REPORT

Isolation and partial purification of a hemolytic sphingomyelin-inhibitable fraction from the sea anemone Anthopleura nigrescens

Javier Alvarado[1], Yeney Álvarez[2], Lohans Pedrera[2], Uris Ros[2], María E Lanio[2], Aisel Valle[2], ✍ Carlos Álvarez[2]

[1] Escuela de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional Apartado 86-300, Heredia, Costa Rica

[2] Centro de Estudio de Proteínas, Facultad de Biología, Universidad de La Habana, UH Calle 25 No. 455 entre I y J, Vedado, CP 10400, La Habana, Cuba

✍ calvarez@fbio.uh.cu

ABSTRACT

Actinoporins are highly hemolytic pore-forming proteins with a molecular mass of around 20 kDa and high affinity for sphingomyelin-containing membranes. On the crude extract of the sea anemone *Anthopleura nigrescens*, the hemolytic activity (HA) was detected. In order to identify the presence of pore-forming proteins similar to actinoporins in this anemone, the fractionation and analysis of its crude extract was carried out. The aqueous extract of the whole body was subjected to gel filtration chromatography on Sephadex G50 medium rendering three resolved peaks (P-I, P-II, and P-III) as measured by their absorbance at 280 nm. Functional characterization of the crude extract and chromatographic fractions was evaluated by HA against human red blood cells. The crude extract and two peaks (P-II and P-III) showed HA. Interestingly, the HA of the crude extract and P-II were specifically inhibited by small unilamellar vesicles of phosphatidylcholine: sphingomyelin (1:1). Both the crude extract and P-II revealed the existence of at least one protein band around 20 kDa by SDS-PAGE. The inhibition by sphingomyelin of the whole body extract HA and the localization of this property in P-II that could be associated with a protein of around 20 kDa suggest the presence of at least one novel actinoporin in *A. nigrescens*. Furthermore, this is the first report of a biochemical activity for this sea anemone. Work is in progress in order to purify and characterize the molecular entities responsible for this HA inhibited by sphingomyelin.

Keywords: actinoporin, *Anthopleura nigrescens*, sphingomyelin, hemolytic activity

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RESUMEN

Aislamiento y purificación parcial de una fracción hemolítica inhibida por esfingomielina de la anémona marina *Anthopleura nigrescens*. Las actinoporinas son proteínas formadoras de poro, altamente hemolíticas, de aproximadamente 20 kDa, con una elevada afinidad por las membranas que contienen esfingomielina. En el extracto crudo de la anémona marina *Anthopleura nigrescens* se detectó actividad hemolítica (AH). Con el fin de identificar la presencia de proteínas formadoras de poros, similares a las actinoporinas, se fraccionó y analizó este extracto crudo. Se sometió a cromatografía de filtración en gel de Sephadex G50 medio, y se obtuvieron tres picos resueltos (P-I, P-II y P-III), según su absorbancia a 280 nm. La caracterización funcional del extracto y las fracciones cromatográficas se evaluaron por la AH frente a eritrocitos humanos. El extracto crudo y los picos II y III mostraron AH. Curiosamente la AH del extracto crudo y del pico II fueron inhibidas por vesículas unilamelares pequeñas de fosfatidilcolina: esfingomielina (1:1). Tanto el extracto crudo como P-II revelaron al menos una banda proteica de alrededor de 20 kDa, en SDS-PAGE. La inhibición por esfingomielina de la AH del extracto y la localización de esta propiedad en P-II, podría asociarse con una banda proteica de alrededor de 20 kDa, lo que sugiere la presencia de al menos una actinoporina en *A. nigrescens*. Este es el primer informe de una actividad bioquímica para esta anémona marina. Se continúa trabajando para purificar y caracterizar las entidades moleculares responsables de esta AH inhibida por esfingomielina.

Palabras clave: actinoporina, *Anthopleura nigrescens*, esfingomielina, actividad hemolítica

*I*ntroduction

Sea anemones are sessile animals that produce venom for defense from predators and capture of their preys. This secretion consists of an ensemble of molecules including phospholipases [1-4], sodium channel blockers [5], protease inhibitors [6], and pore-forming toxins called actinoporins [7-13]. Actinoporins are highly hemolytic, basic cysteine-less proteins with molecular weight around 20 kDa showing high affinity for membranes containing sphingomyelin; therefore, their hemolytic activity (HA) is characteristically inhibited when the toxin is pre-incubated with liposomes containing this phospholipid [11, 14-16]. These toxins have attracted the attention for their potential use in biomedicine and biotechnology, for instance, in the construction of immunotoxins against cancer cells [17, 18] or as a tool for protein-membrane interaction studies [19, 20].

Recently, we described the presence of the sea anemone *Anthopleura nigrescens* on the Pacific coast of Costa Rica [21]. Here, we report the HA against human red blood cells (RBC) of the crude extract and two fractions obtained by molecular exclusion chromatography. The HA detected in the crude extract and in one partially purified fraction were inhibited by sphingomyelin, suggesting the presence of at least one novel actinoporin in *A. nigrescens*.

✍ Corresponding author

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Materials and methods

Specimens were collected during low tide in a rocky area on the Pacific coast of Costa Rica (9º55′01.99′ ′N-84º 42′49.50′′W). Sea anemones were carefully detached from the rock, placed in plastic sample containers, and transported at 4 ºC ± 2 ºC to the laboratory. Crude extracts were obtained by mincing and homogenizing the whole animal body in distilled water (1:2, w/v). Protein concentration was determined according to Bradford [22] by 0.01 % (w/v) Coomassie Brilliant Blue G-250.

The crude extract (4.5 mg of protein) was applied onto a Sephadex G-50 medium (GE-Healthcare) column, equilibrated with 0.02 mol/L sodium phosphate buffer pH 7 at a flow rate of 27.5 cm/h. Fractions of 1 mL were collected and monitored at 280 nm for protein content estimation and HA determination.

The crude extract and fractions homogeneity were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15 % polyacrylamide gel. Samples were dissolved in Laemmli buffer (10 % SDS, 0.02 M 2-mercaptoethanol, pH 6.8) [23] and heated at 100 °C for 10 min. Protein staining was carried out with Coomassie blue dye at 0.05 % (m/v). Molecular weight markers (8 to 220 kDa) ColorBurst™ (Sigma Aldrich) were used.

HA on RBC was evaluated turbidimetrically at 600 nm at room temperature (25 °C ± 2 ºC) as previously described by Martínez *et al*. [24]. Briefly, the erythrocyte suspension was prepared using pooled fresh RBC, washed by centrifugation and resuspended in Tris-buffered saline (TBS; 145 mM NaCl, 10 mM Tris-HCl, pH 7.4). The cell suspension was diluted with TBS to an absorbance of 0.1 at 600 nm and the decrease in absorbance was followed in a microplate reader (MultiSkan FC, Thermo Scientific). The samples were two-fold serially diluted with TBS and the reaction started by adding the same volume of cell suspension to each well (200 µL final volume). The loss of turbidity was quantitatively related to the HA. Negative and positive controls were also run in parallel using RBC without the extract or fractions, and RBC with the actinoporin sticholysin II (St II) from *Stichodactyla helianthus* [9], respectively. HA was quantitatively calculated from a St II standard curve and expressed as protein nanogram equivalents.

Small unilamellar vesicles (SUV) of phosphatidylcholine: sphingomyelin (PC:SM; 1:1) were used for the HA inhibition experiments. The crude extract and fractions that showed HA were incubated at room temperature for 30 min with SUV at different lipid: protein mass ratio (5, 2.5, 1.25, 0.6, 0.3) and in the absence of lipids. HA in incubated samples was determined as described above.

The small unilamellar vesicles (SUV) of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC): egg SM (Avanti Polar Lipids), at 1:1 molar ratio, were prepared by thorough sonication of a multilamellar liposome suspension. For multilamellar vesicle preparation, the appropriate amounts of both lipids dissolved in chloroform: methanol solution 2:1 (v/v) were mixed and evaporated thoroughly at 40 °C. SUV were prepared by sonicating the multillamelar vesicle suspension in buffer (10 mM Tris-HCl, pH 7.4) with a probe ultrasonicator (Branson 450, Danbury, USA) as previously described [25]. Titanium particles released from the probe were removed by further centrifugation at 10 000 *g* for10 min at 22 °C. Phospholipid concentration was measured by determining inorganic phosphate according to Rouser *et al*. [26].

Results and discussion

The body total aqueous extract (1.5 mg/mL estimated on protein grounds) showed HA against RBC in the ng/mL concentration range ($HC_{50} \approx$ 100 ng/mL) similarly to *Stichodactyla helianthus*'s StI and StII ($HC_{50} \approx$ 30-45 ng/mL pure fractions) as reported by Lanio *et al*. [9].

In order to gain some insight into the nature of the proteins responsible for the HA, the effect of incubation with liposomes containing SM on their HA was studied. The HA of the crude extract was partially inhibited upon incubation for 30 min with PC:SM (1:1) SUV (Figure 1A). Two orders of magnitude of protein concentration were necessary to recover the HA of the incubated fraction with SM in respect to the non-incubated fraction (Figure 1B). This suggested that the crude extract HA could be caused by at least one novel actinoporin.

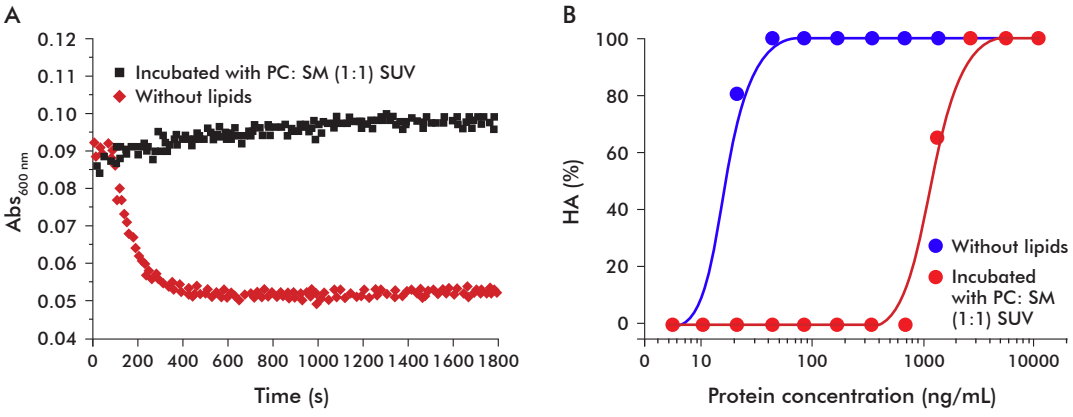

Figure 1. Inhibition of the hemolytic activity (HA) of *Anthopleura nigrescens* total extract by phosphatidylcholine: sphingomyelin (PC:SM; 1:1) small unilamellar vesicles (SUV). A) Time course of HA after incubation for 30 min with SUV PC:SM (1:1) or without SUV addition. Protein concentration was 25 ng/mL. The lipid:protein mass ratio was 5.5. B) Hemolysis (%) as a function of protein concentration.

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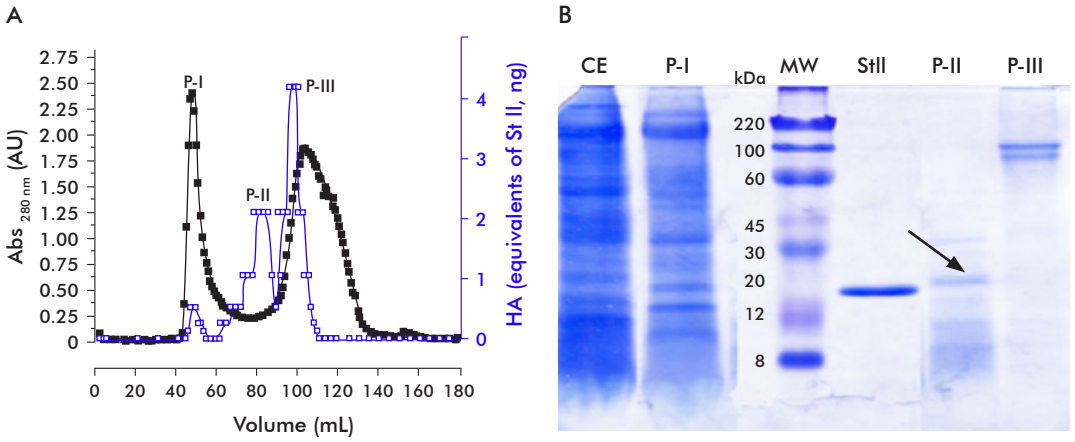

Figure 2. Gel filtration chromatography of *Anthopleura nigrescens* total extract on Sephadex G-50 (medium) and assessment of fraction homogeneity by SDS-PAGE. A) Typical chromatographic profile obtained by gel filtration. Experimental conditions: elution was carried out with 0.02 M sodium phosphate buffer, pH 7 at a flow rate of 27.5 cm/h. Fractions of 1 mL were collected and monitored at 280 nm. HA was expressed as equivalents (ng) of the actinoporin StII. B) SDS-PAGE (15 %) of the total extract and peaks I, II, and III from Sephadex G-50 gel filtration chromatography. Lanes: CE: Crude extract; P-I, P-II and P-III: peaks I, II and III, respectively; MW: molecular weight marker; StII: sticholysin II from *Stichodactyla helianthus*, (20 kDa). The arrow indicates a protein band in peak II with molecular weight of around 20 kDa. Bands were stained with Coomassie Blue 0.05 % (w/v).

The gel filtration chromatographic step of the total body extract rendered two defined peaks of HA (P-II and P-III) while peak I (P-I) was devoid of HA (Figure 2A). As evidenced by SDS-PAGE, P-II showed a band with a molecular weight of around 20 kDa (Figure 2B), a characteristic of the actinoporin family [16, 19, 20]. Peak III showed two bands with molecular weights between 60-100 kDa (Figure 2B). This result is surprising because these bands arise from the last fraction of the gel filtration chromatography (Figure 2A), where small molecules are expected to be found. Probably, such relatively high molecular weight proteins are somehow retained by the Sephadex matrix, and consequently are not properly resolved by gel filtration.

Upon incubation with sphingomyelin-containing vesicles, P-II showed a reduction of its HA that was dependent on the proportion of vesicle: protein assayed (Figure 3) and the time elapsed (inset to Figure 3). In fact, P-II HA inhibition was observed from a very low lipid: protein mass ratio (0.3) and it was completed when a ratio of 5 was achieved. Conversely, P-III HA was not affected by incubation with the same liposomes.

Altogether, the hemolytic activity, the inhibition by sphingomyelin of the whole body extract HA, and the localization of this property in P-II that could be associated with a protein of around 20 kDa, strongly suggests the presence of at least one novel actinoporin in the sea anemone *A. nigrescens*. Furthermore, this is the first report of a biochemical activity for this anemone.

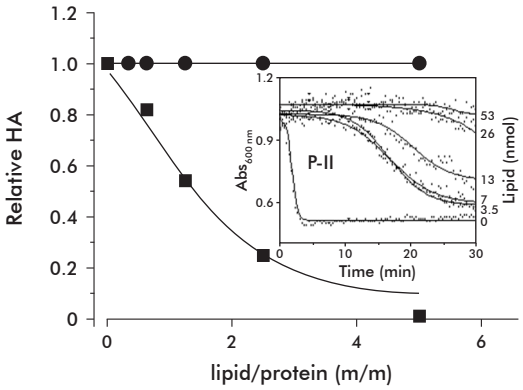

Figure 3. Relative hemolytic activity (HA) of P-II and P-III fractions of *Anthopleura nigrescens* extracts, after incubation for 30 min with PC:SM (1:1, mol:mol) SUV as a function of the lipid:protein mg:mg ratio. Inset: Time-course of hemolysis caused by PII (0.04 mg/mL) in the presence of diferent amount of vesicles. The assay was carried out in Tris-buffered saline (TBS; 145 mM NaCl, 10 mM Tris-HCl, pH 7.4) at 25 ± 2 °C.

Work is in progress in order to purify and characterize the molecular entities responsible for this HA inhibited by sphingomyelin.

Acknowledgments

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