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Antibacterial activity in the hemolymph of the catarina scallop *Argopecten ventricosus*

Actividad antibacteriana en la hemolinfa de la almeja catarina *Argopecten ventricosus*

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Abstract. We conducted a search for antibacterial peptide like activity in hemolymph of *Argopecten ventricosus*. Pre-purification of peptides was done by reverse phase HPLC. Hemolymph acidic supernatant was loaded into a column packed with C_{18} matrix. Stepwise elutions were performed with 5, 50, 80, and 100 % acetonitrile (ACN) in 0.05 % trifluoroacetic acid (TFA) over 550 min at a flow rate of 0.2 ml/min. Absorbance was monitored at 280 nm. Eluted fractions were concentrated under vacuum. *Vibrio alginolyticus* 138-2 was used as a model to test fractions. Growth inhibition zone were observed at 5 and 50 % ACN. This is the first report of antimicrobial peptide-like activity in hemolymph of a pectinid species.

Keywords: Antimicrobial peptide, *Argopecten ventricosus,* hemolymph, immunity, *Vibrio alginolyticus.*

Resumen. Se realizó una investigación para demostrar que la hemolinfa de *Argopecten ventricosus* presenta actividad tipo péptidos antimicrobianos. La prepurificación de los péptidos se realizó por medio de HPLC, con una columna de fase reversa. El sobrenadante ácido de la hemolinfa fue cargado en una columna empacada con matriz C_{18}. Las eluciones escalonadas se realizaron con 5, 50, 80 y 100 % de acetonitrilo (ACN), en 0.05 % de ácido trifluoroacético (ATA), durante 550 min y un flujo de 0.2 ml/min. La absorbancia se monitoreó a 280 nm. Las fracciones eluídas se concentraron al vacío. *Vibrio alginolyticus* 138-2 se usó como modelo para probar la actividad antimicrobiana de las fracciones. Se observaron zonas de inhibición del crecimiento bacteriano en las fracciones con 5 y 50 % de ACN. Este es el primer reporte de actividad tipo péptido antimicrobiano en la hemolinfa de una especie de pectínido.

Palabras clave: *Argopecten ventricosus*, hemolinfa, inmunidad, péptido antimicrobiano, *Vibrio alginolyticus.*

Bacterial diseases, most of which are caused by bacteria belonging to the genus *Vibrio*, have been reported as a limiting factor in mollusc aquaculture (Sainz *et al*., 1999). Efforts have been done in the search of strategies to prevent the infestation of bacteria, as well as in the research of the immunological mechanisms of cultured organisms. The study of antimicrobial peptides (APs) is one of the mayor topics in the field of immunology because they represent a key element of the innate immune response of many organisms (Boman, 1995). APs are small molecules (less than 10 kDa) that tend to display a broad-spectrum antimicrobial activity and possess cationic charge at physiological pH (Boman, 1995). Their positive charge presumably facilitates interactions with the negatively charged bacterial membrane and/or acidic bacterial cell walls, whereas their amphibolic character enables membrane permeabilization (Nissen & Nes, 1997). Here we report the presence of antimicrobial peptide-like activity in the

hemolymph of the scallop *Argopecten ventricosus* (Sowerby II, 1842).

Organisms (150, 56.2 ± 2.1 mm) were collected from Bahia Magdalena, B.C.S., Mexico. In the laboratory, specimens were placed in 1,100 litre fibreglass tank containing areated seawater at 24±1 °C, 36 ‰ salinity and fed with a microalgae mixture.

Individuals were bled 1 ml from the posterior adductor muscle by inserting a 27-gauge needle attached to a 1 ml sterile plastic syringe. Samples from 150 specimens were pooled in sterile glass tubes and kept on ice. Hemolymph was diluted (v/v) with a solution of 0.1 % trifluoroacetic acid (TFA) in the presence of aprotinin (1.5 µM, final concentration) and phenylthiourea (20 µM, final concentration) (Charlet *et al*., 1996). Acidic extraction was performed for 30 min in an ice-cold water bath under gentle stirring, and the extract resultant was centrifuged at 10,000 x g for 20 min at 4 °C (Charlet *et al*., 1996).

Reverse phase HPLC was performed in an Äkta equipment (Pharmacia). After centrifugation, acidic supernatant was loaded into a 50 cm column (Pharmacia) packed with 5 ml of C_{18} matrix and equilibrated with acidified water (0.05 % TFA). Stepwise elutions were performed with 5, 50, 80, and 100 % acetonitrile (ACN) in 0.05 % TFA over 550 min at a flow rate of 0.2 ml/min. Absorbance was monitored at 280 nm. The eluted fractions corresponding to each ACN percentage were concentrated under vacuum (SpeedVac System AES 2010, Savant), reconstituted and pooled in 60 µl MilliQ water.

Cultures of *Vibrio alginolyticus* 138-2 were done in LB broth medium supplemented with 0.5, 1, 3, 5 and 8 % NaCl and grown with constant shaking at 37 °C for 0, 2, 4, 8, 12, 16, and 24 h. Each sample was grown separately in 1 ml volume (990 µl of LB broth plus 10 µl of the stock at –80 °C). Growth was estimated by reading absorbance of the cultures in a Spectronic Genesys 2 spectrophotometer at 580 nm.

Vibrio alginolyticus cells from a 24 h culture in LB broth were harvested by centrifugation (9,000 x *g* for 20 min, Beckman GS-15 R centrifuge), washed twice in a 0.5 % NaCl sterile saline solution and resuspended in the same solution to achieve an optical density (580 nm, Genesys 2 spectrophotometer) of 1. To assess the number of bacteria per mililiter, a count of viable colony forming units (CFU) was performed by 10-fold serial dilutions.

Antibacterial activity of reverse phase chromatography fractions was monitored by radial diffusion assay (Lehrer *et al*., 1991). A volume (50 µl) of diluted bacterial stock containing $\approx 10^6$ CFU was added to 8 ml of melted sterile LB-agarose (LB: 1 % bactotrypton, 0.5 % yeast extract, 0.5 % NaCl, 0.6 % agarose, pH 7.0). After hand shaking, the bacteria with LB-agarose were poured onto a compartment Petri dish. Five microliters of fractions, positive control (penicillin diluted 1:1000 in MilliQ water), and negative control (MilliQ water) were added in small wells (3 mm diameter). The plates were incubated for 24 h at 37 °C and the diameter of the clear zone surrounding the wells was measured with a ruler.

Vibrio alginolyticus can grow over NaCl concentrations from 0.5 to 8 %, showing faster growth response at lower salt content. In view of these results and due to the possible presence of some salt sensitive peptides in the eluted fractions (*Goldman et al., 1997;* Lee *et al*., 1997*)*, LB media supplemented with 0.5 % NaCl was chosen to grow the *Vibrio* strain in fractions tested. Inhibition of antimicrobial activity of cationic peptides by salt is not well known. It is possible that simple charge competition might inhibit the initial interactions between a cationic peptide and the negatively charged bacterial membrane (Lehrer *et al*., 1993).

Results (Fig. 1) showed a 12 mm diameter clear zone in fraction F_1 (5 % ACN), 21 mm in fraction F_2 (50 % ACN) and 30 mm in positive control (penicillin treatment). Negative control (MilliQ water), F_3 (80 % ACN), and F_4 (100 % ACN) fractions did not showed clearing zones. The antibacterial activity found in the hemolymph of *A. ventricosus* indicates that this species could have a marked antimicrobial peptide activity as it has been shown by another bivalve molluscs like *Mytilus edulis* Linnaeus, 1758 (Charlet *et al*., 1996), *Mytilus galloprovincialis* Lamark, 1819 (Mitta *et al*., 1999), *Modiolus modiolus* Linnaeus, 1758 (Haug *et al*., 2004), and *Mytilus edulis chilensis* Hopé, 1854 (Mercado *et al*., 2005). APs from marine bivalves have been normally isolated from the immune cells, haemocytes (Charlet *et al*., 1996; Mitta *et al*., 1999). However, antimicrobial peptide activity has been also detected in gills of *M. edulis chilensis* (Mercado *et al*., 2005) and in extracts from several tissues of *M. modiolus*, including plasma, haemocytes, labial palps, byssus, mantle, and gills (Haug *et al*., 2004).

In this paper we report for first time the presence of antimicrobial peptide-like activity in *A. ventricosus* hemolymph.

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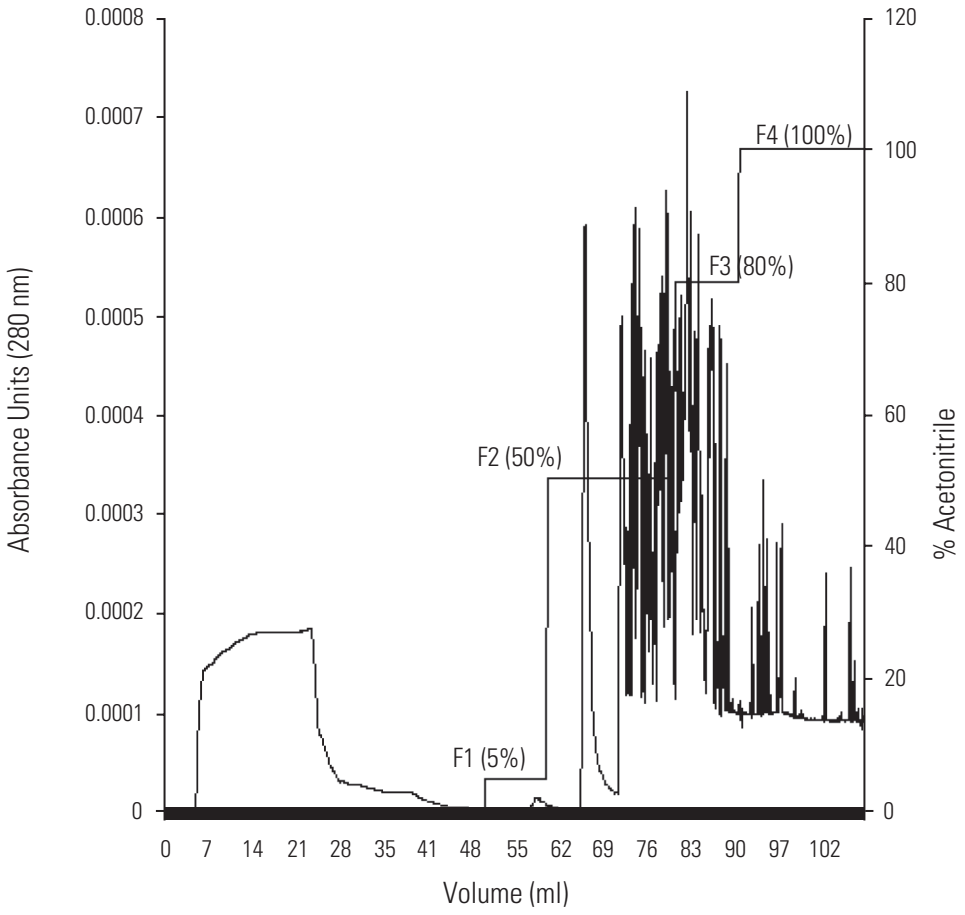

Figure 1. Reverse phase chromatography on packed C_{18} column. Gradient elution step from 0 to 100 % ACN, during 550 min and 0.2 ml/min flow. Fractions of 5 and 50 % ACN showed antibacterial activity against *Vibrio alginolyticus* 138-2.

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