RESEARCH

N-terminal peptide (1-92) of Sticholysin II generated by the cleavage with cyanogen bromide interacts with lipid bilayer but does not promote pore formation

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ABSTRACT

Sticholysin II (St II) is a pore-forming toxin produced by the sea anemone *Stichodactyla helianthus* with a potent hemolytic activity. For an insight into the role of different St II sequence fragments in their interaction with membranes, we carried out protein digestion with cyanogen bromide. This treatment rendered three peptides, one of them (P3) containing the St II N-terminal sequence (amino acids 1 to 92). Neither P2 nor P1 (amino acids 102 to 130, and 131 to 157, respectively) exhibit measurable hemolytic activity or interfere with the native St II function. On the other hand, P3 is able to inhibit toxin hemolytic activity. These differences are discussed in terms of their relative capacity to interact with lipidic membranes.

Keywords: Sticholysin, Pore-forming toxin, actinoporin, CNBr-derived peptides

Biotecnología Aplicada 2006;23:300-304

RESUMEN

El péptido N-teminal (1-92) de Sticholisina II generado por la hidrólisis con bromuro de cianógeno interactúa con la bicapa lipídica pero no promueve la formación del poro. Sticholysina II (St II) es una proteína formadora de poros producida por la anémona *Stichodactyla helianthus* que se caracteriza por su potente actividad hemolítica. Con el fin de profundizar en el papel de los diferentes fragmentos de la secuencia de St II en su interacción con las membranas, se digirió la proteína con bromuro de cianógeno. Se obtuvieron tres péptidos, uno de los cuales (P3) contiene la secuencia N-terminal de St II (aminoácidos 1-92). Los otros dos péptidos: P2 y P1 (aminoácidos 102-130, y 131-157, respectivamente) no presentaron actividad hemolítica medible ni interfirieron con la función de la toxina nativa. Por otra parte, P3 inhibió la actividad hemolítica de la toxina. Estas diferencias se discuten en términos de la capacidad de los tres péptidos para interactuar con las membranas lipídicas.

Palabras clave: Sticholisinas, toxina formadora de poros, actinoporina, péptidos

*I*ntroduction

Sticholysin II (St II; SwissProt accession number: P077845) is a basic cytolysin purified from the Caribbean Sea anemone *Stichodactyla helianthus*, that efficiently permeabilizes natural and model membranes by forming tetrameric pores with an internal radius of about 1 nm [1-4]. The structure of this protein has been recently resolved by X-ray crystallography and electron microscopy [5]. It is based on a β-sandwich fold composed of ten β-strands, flanked on each side by two short α-helices. St II is the most active cytolysin produced by *Stichodactyla helianthus* measured in terms of erythrocyte internal potassium exit and hemolysis rates [6].

A toroidal pore has been suggested for actinoporins [7, 8] consisting of four amphipathic helices (one per molecule) tilted at an angle of approximately 23° from the normal membrane [9]. Recently, it has been demonstrated that the N-terminal segment plays an essential role in pore formation, at least for equinatoxin II (EqT II) from *Actinia equina*, the most extensively studied actinoporin [9, 10]. N terminal truncation by mutagenesis of EqT II showed that the gradual deletion

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of the first 5 or 10 residues reduces the specific hemolytic activity to 89 and 31%, respectively. In addition, the deletion of the first 33 residues resulted in a mutant that is completely unable to produce hemolysis despite its enhanced binding to lipid membranes [11]. Recently, Hong et. al [10] showed that binding of EqT II to the lipid membrane is a two-step process, separately mediated by two regions of the molecule. An exposed aromatic cluster is involved in the initial attachment, a prerequisite for the next step that is promoted by the N-terminal amphiphilic helix, which translocates into the lipid phase.

For an insight into the importance of different segments in the interaction of St II with membranes, we carried out the chemical hydrolysis of this protein using cyanogen bromide (CNBr), which specifically cleaves peptide bonds at the carboxyl groups of the methionine residue [12]. This treatment yielded three peptides, one of them containing the N-terminal fragment (P3). The study of the functional properties of these peptides showed that none of them exhibited hemolytic activity but P3 was able to impair native St II function.

Materials and methods

Hydropathy profile of St II

The plot of the protein was obtained employing the algorithm of Kyte and Doolittle [13] with a window of 19 residues, available in http://bioinformatics.weizmann.ac.il/hyd-bin/plot_hydroph.pl.

Protein hydrolysis and peptide purification

St II was purified as previously described [1]. Protein concentration was determined by the mixture absorption coefficient of 1.87 mL/mg • cm at 280 nm [1]. The concentration of the CNBr peptides was determined by amino acid analysis [14].

St II was purified by high performance liquid chromatography (HPLC) (Pharmacia-LKB, Sweden) on a reversed phase column C4 (4.6 • 250 mm, J.T. Baker, USA). Elution was performed with an acetonitrile gradient from 25% to 85% in water containing 0.1% trifluoroacetic acid (TFA) with a flow rate of 0.8 mL/min at 37 °C for 60 min. Protein peaks were detected by absorbance measurements at 226 nm.

Approximately four milligrams of pure St II were resuspended in 2 mL of TFA (70% v/v), and a 300-fold molar excess of CNBr over methionine residues was included while stirring and it was incubated in the dark under oxygen-free N_2 for 20 hours. The mixture was diluted with 15 volumes of chilled water and freeze-dried. For the complete removal of acid and by-products, freeze-drying was repeated after more water was added. Finally, the sample was resuspended in 6M guanidine hydrochloride and the resulting peptides were purified by reverse phase-HPLC (rp-HPLC) on a C4 column at 37 °C. Elution was performed using an acetonitrile gradient from 0% to 80% in water containing 0.1% TFA for 180 minutes at a flow rate of 0.8 $mL.min^{-1}$. Peptide peaks were detected by absorbance measurements at 214 nm. The pure peptides were freeze-dried in a Speed-Vac centrifuge (Savant, France) and freeze-drying was repeated after two other inclusions of water.

Identification and characterization of the peptides

N-terminal sequence

N-terminal sequencing of peptides was made by automated Edman degradation using a dual phase sequencer model 810/816 (Knauer, Germany) equipped with an on-line phenylthiohydantoin amino acid analyzer.

Amino acid analysis

Amino acid analysis of the purified peptides was performed after acid hydrolysis with 6M HCl at 110 °C for 24 hours according to Allen's method [14]. The peptides were quantified with an internal standard (nor-Leucine). The chromatographic procedure was carried out with an automatic analyzer Alpha Plus 4151 (Pharmacia-LKB, Sweden) operated according to the manufacturer's instructions. The dried CNBr peptides were dissolved in water and one aliquot was quantified by amino acid analysis and the rest was used in the biological assay.

Mass spectrometry

Molecular weights of the peptides were determined by Electrospray Ionization-Mass Spectrometry (ESI-MS). Measurements were carried out on an API III triple quadrupole MS system (PE Sciex Instruments, Canada) using nanospray needles Econo12 (1+/-05 µm) (New Objective pico Tip™, USA). The instrument was calibrated with polypropylenglycol (MW of 425, 1000 and 2000) solutions at concentrations of 0.4, 0.1 and 0.014 g/L, respectively. Samples were infused in an aqueous solution of 50% acetonitrile and 0.5% formic acid.

Hemolytic activity assay

Hemolysis was tested by measuring the decrease in turbidity of a human red blood cell suspension at 650 nm with a microplate reader (Molecular Devices, USA) as described by Tejuca et al. [4]. Human red blood cells were prepared from total blood obtained intravenously from healthy volunteers, by washing twice (10 minutes centrifugation at 700g, at room temperature) in 10 mM Tris-HCl, 140 mM NaCl, pH 7.5 (TBS) containing 0.1 mg/mL Prionex, which strongly reduces unspecific binding of proteins to plastic [15]. In each well, a fixed concentration of St II was present, in a final volume of 100 mL of TBS with or without variable amounts of each peptide. The reaction was started by adding 100 mL of red blood cells at a titer corresponding to an initial A_{650} value of around 0.1. The microplate was shaken and read every 8 seconds for the first 30 minutes.

Vesicle preparation

Egg phosphatidylcholine and brain sphingomyelin were purchased from Avanti Polar Lipids (USA). Phosphatidylcholine was dissolved in chloroform, sphingomyelin in chloroform:ethanol (4:1, V:V), and aliquots mixed to attain a 50:50 molar ratio. The lipid mixture was thoroughly evaporated under vacuum until a dry film was obtained on the bottom of a round flask. Multilamellar vesicles were prepared by hydration of the lipid film with TBS and intensive

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vortexing. Small unilamellar vesicles were obtained by sonication of multilamellar vesicles (2 minutes total time, cycles of 15 s irradiation and 15s break) with a Soniprep ultrasonicator (MSE, UK)

Fluorescence assays

Fluorescence was measured in a spectrofluorimeter Shimadzu RF-540 (Japan) using a 1 cm path length quartz cuvette. Slit widths of a nominal band pass of 10 nm were used for both excitation and emission beams. Intrinsic fluorescence emission spectra of the peptides were recorded from 310 to 450 nm after excitation at 295 nm (16). Background intensities measured in samples non-containing the peptide were always subtracted.

In order to obtain the signal exclusively due to tryptophanyl residues (Trp), peptide samples were excited at 295 nm (16) and their fluorescence intensity measured at 350 nm, corresponding to the emission maximum of the peptides. Increasing amounts of lipid vesicles were added to the peptide solutions, and measured the changes in the fluorescence intensity. Dilution and scattering effects associated to the added vesicles were corrected. Selective quenching of the fluorescence emitted by Trp moieties was performed by adding increasing acrylamide concentrations (16). The quenching constant (K_{sv}) was calculated according the Stern-Volmer equation described by Lakowicz (16).

Circular dichroism

UV circular dichroism (CD) spectra of St II derived peptides were recorded on a CD6 Jobin Yvon spectropolarimeter (Division Instruments S.A., USA). Spectra were base-line corrected by using control samples of similarly prepared solutions. CD spectra of the peptides were measured in the far-UV region (190-250 nm) in 1 mm path length quartz cuvettes.

Results and discussion

St II is essentially a hydrophilic protein (GRAVY -0.233) however its N-terminal sequence presents significant hydrophobic properties (Figure 1). The profile displays a stretch with a high hydropathy index according to the scale of Kyte and Doolittle [13] from the N-terminus to approximately Lys_{26}. This sequence also contains a segment with amphiphilic properties folded as an α-helix [5], a common structural motif in all actinoporins [17] that plays an essential role in pore formation.

Among its 175 amino acids, St II contains six Met residues located at positions 92, 101, 130, 157, 161 and 169 [17]. According to this, the CNBr hydrolysis renders an N-terminal bearing peptide whose molecular size would correspond to approximately half of the protein molecular weight. On the other hand, the low number of methionine residues and the use of rp-HPLC to separate fragments could help obtain pure peptides, which is essential to be able to attribute the property to a unique sequence. Another interesting characteristic of the N-terminal bearing peptide is the presence of a Trp residue that enables intrinsic fluorescence studies.

The CNBr treatment of native Sticholysin II failed to hydrolyze products (data not shown), probably due to the compact three-dimensional structure of this protein

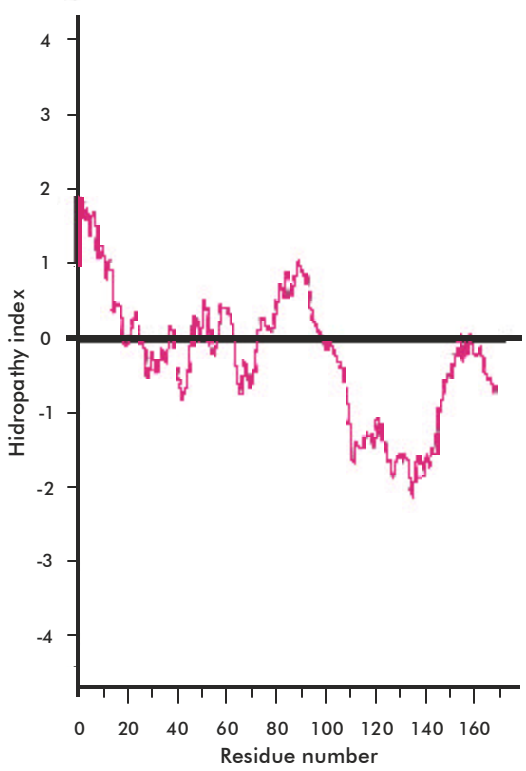

Figure 1. Hydropathy profile of St II. The profile was obtained with the algorithm of Kyte and Doolittle (1982) with a window of 19 residues, available in http://bioinformatics.weizmann.ac.il/hyd-bin/plot_hydroph.pl.

[5]. To circumvent this difficulty, we decided to include an initial purification of St II on rp-HPLC before CNBr hydrolysis. Additionally this chromatographic step avoids the slight contamination of St II with St I, previously reported [1].

The products form the CNBr hydrolysis of St II were separated by rp-HPLC (Figure 2). This treatment yielded three main peptides designated as P1, P2 and P3, according to the elution order from the reversed phase column. Mass spectrometric analysis revealed that P1 comprises residues from Y_{131} to M_{157}, P2 from

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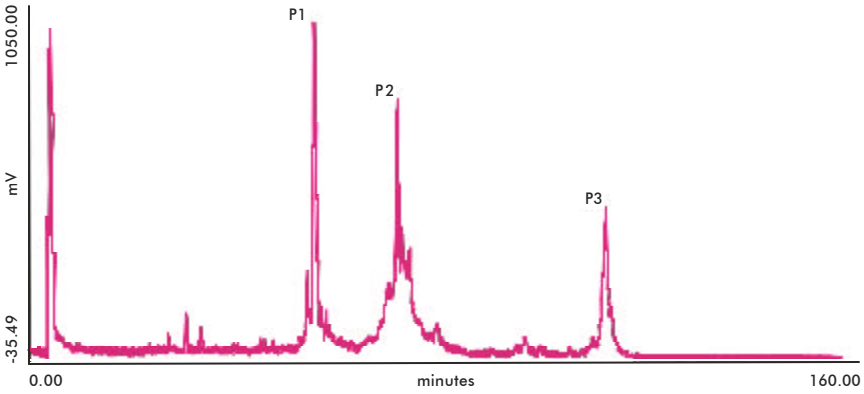

Figure 2. Reverse phase-HPLC profile of St II CNBr derived peptides. Chromatographic conditions: Baker rp-C4 column (4.6 X 250 mm); gradient: from 0% to 80% B in A, where solvent A was 0.1% (by vol.) aqueous TFA and solvent B was acetonitrile containing 0.05% (by vol.) aqueous TFA. Flow rate and column temperature were 0.8 mL.min-1 and 37 °C, respectively.

Table 1: Mass spectrometric characterization of the CNBr fragments of St II resolved by rp- HPLC. The molecular masses correspond to the average values considering the conversion of methionine into homoserine lactone

Peptide	TM (Da)	EM (Da)	Assignment of sequence in St II
P1	3232.49	3232.73	$_{131}$YEDLYYGNPYRGDNG**W**HEKNLGYGLRM$_{157}$
P2	3567.70	3567.75	$_{102}$FSVPFDYN**W**YSN**WW**DVKIYSGKRRADQGM$_{130}$
P3	9589.06	9588.9	$_{1}$ALAGTIIAGASLTFQVLDKVLEELGKVSRKIAVGIDNESGGT**W**TAL NAYFRSGTTDVILPEFVPNTKALLYSGRKDTGPVATGAVAAFAYYM$_{92}$

TM: theoretical mass

EM: experimental mass

F_{102} to M_{130} and P3 from A_1 to M_{92} (Table 1). P3 is the largest of the three peptides, representing more than 55% of the St II amino acid sequence [17], and containing the hydrophobic and amphiphilic regions most probably involved in pore formation [8].

To determine if the isolated peptides retain the biological activity of St II, we evaluated their capacity to permeabilize human erythrocytes. None of the peptides or their combinations showed a measurable hemolytic activity, at least in the micromolar concentration range (data not shown). This could be due to an intrinsic lack of activity and/or to an irreversible denaturation during their preparation or hydrolysis. In fact, a peptide obtained by chemical synthesis simulating the St II N-terminal sequence up to the 30th amino acid showed hemolytic activity although at a lower extent compared with that of the protein [18]. Furthermore, St II submitted to rp-HPLC conditions significantly losses its hemolytic activity (data not shown). Moreover, the lack of hemolytic activity for P1 and P2 could be due to the fact that they do not contain the amphiphilic sequence supposedly involved in pore formation [10]. Experiments of N terminal truncation mutagenesis for the closely related actinoporin EqT II showed that the deletion of the first 33 residues resulted in a mutant that is completely unable to produce hemolysis [11].

To evaluate whether the presence of peptides could modify the hemolytic activity of the whole toxin, we proceeded to test the hemolytic activity St II of in the presence of the peptides at different peptide:toxin ratios. Neither P1 nor P2 elicit any variation in the hemolytic activity of St II (up to 50 mol/mol, data not shown). However, P3 decreases native St II hemolytic activity. Figure 3 shows the time course of hemolysis induced by St II in the presence of P3. There is a decrease in the activity when the proportion of P3 increased in the incubation medium suggesting that the N-terminal bearing peptide associates with St II probably forming non-functional heteroligomers. If we consider that the lytic activity is exclusively due to the protein, an increase in P3 concentration could limit the number of St II molecules able to form functional pores. A similar phenomenon has been described by Walker *et al.* [19] for truncated mutants of bacterial alpha-toxin from *Staphylococcus aureus*.

With the purpose of evaluating the interaction of the peptides with model membranes, the intrinsic fluorescence was measured in the presence of small unilamellar vesicles composed of equimolar amounts of phosphatidylcholine and sphingomyelin. This mixture is considered to be the optimal composition for studies of actinoporins in model membranes [3, 20, 21]. The three peptides derived from the CNBr hydrolysis of St II contain Trp residues (Table 1), making it possible to evaluate their interaction with membranes by following the increase in intrinsic fluorescence as a consequence of the transition of Trp residues to a more hydrophobic environment [16].

The maximum fluorescence emission of the three peptides takes place at ca. 350 nm, consistent with a relatively high exposure of the Trp groups to the external medium. Furthermore, the position of the band did not change significantly when vesicles (60 M lipids) were added (data not shown). This would indicate that the Trp groups of the peptides are not incorporated into the bilayers. This contrasts with the blue shift of the St II emission in the presence of liposomes [6].

Fluorescence intensity of P1 and P2 is not modified by the presence of vesicles, up to 60 mM lipids, a result compatible with the lack of a deep penetration of the Trp groups in the bilayers (Figure 4). However, a small decrease of 30% in the Ksv value by acrylamide observed for P2 would indicate that this peptide is somehow associated to the liposomes. This could be explained in terms of the presence in P2 of the conserved stretch of clustered aromatic amino acids (WYSNWW) that participates in binding to lipid bilayer [5, 10]. It is interesting to note that, in spite of this association; P2 does not present a measurable hemolytic activity or does it interfere with the hemolytic activity of St II. This is compatible with the proposal that, even when this zone is involved in EqT II binding, it does not participate in pore structure [10].

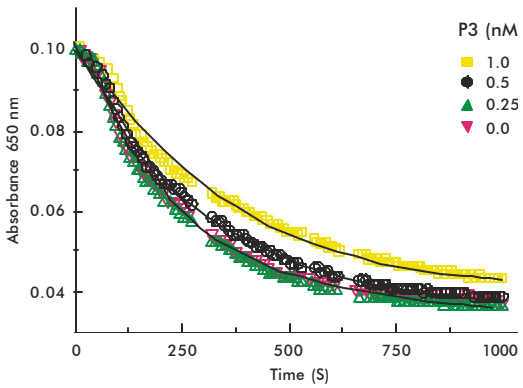

Figure 3: Hemolysis induced by St II in the presence of P3. The toxin-induced hemolysis of human erythrocytes in the presence of different amounts of P3 was determined by measuring the decrease in turbidity at 650 nm after the addition of 1 nM of St II. P3 concentrations: 1 nM (squares); 0.5 nM (circles); 0.25 nM (triangles); 0 nM (down triangles). Each curve corresponds to the average of three experiments.

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In contrast to P1 and P2, the addition of lipid vesicles to P3 (ca. 1 μM) produced an increase in fluorescence intensity up to 1.4 fold over the initial fluorescence, measured at 350 nm (Figure 4), even at low lipid concentrations (ca. 6.6 mM). This would indicate a favorable interaction between P3 and the lipid membrane. This is supported by the reduced acrylamide quenching (40%) in the presence of liposomes.

The Far-UV circular dichroism spectra of St II-derived peptides were recorded. The spectrum of P3 in the absence of vesicles displays one main negative band c.a. 200 nm followed by a broad positive band between 205 and 225 nm that could indicate that this fragment is partially aggregated and/or adopts an extended conformation (data not shown). Although the addition of vesicles reduced the negative band in about 200 nm suggesting that the peptide somehow binds to vesicles, it did not promote the slight increase in the secondary structure previously observed for St II [22, 23]. Probably, due to conformational differences, P3 is not able to lyse red blood cells albeit it keeps its membrane binding ability.

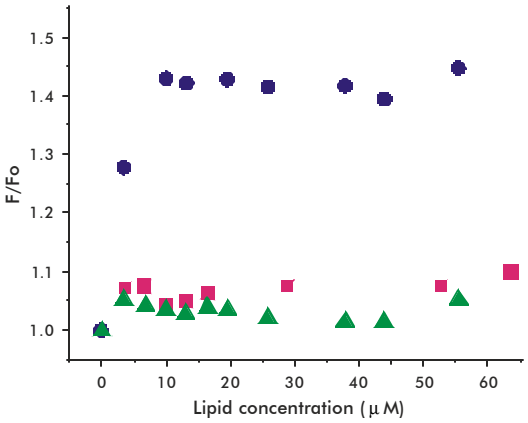

Figure 4: Interaction of St II derived peptides with lipid vesicles. Small unilamellar vesicles were added to a peptide solution and the fluorescence intensity was measured (excitation: 295 nm; emission: 350 nm). Corrections were applied for the dispersion introduced by the vesicles. P1 (triangles), P2 (squares), P3 (circles).

Received in july, 2006. Accepted for publication in october, 2006.

The three-dimensional structure of St II determined by Mancheño *et al.* [5] showed that it is based on a β-sandwich fold composed of ten β-strands, flanked on each side by two short α-helices. The two β sheets of the sandwich structure consist of five and four strands, respectively. In a recent study, Kristan *et al.* [24] demonstrated that a flexible N-terminal region and a stable β-sandwich are pre-requisites for proper pore formation by the actinoporin family.

The N-terminal bearing peptide P3 comprises the amino acid residues that correspond to the anphiphilic helix a1 (Phe14-Glu23), and five β-strands (b1:Ile6-Ala8); (b2:Lys30-Glu38); (b3:Trp43-Ser52); (b4:Lys67-Arg74) and (b5:Ala84-Met92). Three of these β-strands (b1, b2 and b4) belong to the same b-sheet, while b3 and b5 belong to the second sheet. As demonstrated by CD experiments, the N-terminal bearing peptide P3 probably adopts an extended conformation, and the addition of vesicles do not promote changes in its secondary structure. These findings support the hypothesis that in spite of P3 having the amino acid sequence required for pore formation including the first 30 residues [18], it is not able to permeabilize cells probably due to its size and its binding to the membrane in a non-functional conformation.

Further studies should explore the behavior of localized fragments of the actinoporin sequence eliminating the contribution of other molecule segments. This approach would define whether the N-terminal fragment, including both the hydrophobic and amphiphilic segments, is able to form functional pores on the lipid bilayer as in certain antimicrobial peptides, such as magainin that forms toroidal multimeric pores on membranes like the actinoporins [25, 26].

Acknowledgements

This research was financially supported by a grant from the Instituto Trentino di Cultura , Italy (to C.A.V.) and a grant from Alexander von Humboldt Foundation IV KUB 1064773STP - Germany (to V.M.C.). We also acknowledge CITMA (Cuba)- CONYCIT (Chile) as well as CNPq-MES (Brazil-Cuba) collaboration programs.

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