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Note

Evaluation of antiviral activity in hemolymph from oysters *Crassostrea rhizophorae* and *Crassostrea gigas*

Márcia C. Carriel-Gomes[2], Jadel M. Kratz[1], Vanessa D.M. Müller[1], Célia R.M. Barardi[2] and Cláudia M.O. Simões[1,a]

[1] Laboratório de Virologia Aplicada, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, UFSC, Campus Universitário Trindade, Florianópolis, SC, Brazil
[2] Laboratório de Virologia Aplicada, Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina, UFSC, Campus Universitário Trindade, Florianópolis, SC, Brazil

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Abstract – This study describes the in vitro antiviral activity of two species of oyster hemolymph (*Crassostrea rhizophorae* and *C. gigas*) collected on Southern Brazilian coastline. Either the acellular and cellular fractions were tested against herpes simplex type-1 (HSV-1), human adenovirus respiratory strain (AdV-5) and simian rotavirus (RV-SA11) by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The evaluation of viral inhibition was performed using four different strategies: simultaneous, pre-infection and post-infection treatments and virucidal assays. The cellular fraction from *Crassostrea rhizophorae* showed the most prominent inhibition of HSV-1 and AdV-5 replication, particularly in post-infection treatment assay. No inhibition of rotavirus replication was observed. Both oyster hemolymph fractions showed virucidal activity against all tested virus in non-cytotoxic concentrations.

Key words: Antiviral / Oyster hemolymph / *Crassostrea rhizophorae* / *Crassostrea gigas* / Brazil

1 Introduction

Viral infections are common in crustaceans, but little is known about the ability of marine invertebrates to control infections. Differing from vertebrates, invertebrates do not produce specific antibodies, and may rely on innate defense for host protection against microorganism infections, including viral infections (Schapiro 1975; Schnapp et al. 1996).

Some reports have shown that several substances extracted from marine organisms, including bivalves, possess broad-spectrum antimicrobial activities (Nakamura et al. 1988; de Vries and Beart 1995; Mitta et al. 2000; Belaid et al. 2002; Zasloff 2002; Blunt et al. 2004). For instance, paolin, a substance isolated from alcoholic extracts of oyster tissues showed antiviral activity against herpesvirus (Prescott et al. 1966) and tachyplesin, a peptide isolated from horseshoe crabs, inhibited influenza A virus, vesicular stomatitis virus and human immunodeficiency virus (HIV-1), but not herpes simplex virus, adenovirus, reovirus and poliovirus (Murakami et al. 1991).

Additionally, Lee and Maruyama (1998) described a peptide isolated from *Crassostrea gigas* hydrolysate that inhibited HIV-1 protease, and a recent study demonstrated the antiviral activity of *C. gigas* acellular hemolymph against herpes simplex virus type-1 (HSV-1) and two fish viruses (Olicard et al. 2005a,b).

The present study describes the antiviral evaluation of oyster hemolymph fractions (cellular and acellular) derived from *Crassostrea rhizophorae* and *Crassostrea gigas* against HSV-1, human adenovirus respiratory strain (AdV-5) and simian rotavirus SA11 (RV-SA11).

2 Materials and methods

2.1 Sources of oysters and hemolymph preparation

One year-old oysters (*C. gigas and C. rhizophorae*) were obtained at Florianópolis City, Santa Catarina State, Brazil, respectively, from shellfish farms and from Laboratório de Cultivo de Moluscos Marinhos (LCMM) CCA/UFSC experimental cultive at the Brazilian Atlantic coastline. Both species were collected during the summer (March/2004).

Hemolymph collection: a small notch was carved in the dorsal side of the oyster shell, adjacent to the adductor muscle, and approximately 1 ml of hemolymph was collected from the adductor muscle sinus with a 27-gauge needle. Samples

[a] Corresponding author: claudias@reitoria.ufsc.br

were placed on ice immediately following collection to avoid aggregation.

The acellular fractions were obtained by centrifugation (2000 Xg, 4 °C, 2 min) and supernatant harvesting. The cellular fractions were obtained by suspension of the pellets in PBS buffer, sonication for 15 min (F60 Sonic Dismembrator, Fisher Scientific), centrifugation at 2000 Xg, 4 °C, for 2 min. The pellets (cell lysates) were collected and used as the cellular fractions.

Both acellular and cellular samples were filtered through a 0.22 μm filter (Millipore) and stored at −80 °C until use. Total concentrations of proteins in the extracts were determined by the Bradford method (Bradford 1976).

2.2 Cell culture and viruses

The following cell lines were used: Vero (African green monkey cells), HEp-2 (Human caucasian larynx carcinoma cells) and MA104 cells (Rhesus monkey kidney cells). All cell lines were grown in Minimum Essential Medium (MEM, Sigma) supplemented with 10% fetal bovine serum (FCS, Gibco BRL) and 1% of antibiotics PSA (100 IU ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin and 0.025 mg ml^{-1} amphotericin B; Gibco BRL). The cell cultures were maintained in a humidified atmosphere under 5% CO_2 at 37 °C.

The following viruses were used: Herpes Simplex Virus type 1 (HSV-1), KOS strain (Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Rennes, France); human adenovirus respiratory strain (AdV-5) and simian rotavirus SA11 (RV-SA11) (both from Biological Sciences Institute, University of São Paulo/USP, Brazil). HSV-1 and AdV-5 were propagated in Vero and HEp-2 cells, respectively; RV-SA11 was propagated in MA104 cells in the presence of trypsin (5 μg ml^{-1}, Sigma) (Estes et al. 1981). Stock viruses were prepared as described previously by Simões et al. (1999), and cell culture media were harvested from infected cells, titrated and stored at −80 °C until used. HSV-1 and AdV-5 titers were obtained by the plaque method and were expressed as plaque forming units per ml (pfu ml^{-1}) (Burleson et al. 1992). RV-SA11 titer was obtained by immunofluorescence assay and expressed as focus forming units per ml (ffu ml^{-1}) (Barardi et al. 1998).

2.3 Cytotoxicity evaluation

The cell viability was evaluated by a standard colorimetric assay for measuring cellular proliferation: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Mossmann 1983; Sieuwerts et al. 1995), with minor modifications.

Vero, HEp-2, and MA104 cell cultures (2×10^5 cells ml^{-1}) were prepared in 96-well tissue culture plates. After a 24 h incubation period at 37 °C in a humidified 5% CO_2 atmosphere, cell monolayer was confluent. The medium was removed from each well and replenished with 200 μl of different concentrations of oyster hemolymph fractions per well ranging from 7.81 to 1000 μg ml^{-1} of protein in medium. For cell controls, 200 μl of medium were added.

After 72 h, 96 h and 120 h of incubation for MA104, Vero and HEp-2 cells at 37 °C, respectively, the medium was removed and 50 μl of MTT solution (1 mg ml^{-1}, Sigma; prepared in MEM) were added to each well. Plates were incubated for 4 h at the same conditions cited above. The MTT solution was removed and 100 μl of dimethyl sulphoxide (DMSO, Nuclear) were added to each well to dissolve formazan crystals. After gently shaking the plates for 10 min, absorbance was read on a multiwell spectrophotometer (Bio-Tek, El × 800, USA) at 540 nm. The percentages of cytotoxicity were calculated as $[(A - B)/A \times 100]$, where A and B are the absorbances of control and treated cells, respectively. For each oyster hemolymph fractions, the concentration that reduced the absorbance of treated cells by 50% when compared to cell control was defined as 50% cytotoxic concentration (CC_{50}).

2.4 Antiviral assays

Antiviral assays were based upon cell viability using the MTT method. The percentages of protection were calculated as $[(A - B)/(C - B) \times 100]$, where A, B and C indicate the absorbance of the oyster hemolymph fractions, virus and cell controls, respectively. The concentration that reduced the absorbance of infected cells to 50% when compared to cell and virus controls was defined as the 50% effective concentration (EC_{50}).

Acyclovir [9-(2-hydroxyethoxymethyl) guanosine, Sigma, 10 μg ml^{-1}] was used as positive control for HSV-1 (KOS strain) inhibition, and no positive controls were used for the other two viruses inhibition since the treatment is only symptomatic.

The CC_{50} and EC_{50} concentrations were calculated from concentration-effect curves after linear regression analysis. The results were expressed using the selectivity index (SI = CC_{50}/EC_{50}).

2.4.1 Simultaneous treatment assay

Vero, HEp-2 and MA104 cultures (2×10^5 cells ml^{-1}) were prepared in 96-well tissue culture plates. After 24 h period of incubation at 37 °C in a humidified 5% CO_2 atmosphere, cell monolayers were confluent and the simultaneous treatment assay was performed (Takeuchi et al. 1991). The medium was removed from each well and replenished with 100 μl per well of non-cytotoxic oyster hemolymph fractions at different concentrations (below CC_{50} values) and 100 μl per well of each one of the following viruses (Multiplicity Of Infection, i.e. ratio of infectious virus particles to cells, MOI = 0.5): HSV-1, KOS strain; AdV-5; and RV-SA11 in the presence of trypsin (5 μg ml^{-1}). For cell and viral controls, 200 μl of MEM medium or viral suspension were added, respectively. Plates were incubated for different periods: 72 h, 96 h and 120 h for RV-SA11, HSV-1 and AdV-5, respectively.

2.4.2 Pre-infection treatment assay

This assay was carried out to determine whether oyster hemolymph fractions inhibited viral replication by affecting their adsorption or penetration on the host cells. Vero, HEp-2

Table 1. Cytotoxicity and antiviral activity of oyster hemolymph fractions, obtained by MTT assay, using different strategies of evaluation (simultaneous, pre- and post-infection treatments assays). Results are expressed in mg of protein per ml. CC_{50}: cytotoxic concentration for 50% of cells; EC_{50}: effective concentration to 50% of cells; SI: selectivity index (CC_{50}/ EC_{50}).

Oyster Hemolymph fractions	CC_{50}	EC_{50} Simul.	SI Simul.	EC_{50} Pre-	SI Pre-	EC_{50} Post-	SI Post-
Vero / HSV-1							
C. rhizophorae **c**	0.35 ± 0.06	-	-	-	-	0.02 ± 0.00	17.50
C. rhizophorae **a**	0.88 ± 0.46	-	-	-	-	0.12 ± 0.02	7.33
C. gigas **c**	0.34 ± 0.06	-	-	-	-	0.07 ± 0.02	4.86
C. gigas **a**	0.55 ± 0.08	-	-	-	-	0.39 ± 0.18	1.41
HEp-2 / AdV-5							
C. rhizophorae **c**	0.34 ± 0.04	0.18 ± 0.01	1.89	0.16 ± 0.07	2.13	0.06 ± 0.03	5.67
C. rhizophorae **a**	0.36 ± 0.03	0.18 ± 0.03	2.00	0.15 ± 0.02	2.40	0.09 ± 0.05	4.00
C. gigas **c**	0.19 ± 0.01	0.10 ± 0.01	1.90	0.11 ± 0.01	1.73	0.05 ± 0.01	3.80
C. gigas **a**	0.32 ± 0.06	0.16 ± 0.06	2.00	0.20 ± 0.01	1.60	0.06 ± 0.02	5.33

(-): Percentage of inhibition < 50%, consequently the SI values were not calculated; c: cellular fraction; a: acellular fraction. Values represent the mean ± standard deviation of three separate experiments.

and MA104 cultures (2×10^5 cells ml^{-1}) were prepared in 96-well tissue culture plates. After 24 h period of incubation at 37 °C in a humidified 5% CO_2 atmosphere, cell monolayers were confluent and the pre-infection treatment assay was performed. Non-cytotoxic concentrations (below CC_{50}) of oyster hemolymph fractions (100 μl per well) were added to the cells and incubated for 3 h prior to virus infection (100 μl of each virus per well, MOI = 0.5) (Bettega et al. 2004). For cell and viral controls, 200 μl of MEM medium or viral suspension were added, respectively. Plates were incubated for different periods: 72 h, 96 h and 120 h for RV-SA11, HSV-1 and AdV-5, respectively.

2.4.3 Post-infection treatment assay

This assay was performed to determine if the oyster hemolymph fractions inhibited viral replication when viruses have already infected cells. Cells were prepared using the same method described above and the post-infection treatment was performed. After cell monolayers confluence (24 h, 37 °C, 5% CO_2), viruses were added to the cells (200 μl per well of viral suspension, MOI = 0.5) and incubated during 2 h at 37 °C (Hudson 1999). Viral inocula were then removed and 200 μl of non-cytotoxic concentrations of oyster hemolymph were added. For cell and viral controls, 200 μl of MEM medium or viral suspension were added, respectively. Plates were incubated for different periods: 72 h, 96 h and 120 h for RV-SA11, HSV-1 and AdV-5, respectively.

2.4.4 Virucidal assay

In order to evaluate possible extracellular viral inactivation by oyster hemolymph fractions, a virucidal assay was performed according to Bettega et al. (2004). Equal volumes (100 μl) of the virus stock suspension and different concentrations below the CC_{50} of the oyster hemolymph fractions were mixed and incubated for 2 h at 37 °C. Each mixture was then serially diluted 10-fold and 200 μl of each dilution were added to the cell monolayer. Plates were incubated for different periods: 72 h, 96 h and 120 h for RV-SA11, HSV-1 and AdV-5, respectively, and the virus titers were calculated using the end-point determination (Reed and Muench 1938).

2.5 Data analysis

The CC_{50} and EC_{50} values were estimated from concentration-effect curves after linear regression analysis, and represent mean ± standard deviation of the mean values of three independent experiments. The percentages of viral inhibition from oyster hemolymph fractions in relation to each tested virus represent mean ± standard deviation of the mean values of three different experiments. ANOVA/Tukey tests ($p < 0.05$) were carried out as appropriate.

3 Results

3.1 Cytotoxicity evaluation

The assessment of cytotoxicity was performed with oyster hemolymph fractions ranging from 0.008 to 1 mg of protein ml^{-1}. Plates were incubated for a period corresponding to four cycles of viral replication (72 h, 96 h and 120 h for MA104, Vero and HEp-2 cells, respectively). The CC_{50} values for Vero and HEp-2 cells are listed (Table 1). All

oyster hemolymph fractions evaluated showed no cytotoxicity to MA104 cells, except for the acellular fraction from *C. rhizophorae* (CC_{50} = 0.86 ± 0.15 mg ml^{-1}).

3.2 Antiviral evaluation

3.2.1 Simultaneous and Pre-infection treatment assays

Both hemolymph fractions from *C. rhizophorae* and *C. gigas* did not inhibit 50% of HSV-1 and RV-SA11 replication. SI values were not calculated. After 120 h of treatment, the maximum percentage of AdV-5 inhibition was obtained with 0.20 mg ml^{-1} of the cellular fraction from *C. rhizophorae*, which inhibited 63.93% (simultaneous) and 63.84% (pre-infection) of AdV-5 replication (Fig. 1). In simultaneous treatment assay, SI values ranged from 1.89 (*C. rhizophorae* cellular fraction) to 2.00 (*C. rhizophorae* and *C. gigas* acellular fraction), and in pre-infection treatment assay, SI values ranged from 1.73 (*C. gigas* cellular fraction) to 2.40 (*C. rhizophorae* acellular fraction) (Table 1).

3.2.2 Post-infection treatment assay

Both hemolymph fractions from *C. rhizophorae* and *C. gigas* did not inhibit 50% of RV-SA11 replication. SI values were not calculated. After 96 h of treatment, the maximum percentage of HSV-1 inhibition was obtained with 0.20 mg ml^{-1} of the cellular fraction from *C. rhizophorae*, which inhibited 75.47% of HSV-1 replication (Fig. 1), with a low EC_{50} value (0.02 ± 0.004 mg ml^{-1}) resulting in a SI value of 17.50 (Table 1). After 120 h of treatment, the maximum percentage of AdV-5 inhibition was obtained with 0.20 mg ml^{-1} of the cellular fraction from *C. rhizophorae*, which inhibited 76.33% of AdV-5 replication (Fig. 1). SI values ranged from 3.80 (*C. gigas* cellular fraction) to 5.67 (*C. rhizophorae* cellular fraction) (Table 1).

3.2.3 Virucidal assay

The preincubation of viruses with both oyster hemolymph fractions for 2 hours at 37 °C prior to addition to the cell monolayers protected >90% of Vero, HEp-2 and MA104 cells against HSV-1, AdV-5 and RV-SA11 infection (Fig. 1), respectively.

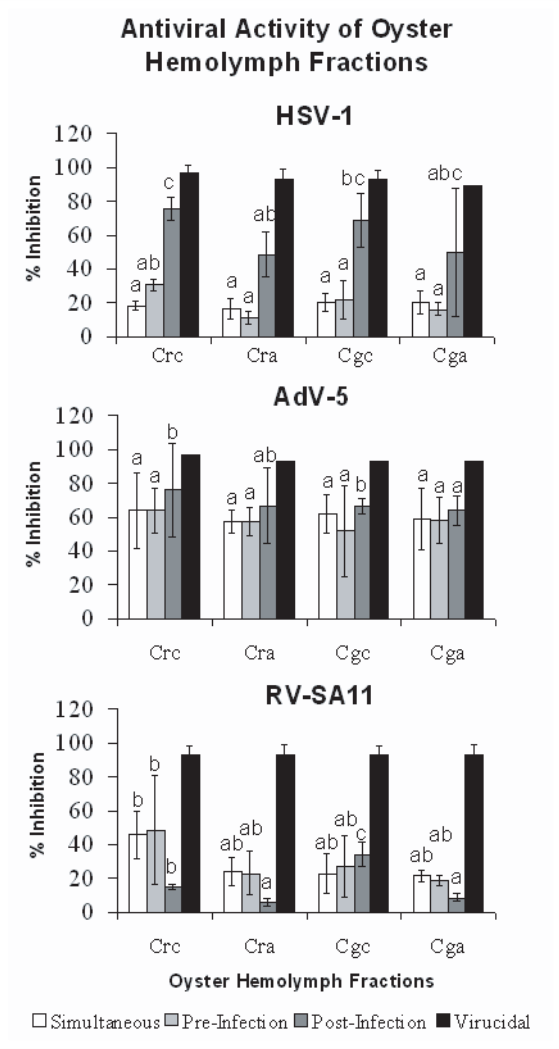

Fig. 1. Comparison among the percentages of inhibition of the different tested viruses by oyster hemolymph fractions, obtained by MTT assay, using different strategies of evaluation (simultaneous, pre- and post-infection treatments and virucidal assays). Each bar represents the mean of percentage of inhibition of the highest concentration evaluated ± standard deviation of the mean values of three separate experiments. ANOVA/Tukey tests ($p < 0.05$) were carried out as appropriate. Same letters indicate no significant statistical difference among means of the same treatment group. Cr: *Crassostrea rhizophorae*; Cg: *Crassostrea gigas*; c: cellular fraction; a: acellular fraction.

4 Discussion

The search for antiviral molecules from marine organisms can be based on the evaluation of the inhibition of viral replication by extracts of the whole organism or part of it, for instance, the hemolymph (Bergé et al. 1999; Olicard et al. 2005a,b).

In our study we observed that cellular fraction from *Crassostrea rhizophorae* hemolymph showed the most prominent inhibition of HSV-1 and AdV-5 replication, particularly in post-infection treatment assay. The putative compounds within this fraction probably act to inhibit the late stages of viral replication (Hudson 1999), but the reason why SI values are higher in the post-infection treatment rather than in the simultaneous treatment is unknown.

Oyster hemolymph fractions did not inhibit RV-SA11 replication when viruses infected cell monolayers, although they showed an important virucidal activity, as also observed for HSV-1 e AdV-5 (inhibition above 90%) suggesting that oyster hemolymph fractions may exert viral inactivation.

Regarding *Crassostrea gigas* hemolymph, a recent study (Olicard et al. 2005a) described the anti-HSV-1 activity from an acellular fraction of hemolymph, evaluated by the neutral red dye method, using the Vero cell/HSV-1 model. The authors discussed that the mode of action could not be precisely

determined due to a high degree of cytotoxicity, resulting in a low selective index (1.76). After 48 h of treatment, the CC_{50} and EC_{50} values were 0.75 and 0.42 mg ml^{-1}, respectively. In our study, the acellular fraction from *C. gigas* hemolymph showed a very similar SI value (Table 1) when added to the culture 2h after the infection, in despite of the different period of incubation and MOI used. After 96 h of treatment, the CC_{50} and EC_{50} values were 0.55 and 0.39 mg ml^{-1}, respectively. The major disparity was observed in the virucidal assay. Although the preincubation periods were different (1 h in the mentioned study and 2 h in ours), such contrasting results (no protection and >90% of protection, respectively) were not expected. Consequently a confirmatory evaluation, comparing both approaches, is desirable.

In conclusion, oyster hemolymph fractions from *C. gigas and C. rhizophorae* demonstrated antiviral activity for three different viruses especially in the virucidal assay. Since the specific antiviral mechanism is unknown, further investigations intending to fractionate and identify the nature of the molecules responsible for the observed activities seem warranted.

According to our survey in the literature, this is the first report of a biological activity from *C. rhizophorae*, a native Atlantic species that occurs among the roots of mangrove trees in Brazil (Ignacio et al. 2000; Lapégue et al. 2002).

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