Rev. Invest. Mar. 27(1):41-48, 2006

STICHOLYSIN II, A CYTOLYSIN ISOLATED FROM THE CARIBBEAN SEA ANEMONE *Stichodactyla helianthus*, INTERACTS WITH SERUM LIPOPROTEINS, FREUND ADJUVANT AND SPECIFIC ANTIBODIES TO THIS PROTEIN.

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SUMMARY

Sticholysin II (St II) is a pore forming cytolysin isolated from the Caribbean Sea anemone *Stichodactyla helianthus*. This 20 kDa hydrophilic protein forms oligomeric pores in biological membranes provoking osmotic imbalance that eventually leads to lysis. St II represents an useful tool in biomembrane and protein studies and a potential weapon against dangerous cells. In this study the inhibition of St II's hemolytic activity by two structurally and physiologically different vertebrate proteins and by the well known Freund adjuvant. 1.25 % of normal serum was able to neutralize completely St II's hemolytic activity as well as 40 % of Freund adjuvant and 10 µg of purified antibodies specific to St II. These results provide new insight of St II interactions with important implications in future applications of sea anemone cytolysins.

Key words: cytolysin; toxin; inhibition; biomembrane; *Stichodactyla helianthus*

RESUMEN

Sticholisina II (St II) es una citolisina aislada de la anémona marina *Stichodactyla helianthus*. Esta proteína hidrofilica forma poros en las membranas celulares, lo que provoca el desbalance osmótico que conduce a la lisis celular. St II se ha convertido en una herramienta muy útil en el estudio de las biomembranas y es un arma potencial contra células cancerosas. En este trabajo se evalúa la inhibición de la actividad hemolítica de St II por dos proteínas estructural y funcionalmente diferentes, así como por el conocido adyuvante de Freund. 1.25 % del suero normal fue capaz de neutralizar completamente la actividad hemolítica de St II al igual que 40 % del adyuvante de Freund y 10 µg de anticuerpos anti-St II purificados por afinidad. Estos resultados proveen nuevos elementos sobre las interacciones de St II con proteínas de vertebrados, así como sus implicaciones en las aplicaciones futuras de las citolisinas de anémonas marinas.

Palabras clave: citolisina; toxina; inhibición; biomembrana; *Stichodactyla helianthus*

Sea anemone cytolysins are produced by specialized cells called nematocytes. These cells are fairly complex and contain a specialized organelle called nematocyst (Kass-Simon and Scappaticci, 2002). The nematocyst harpoons and their toxic products are used to capture prey, digestion, defense and intraspecific competition (Hessinger and Lenhoff, 1988). Sticholysin II is a cytolysin isolated from the Caribbean Sea anemone *Stichodactyla helianthus* which could act as a toxin in the physiology of the sea anemone (Basulto *et al.*, 2006). It forms oligomeric pores in biological membranes provoking osmotic imbalance that eventually leads to lysis (Tejuca *et al.,* 2001). St II exists in a monomeric soluble state but forms tetramers in presence of a lipidic interface (Mancheño *et al.*, 2003). Structurally, it is a 20 kDa hydrophilic polypeptide that uses a unique single helix insertion mechanism for pore formation and permeabilization (Malovrh *et al.*, 2003).

Interactions of sea anemone cytolysins with vertebrates could be important since casual stings could be life threatening or provoke anaphylactic (allergic) reactions resulting in death. Relationship between cytolysins from different sources (vertebrate and invertebrate, plants and bacteria) has not been established despite the homologies found in their primary protein sequence (Turk, 1991; Samejina *et al.,* 2000), structure (Keith and Luzio, 1988; Laine *et al.*, 1988) and mechanism of membrane permeabilization (Kem, 1988; Tejuca, 1996). When proteins from different sources have homologies or the same behavior during interactions, relationships could be established providing valuable information regarding their evolution, mechanism and physiological role. It could be the case of pore forming proteins. The dynamic of protein interactions could be also relevant for their therapeutical applications and research. Especially, St II has been used as a model protein in biomembrane studies and St I is being currently evaluated against cancer cells (Tejuca *et al.*, 2004). Researches on interactions of St II with other molecules could contribute widely to these studies being a useful approach for the structural and functional characterization of sea anemone cytolysins. Here we report inhibition of the hemolytic activity of the sea anemone toxin St II by two structurally and physiologically different groups of vertebrate proteins and by the well known Freund adjuvant (FA).

MATERIALS AND METHODS

Sticholysins

Stichodactyla helianthus was collected in the coast of Habana city and sticholysins were purified according to Lanio *et al.* (2001). Briefly, 500 mg of crude extract of the anemone were applied to a Sephadex G-50 (Medium) column (dimensions 4 x 100 cm, Pharmacia-LKB, Sweden) equilibrated with 0.02 M sodium acetate buffer, pH 5. The second peak was chromatographed on a CM-52 cellulose (Pharmacia-LKB, Sweden) equilibrated with 0.1 M sodium acetate buffer, pH 5. St II was filtered through Diaflo PM 10 (AMICON) membrane and stored at -20°C. Its concentration was determined spectrophotometrically (280 nm) using an $E^{0.1\%,1cm}$ of 1.87 (Lanio *et al.* 2001).

Hemolysis by St II.

Hemolysis caused by St II was assayed on fresh adjusted human erythrocyte suspension (10^8 cells/mL) according Alvarez *et al.* (1998). 100 µL of blood cells in TBS (NaCl 0.145 M, Tris-HCl 0.01 M, pH 7.4) were incubated 30 min at 37°C with St II (3.2 to 30 ng). Turbidity decrease caused by lysis was monitored at 600 nm using non-treated cells as negative experimental controls.

Inhibition of St II's hemolytic activity.

Inhibition of St II's hemolytic activity by rabbit, mouse and human sera, polyclonal antibodies and Freund adjuvant (FA)(Sigma, cat. no. A-6258) was studied by introducing 10 µL of inhibitor in the assay mixture. Were assayed 1.25% of normal serum, 2.5 to 16 ng of anti-St II gamma rich purified fraction from immunosera produced with FA by Pico and collaborators (2000). Were also assayed 5, 20, and 40% of FA. The effect of preincubation 30 min at 37°C was also assayed by preincubating inhibitor with 22.5 ng of St II prior addition of cells. Inhibition was regarded as Inhibition % to which absorbances of non treated erythrocytes were considered as 100% inhibition.

Turbidimetric assay

The non-specific interaction between St II and serum components was performed by incubating increasing volumes of rabbit non-immune serum with 32 ng of St II diluted in PBS. After 30 min at 37°C was measured the absorbance at 600 nm. St II in PBS and serum in PBS were used as controls.

Immunoelectrophoresis

Rabbit serum was run out in 1.5% (w/v) agarose gel (Merck) using 0.075 M Veronal buffer, pH 8.6, 0.1% NaN_3. After 3 H at a constant voltage of 10 Volt/cm, 125 µg de St II was placed in the upper channel. In the bottom channel was placed mouse anti-rabbit serum. Slides were placed in a moist chamber for 24 h at room temperature. Following washing out of soluble components precipitation lines were stained with amido black in methanol-glacial acetic acid (9:1 v/v).

Electrophoresis and Immunoblotting

SDS-PAGE of anemone's crude extract and purified St II was performed according to the modified Laemmli gel system (Bio-Rad Laboratories, Richmond, CA) in 8% resolving gels run at 200 Volt. Protein bands were visualized by staining with Coomassie brilliant blue R-250 (CBB) (Serva, Heidelberg, Germany). Western immunoblotting was performed by transferring proteins onto a nitrocellulose membrane with Multiphor II (Pharmacia LKB Biotechnology, Uppsala, Sweden). Remaining sites on membrane were blocked with BSA 1% and antigen-antibody interactions were revealed with anti-St II antibodies produced in rabbits (Pico *et al.*, 2000), a biotin labeled anti-rabbit IgG and avidin-horserabbit peroxidase conjugate (Sigma, USA, cat. no. A-6156). Color was revealed with diaminobenzidine plus H_2O_2 and reaction stopped by washing out substrate.

Sampling and statistic

Each experiment include three intra-assay and two inter-assay replicas. Statistical differences between groups was performed with the Student t-test on Statistica 5.0 supported by Window Millennium Edition.

RESULTS

Relevant information regarding sticholysins have been obtained from structural and functional analysis on biological and artificial membranes (Tejuca *et al.*, 1996, 2001; Alvarez *et al.*, 2001; Mancheño *et al.*, 2002). These basic polypeptides interact with lipids bilayers, especially those containing sphingomyelin (Alvarez *et al.*, 1994; Tejuca *et al.*, 1994) and no other protein-protein interaction have been characterized.

Serum has shown to inhibit the hemolytic activity of sea anemone cytolysins indicating the presence of some unspecific inhibitor (Bernheimer and Avigad, 1976; Giraldi *et al.*, 1976; Batista *et al.*, 1990). As shown in Fig. 1, 1,25% of rabbit normal serum was able to neutralize completely St II's hemolytic activity, as mouse and human sera also did (data not shown). In order to characterize and differentiate serum components implicated in this unspecific interaction, were carried out turbidimetric and immuno-electrophoretic assays. When 32 ng of St II were mixed with normal serum was visible the formation of a precipitate that was also followed at 600 nm (Fig. 2a). Immunoelectrophoresis of serum faced to St II revealed the formation of a precipitation arch only in the position corresponding to β-lipoproteins (Fig. 2b). When immune sera specific to St II were employed, there was no precipitation in the usual position of globulins. Similar studies with equinatoxin II (Eqt II) from the sea anemone *Actinia equina* revealed the possible implication of β-lipoproteins in the interaction (Turk *et al.*,1989; Batista *et al.*, 1990). With Eqt II have demonstrated the quick formation of an insoluble precipitate when incubated with serum (Narat *et al.*, 1994). This insoluble precipitate have been additionally reported as poorly cytotoxic (Batista *et al.*, 1990). The interaction with β-lipoproteins has been prevented by modifying Eqt II with tetraminometane that transforms tryptofan residues into charged derivates, indicating the hydrophobic nature of this interaction (Turk *et al.*,1989).

Other results of our laboratory indicates the high affinity of St II for hydrophobic environments not precisely resembling lipid bilayers. Freund adjuvant, a mineral oil based immunostimulator extensively used in antibody production, neutralized completely the hemolytic activity of St II as shown in Fig. 3. Increasing among of FA added simultaneously with St II to cells, inhibit St II's hemolytic activity until the complete inhibition reached with 40% of FA. When FA was preincubated with St II for 30 minutes at 37°C it was obtained the same degree of inhibition as in simultaneous addition, indicating the high affinity of St II for hydrophobic environments (Fig. 3).

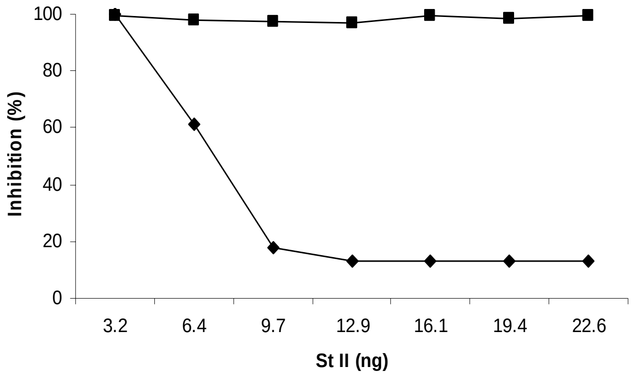

Fig. 1. Inhibition of St II's hemolysis by rabbit normal serum. 1.25% of rabbit's serum was able to neutralized completely St II hemolytic activity (■) as compared with positive control of hemolysis. There was a considerable statistical difference between treatments (data not shown). Each experiment include three replicas and was repeated twice according Materials and Methods

Considering the strong and fast interaction of St II with hydrophobic environments we still argue in its implication in further interactions of St II with proteins. Since no other protein-protein interaction have been reported to St II here we refer to the antigen-antibody interaction. St II has been used to generate polyclonal antibodies in rabbits obtaining good antibodies titers higher than 1/64000 when Freund's adjuvant was used (Pico *et al.*, 2000). As it is shown in Fig. 4a, polyclonal antibodies to St II recognized specifically the cytolysin when faced to a complex protein mixture represented by the crude extract of the sea anemone *S. helianthus*. Although immunizations were carried out with 50% FA there was a specific recognition of St II by rabbit's immune system resulting in a strong antibody response maybe due to the long phylogenetic distance of sea anemone proteins from rabbit's ones. However the specific recognition and high titer of anti-St II serum could have not correlation with the functionality of

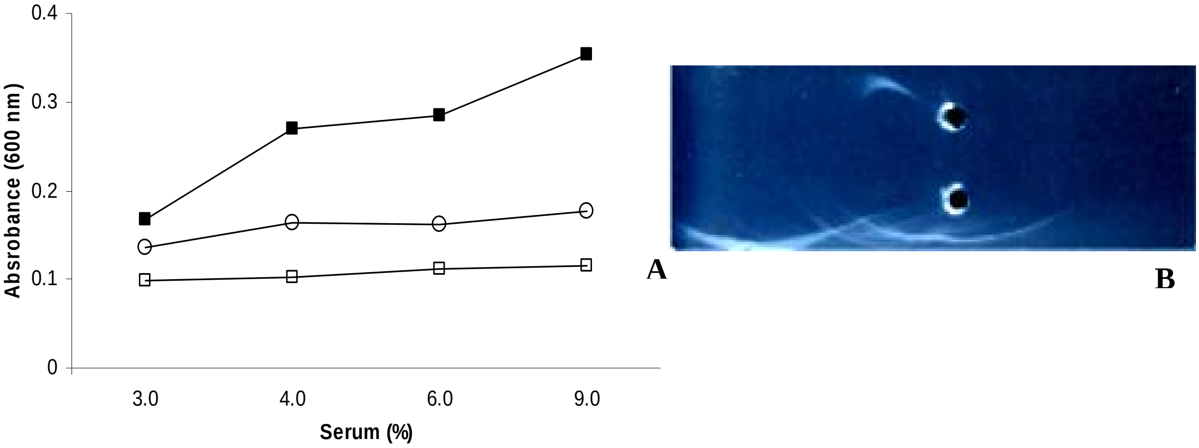

Fig. 2. Unspecific interaction of St II with serum components. (**A**) Increase of turbidity by addition of rabbit serum to 32 ng of St II (■); St II-buffer (□) and serum-buffer (○) controls. Statistical difference was obtained only with the treatment St II-Serum (Data not shown). (**B**) Immunoelectrophoresis of rabbit serum faced to St II (top channel) and anti-rabbit serum (inferior channel) revealing a precipitation arch only in the position corresponding to β-lipoproteins.

specific antibodies to St II, that is the antibody capacity of inhibit St II's lytic activity, strongly depending on the particular epitope from which antibodies are derived. A previous report indicates a poor neutralizing antibody response when mice were immunized with lipid-attenuated Eqt II (Narat *et al.*,1994). To determine the functionality of polyclonal antibodies to St II, purified antibodies were used in inhibition assays. As shown in Fig. 4b, increasing the amounts of anti-St II IgG neutralize the hemolysis provoked by St II as compared with the effect of non-specific purified IgG (up to 80 µg) (data not shown).

DISCUSION

Cnidaria (hydras, jellyfish, sea anemones and corals) comprises an early phylum that colonizes successfully oceans and coasts, maybe correlated with the presence of a wide array of toxic molecules harbored by these organisms (Fautin and Mariscal, 1991; Basulto *et al.*, 2006). The characterized toxins have neurotoxic, cytolytic or enzymatic activities (Kem, 1988) and have been of interest in recent years due to their interesting biology and potential uses as therapeuticals. However, knowledge on cnidarian's toxin action mechanism, pharmacology and toxicology are still under profound research. Here three different interactions of St II with serum components, a commonly used adjuvant and specific antibodies to St II were studied. The quick formation of an insoluble precipitate during the interaction of St II with serum lipoproteins could represent an important step in the protection *in vivo* against actinoporins, maybe representing a more general mechanism against cytolysins. It could be the case of human perforin from citotoxic T lymphocytes and natural killer cells (NK), two mammals pore forming proteins implicated in the immune response.

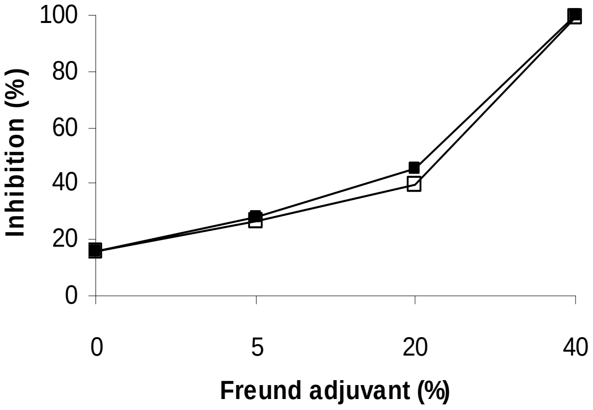

Fig. 3. Inhibition of St II's hemolytic activity by Freund adjuvant. FA was added simultaneously (□) with 22.5 ng of St II to cells or preincubated (■) with the toxin for 30 min at 37 °C. Inhibition (%) was calculated considering as 100 % inhibition the absorbance (600 nm) of erythrocyte suspension alone. There was no statistical difference between inhibition achieved with simultaneously addition and preincubation (data not shown).

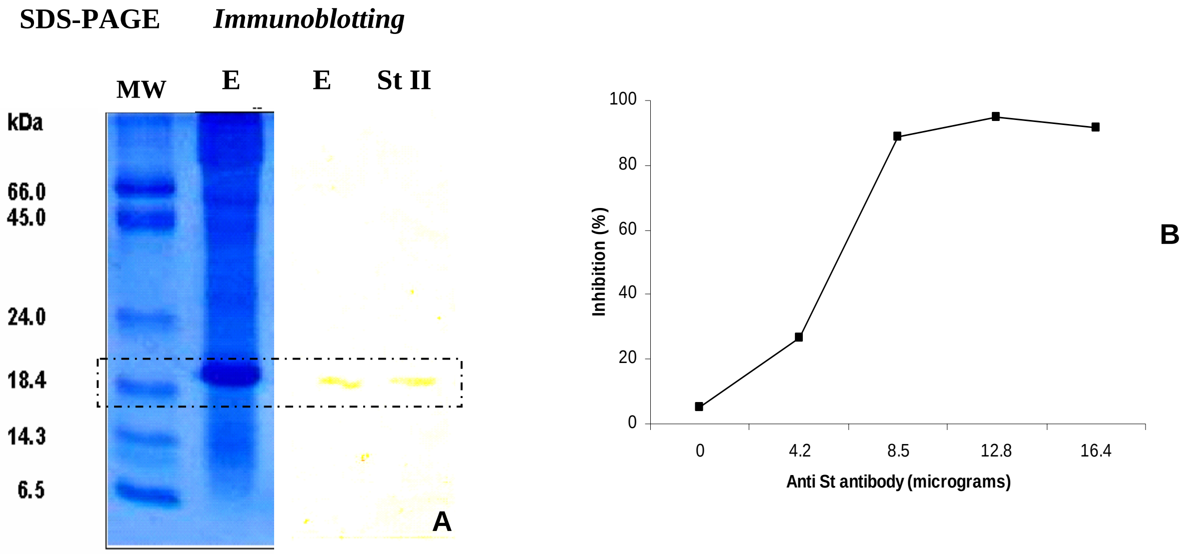

Fig. 4. Interaction of St II with specific antibodies produced in rabbits. (**A**) Specificity of antibodies to St II by immunoblotting on nitrocellulose of crude extract (E) and purified St II. (**B**) Inhibition of St II's hemolytic activity by specific IgG. An excess of non-specific purified IgG had no effect in the hemolysis produced by St II (data not shown). Each experiment include three replicas and was repeated twice according Materials and Methods.

Perforin have been related structurally and functionally with the terminal components of the complement, C9 (Keith and Luzio, 1988). The lytic activity of these proteins is inhibited for less than 1% of normal human serum. Two serum lipoproteins, the human low density lipoproteins (LDL) and high density lipoproteins (LDL) produce a striking inhibition of the lytic activities of perforin and C9, strongly correlated with inhibitor concentration (Ding-E Young J. *et al,* 1987). It could be established some relationship between the inhibition of vertebrate pore forming proteins and the inhibition of actinoporins by serum lipoproteins?. Some structural and immunochemical relationships have been established. The N-terminal portion of HmT, a cytolysin isolated from the sea anemone *Heteractis magnifica*, which exhibits homology with other sea anemone cytolysins from *A. equina* (Belmonte *et al.*, 1994), *A. tenebrosa.* (Simpson *et al.*, 1990) and *S. helianthus* (Blumental and Kem, 1983), have also structural homology with melitin from the bee poison (Samejina *et al.*, 2000) and melittin has been immunochemically related with C9 (Laine *et al.*, 1988; Laine and Esser, 1989). Policlonal antiserum to melittin recognizes and slows the hemolysis produced by C9 (Laine and Esser, 1989).

From these results we could speculate on the relationship between actinoporins and mammal pore forming proteins and further experimental data will be necessary to confirm it. Endogenous and exogenous cytolysins could be "trapped" within serum lipoproteins preventing further tissular damage. This protection mechanism has been supported for perforin and C9 whose targets are basically the closer standing cells additionally attached by Fc receptor-antobody interactions (Ding-E Young J. *et al,* 1987).

On the other hand, interaction of St II with FA could have serious implication in the production of antibodies to this cytolysin. Freund adjuvant has been extensively used in the production of antibodies against cytolysins (el Ayeb *et al.,* 1986; Laine *et al.,* 1988; Baharoui *et al.,* 1989). Looking for a protective immune response, Narat and collaborators (1994) immunized mice with Eqt II mixed with autologous lipids obtaining a poor protection. Looking forward for an insight in the topology of the interaction of St II with oil based adjuvants, further empirics analysis of the contribution of other hydrophilic and hydrophobic immunostimulators must be accomplished. Our results suggest that immunization with FA was carried out with total or partially inactivated sticholysin. Despite this unspecific interaction resulting in total inhibition, a good immune response was raised against St II in rabbits (Pico *et al.,* 2000, 2004). Relating the high affinity of St II for serum lipoproteins and FA and the functionality of antibodies to St II, we could hypothesize that St II accommodates in such environments by mean of its amphipathic N-terminal or its hydrophobic core of tryptophan residues and exposes the remaining hydrophilic moieties as targets for the immune system. On the other hand, the molecular interaction of St II with specific antibodies must depend on different forces pattern from those involucrated in the interaction with lipoproteins and Freund adjuvant. In the antigen-antibody interaction major antigenic moieties are hydrophilic regions exposed to the solvent (Berzofski and Berkower, 2003). In the future, further studies must be addressed toward the examination of anti-St II antibodies functionality including other actinoporins, beside the understanding of the St II interaction topology with lipoproteins and Freund adjuvant.

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Aceptado: 2 de octubre de 2005