J. Matousek, T. Podzimek, P. Pocuckova, J. Stehlik, J. Skvor, J. Soucek, Antitumor effects and cytotoxicity of recombinant plant nucleases, Oncol. Res. 18 (2000) 163–171.

S. Podarzak, R. Averineni, M. Alqahtani, O. Perumal, Synthesis of novel biodegradable methoxy poly(ethylene glycol)-zein micelles for effective delivery of curcumin, Mol. Pharm. 9 (2012) 2778–2786.

M.G. Guevara, G.R. Daleo, P. Hrelia, M.G. Guevara, Cytotoxic effect of potato aspartic proteases (StAPs) on Jurkat T cells, Fitoterapia 81 (2010) 321–326.

V. Neuhoff, N. Arolf, D. Taube, W. Erhardt, Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250, Electrophoresis 9 (1988) 255–262.

M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

O.B. Kinstler, D.N. Brems, S.L. Lauren, A.G. Paige, J.B. Hamburger, M.J. Treuheit, Characterization and stability of N-terminally PEGylated rhG-CSF, Pharm. Res. 13 (1996) 996–1002.

M.M. Kurfurst, Detection and molecular weight determination of polyeth-yylene glycol-modified birudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Anal. Biochem. 200 (1992) 244–248.

D. da Silva Freitas, J. Abrahao-Neto, Biochemical and biophysical characterization of lysozyme modified by PEGylation, Int. J. Pharm. 392 (2010) 111–117.

P. Balon, W. Berthold, PEG-conjugated pharmaceutical proteins, Pharm. Sci. Technol. Today 1 (1998) 352–356.

F. Caliceti, F.M. Veronese, Pharmacokinetic and biodistribution properties of polyethylene glycol-protein conjugates, Adv. Drug Deliv. Rev. 55 (2003) 1261–1277.

D. Collen, P. Simanne, E. Demarsin, H. Moreau, M. De Maeyer, L. Jespers, Y. Laroche, F. Van de Werf, Polyethylene glycol-derivatized cysteine-substitution variant of recombinant staphylokinase for single-bolus treatment of acute myocardial infarction, Circulation 102 (2000) 1766–1772.

M. Planas, E. Cres, R.A. Rodriguez, R. Ferre, E. Bardaji, Solid-phase synthesis of new peptide–enzyme hybrids from N-TCP amino acids, Tetrahedron Lett. 43 (2002) 4431–4434.

K. Thevis, T. Verras, W.F. Broekhout, Permeabilization of fungal membranes by plant defensins inhibits fungal growth, Appl. Environ. Microbiol. 65 (1999) 5451–5458.

Y. Inatra, M. Nishida, Y. Ogawa, Y. Takakura, K. Matsuuzaki, Action mechanism of tachyplesin I and effects of PEGylation, Biochim. Biophys. Acta. Int. J. 20 378 (2007) 1160–1169.

R.M. Epand, R.F. Epand, Lipid domains in bacterial membranes and the action of antimicrobial agents, Biochim. Biophys. Acta. Int. J. 20 378 (2007) 289–294.

H. Nikiado, Outer membrane barrier as a mechanism of antimicrobial resis-tance, Antimicrob. Agents Chemother. 33 (1989) 1831–1836.

A. Giusotto, M. Pozzohno, M. Canevati, R. Manganelli, M. Scarin, F.M. Veronese, PEGylation of the antimicrobial peptide nisin A: problems and perspectives, Farmaco 58 (2003) 45–50.

Y. Inatra, M. Nishida, K. Matsuuzaki, Action mechanism of PEGylated magainin 2 analogue peptide, Biochim. Biophys. Acta. Int. J. 20 378 (2007) 2578–2585.

Z. Wu, X. Li, B. Ericksen, E. de Leeuw, G. Zou, P. Zeng, C. Xie, C. Li, J. Lukowicki, W.Y. Lu, W. Lu, Impact of pro segments on the folding and function of human polyglycine alpha-defensins, J. Mol. Biol. 368 (2007) 537–549.

N. Asthana, S.P. Yadav, J.K. Ghosh, Dissemination of antibacterial and toxic activity of melittin: a leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity, J. Biol. Chem. 279 (2004) 55042–55050.

M.A. Fox, J.I. Thwaites, D.O. Utao, T.P. Atkins, H.S. Atkins, Design and charac-terization of novel hybrid antimicrobial peptides based on cecropin A, LL-37 and magainin II, Peptides 33 (2012) 197–205.

L.H. Kondev, J.S. Farmer, D.S. Wishart, R.E. Hancock, R.S. Hodges, Gram-icin S is active against both gram-positive and gram-negative bacteria, Int. J. Pept. Res. Protein Res. 47 (1996) 460–466.

M.A. Ryan, H.T. Akinbi, A.C. Gerry, J. Perez-Gil, H. Wu, F.X. McCormack, T.E. Weaver, Antimicrobial activity of native and synthetic surfactant protein B peptides, J. Immunol. 176 (2006) 416–425.

T. Abraham, R.N. Lewis, R.S. Hodges, R.N. McElhaney, Isothermal titration calorimetry studies of the binding of the antimicrobial peptide gramicidin S to phospholipid bilayer membranes, Biochemistry 44 (2005) 11279–11285.

R.M. Verly, M.A. Rodriguez, K.R. Daghastani, A.M. Denadai, I.M. Cuccovia, C. Bloch Jr., F. Frezard, M.M. Santoro, D. Pilo-Veloso, M.P. Benqueira, Effect of cholesterol on the interaction of the amphipathic antimicrobial peptide Dd K with liposomes, Peptides 29 (2008) 15–24.

W.M. Yau, W.C. Wimley, K. Gawrisch, S.H. White, The preference of tryptophan for membrane interfaces, Biochemistry 37 (1998) 14713–14718.

P. Jedlovszy, M. Mezei, Effect of cholesterol on the properties of phospholipid membranes. 1. Structural features, J. Phys. Chem. B 20 (2003) 5311–5321.

J.R. Mendieta, C. Fimognari, G.R. Daleo, F. Hrelia, M.G. Guevara, Cytotoxic effect of potato aspartic proteases (SAPs) on Jurkat T cells, Fitoterapia 81 (2010) 329–335.