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Toxic, antimicrobial and hemagglutinating activities of the purple fluid of the sea hare *Aplysia dactylomela* Rang, 1828

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Abstract

The antimicrobial, hemagglutinating and toxic activities of the purple fluid of the sea hare *Aplysia dactylomela* are described. Intact or dialyzed purple fluid inhibited the growth of species of Gram-positive and Gram-negative bacteria and the action was not bactericidal but bacteriostatic. The active factor or factors were heat labile and sensitive to extreme pH values. The fluid preferentially agglutinated rabbit erythrocytes and, to a lesser extent, human blood cells, and this activity was inhibited by the glycoprotein fetuin, a fact suggesting the presence of a lectin. The fluid was also toxic to brine shrimp nauplii (LD_{50} 141.25 µg protein/ml) and to mice injected intraperitoneally (LD_{50} 201.8 ± 8.6 mg protein/kg), in a dose-dependent fashion. These toxic activities were abolished when the fluid was heated. Taken together, the data suggest that the activities of the purple fluid are due primarily to substance(s) of a protein nature which may be involved in the chemical defense mechanism of this sea hare.

Key words
- *Aplysia*
- Antimicrobial
- Purple fluid
- Toxicity
- Hemagglutinating activity

Introduction

Many sea hares, which are opistobranch molluscs, discharge a fluid from the purple gland when disturbed. This reaction suggests that this fluid contains bioactive factors which may act against potential enemies since the defense mechanisms of the sea hare differ from those of highly developed vertebrates (1). Sea hare species have attracted the interest of many workers investigating the chemical compounds secreted by the purple gland or present in different tissues, possibly involved in the defense of these invertebrates. Thus, some sea hare species have been shown to contain low molecular mass substances with antimicrobial (2-6) and antitumor activities (7-10) and also high molecular mass compounds such as those from *Aplysia kurodai*, *Aplysia juliana* and *Dolabella auricularia* with similar activities, and which were named aplysianins (11,12), julianins (13,14) and dolabellanins (15,16), respectively. This study describes some biological properties (antibacterial, antifungal, hemagglutinating and toxic activities) of the purple fluid of the sea hare *Aplysia dactylomela*, which is an opistobranch widespread along the Brazilian coast and also occurring in the intertidal zones from Southern Florida in the United States to Eastern India (17).

Material and Methods

Collection of the purple fluid

Specimens of *Aplysia dactylomela* Rang, 1828 were collected at Pacheco Beach, Caucaia, State of Ceará, Brazil, in June and July. The purple fluid was obtained by irritating the hare and squeezing it gently for a few minutes outside the water. The secretion was collected into a sterile bottle and subsequently frozen at -10°C until used.

Protein determination

A manual colorimetric procedure for measuring ammonium nitrogen in Kjeldahl digests (18) was used for the determination of total nitrogen and protein content, which was calculated using a nitrogen conversion factor of 6.25.

Antibacterial assays

Inhibition of bacterial growth by the purple fluid samples was determined as described by Bauer et al. (19). Briefly, bacterial cultures were maintained in Müller-Hinton broth (Difco Laboratories, Detroit, MI). Sterile swabs were immersed in the microbial suspensions (10^8 cells/ml) and evenly applied to Petri dishes containing Müller-Hinton agar. Sterile Whatman AA filter paper disks (6 mm in diameter) were fully imbibed with 30 µl of the purple fluid samples and placed over the agar in the plates. Tobramicin disks (10 µg; Cecon, São Paulo, SP) were used as positive control. The plates were incubated overnight at 35°C and then examined for zones of growth inhibition around each disk.The bacteria used were *Serratia marcescens, Citrobacter freundii, Vibrio cholerae, Salmonella thyphimurium* and *Proteus vulgaris* (all from the collection of Universidade Federal do Ceará), *Bacillus subtillis* (ATCC 6633), *Escherichia coli* (ATCC 13863), *Staphylococcus aureus* (ATCC 6538), and *Pseudomonas aeruginosa* (ATCC 25619). To investigate whether the antibacterial action was bacteriostatic or bactericidal, fluid samples were serially diluted in 1% peptone broth and incubated with cells of *Staphylococcus aureus* and *Pseudomonas aeruginosa* for 18 h at 35°C. After this period, the minimum inhibitory concentration (MIC) was determined (20) and the mechanism of growth inhibition evaluated by subculturing the cells in media without purple fluid.

Antifungal assays

Growth inhibition of *Candida albicans* and *Saccharomyces cerevisiae* (all from the UFC collection) by the purple fluid was determined as described by Roberts and Selitrennikoff (21). Briefly, agar assay plates were prepared by autoclaving agar Sabouraud medium (Difco Laboratories). After cooling to 45°C, the yeasts were added to a final concentration of 10^7 cells/ml. Fifteen-milliliter aliquots of the suspension were dispensed into 100-mm diameter Petri dish and allowed to solidify before placing 6-mm diameter sterile paper disks on the surface of the agar. Thirty microliters of the purple fluid was added to each disk, and plates were incubated overnight at 35°C. Plates were examined as described for the antibacterial assay. Hyphal extension inhibition assays were done essentially as described by Mirelman et al. (22). Thus, hyphae-containing agar plugs (*Aspergillus niger*, *Penicillium herguei* and *Trichophytum mentagrophytes* - all from UFC collection) were placed in the center of agar plates and the test samples were added to wells surrounding the plugs. Plates were incubated at 30°C for 48-72 h and examined for crescents of hyphal inhibition. Nistatin (10,000 IU; Neoquímica, Anápolis, GO) was used as positive control.

Effects of dialysis, heat treatment and pH on antibacterial activity

Aliquots of the fluid were dialyzed (cut-off 12,000) thoroughly against water, at 4ºC, and subsequently tested for antibacterial activity (*Pseudomonas aeruginosa* and *Staphylococcus aureus*), as described before. Fluid samples were heated at 80ºC for 2, 5 and 15 min. After heating, the samples were cooled and centrifuged and the supernatants tested for activity. For the pH stability test, aliquots were adjusted to pH 2.0 (HCl) and 12.0 (NaOH) and kept in a refrigerator for up to 30 min. After this period, the fluid had its pH adjusted back to its original value of 6.4 and was tested for antibacterial activity.

Erythrocyte agglutination and inhibition assays

The hemagglutinating activity was assayed according to Vasconcelos et al. (23). Serial 1:2 dilutions of the fluid dialyzed against 25 mM Tris-HCl, pH 7.5, were mixed in small glass tubes with 0.25 ml of a 2% suspension of untreated or trypsin-treated erythrocytes (horse, chicken, pig, cow, rabbit or human). The enzyme-treated cells were obtained by incubation of trypsin (0.1 mg; Type I, Sigma Chemical Co., St. Louis, MO) with 25 ml of a 2% suspension of cells in 150 mM NaCl for 60 min at 4ºC. After washing six times, a 2% suspension was prepared in 150 mM NaCl. The extent of agglutination was monitored visually after the tubes had been left at 37ºC for 30 min and subsequently at room temperature for a further 30 min. The results are reported as the number of hemagglutination units (HU) per mg of fluid protein able to induce visible erythrocyte agglutination. One HU was defined as the minimum protein concentration required to produce visible agglutination. The carbohydrate-binding specificity of the protein was assessed by the ability of sugars or glycoproteins in 150 mM NaCl to inhibit agglutination of rabbit erythrocytes. The fluid was added to each tube at a concentration of 0.4 µg protein/ml, the minimum concentration required to produce visible agglutination. The lowest glycoprotein or sugar concentration giving full inhibition of agglutination was determined by two-fold serial dilution of solutions at 1 mg/ml initial concentration.

Toxicity bioassay against brine shrimp nauplii

A method using brine shrimp (*Artemia* sp), proposed as a simple bioassay for research on natural products, was employed (24). Brine shrimp eggs (5 mg) were hatched in a rectangular dish (32 x 22 x 10 cm) filled with 5 l of sea water. A plastic sieve was clamped to the dish to form two unequal compartments. The eggs were sprinkled into the larger one which was darkened, while the smaller one was illuminated. After 48 h, the phototrophic larvae (nauplii) were collected with a pipette from the lighted side and transferred (10 shrimps) to vials filled with sea water (5 ml) containing 1 drop of casein peptone solution (3 mg/5 ml) as food. Dialyzed fluid was added to the vials to final concentrations of 50, 500 and 5000 µg protein/ml. As control, a group of vials was filled with sea water containing the casein peptone solution. The vials were kept illuminated during 24 h of contact with the substances, and survivals were counted with the aid of a magnifying glass. This assay was carried out three times with five replicates for each fluid concentration tested. To calculate the LC_{50} (mean lethal concentration) the results were plotted as logit % mortality *vs* log concentration. Logit is defined as ln (% mortality/% survival) (25).

Mouse toxicity assay

Toxic activity was defined as mortality observed in mice within 24 h after intraper-

itoneal injections of the fluid exhaustively dialyzed against 25 mM Tris-HCl, pH 7.5. One LD_{50} unit (26) was taken as the amount of protein (in mg protein/kg body weight) producing death of 50% of the tested animals (six doses; six mice per dose).

SDS-polyacrylamide gel electrophoresis

Electrophoresis of the crude and dialyzed purple fluid was carried out by the method of Laemmli (27) on a 2-mm vertical slab gel consisting of stacking gel mix, 3.95% total acrylamide, and main running gel mix, 12.0% acrylamide. Fluid samples containing 2% SDS and 1% 2-mercaptoethanol were incubated at 100°C for 10 min. A few sucrose crystals were dissolved in the samples before being applied (30 µl) to the gel. Electrophoresis was carried out at 20-mA constant current for 60 min and protein bands were visualized by silver staining (28). The molecular mass markers used were α-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24.0 kDa), carbonic anhydrase (29.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), egg albumin (45.0 kDa) and bovine serum albumin (66.0 kDa).

Table 1 - Antibacterial activity of the crude (0.48 mg protein) and dialyzed (0.30 mg protein) purple fluid of *Aplysia dactylomela.*

Data are reported as the mean ± SD for 3 experiments carried out in duplicate. [a]Nitrofurantoin for *Vibrio cholerae* and tobramicin for all the others.

Bacteria	Inhibition zone diameter (mm)		
	Crude fluid	Dialyzed fluid	Control[a]
Gram-positive			
Bacillus subtilis	12.0 ± 2.6	9.3 ± 1.2	31.0 ± 1.3
Staphylococcus aureus	16.8 ± 4.6	16.6 ± 4.2	25.0 ± 1.1
Gram-negative			
Salmonella thyphymurium	10.7 ± 1.5	10.7 ± 2.3	22.0 ± 1.5
Citrobacter freundii	12.5 ± 0.7	11.5 ± 0.7	23.0 ± 1.1
Vibrio cholerae	13.5 ± 0.6	13.5 ± 0.6	26.0 ± 1.3
Escherichia coli	14.0 ± 1.7	11.6 ± 3.2	22.0 ± 1.6
Serratia marcescens	14.0 ± 1.2	11.4 ± 3.0	17.0 ± 1.2
Proteus vulgaris	17.1 ± 3.8	14.3 ± 2.9	24.0 ± 2.2
Pseudomonas aeruginosa	19.7 ± 2.1	11.5 ± 0.7	34.0 ± 2.4

Results and Discussion

Both the crude (16.0 ± 0.7 mg protein/ml) and the dialyzed purple fluid (10.1 ± 0.6 mg protein/ml) inhibited the growth of all species of Gram-positive and Gram-negative bacteria tested, with *Staphylococcus aureus, Pseudomonas aeruginosa* and *Proteus vulgaris* being the most sensitive (Table 1). The antibacterial activity of the fluid against *P. aeruginosa* was reduced by 40% after dialysis, suggesting that low molecular mass components are involved in this activity. The fluids stopped the growth of *S. aureus* and *P. aeruginosa* but the bacteria grew again after their removal from culture, indicating a bacteriostatic and not a bactericidal action of the fluids, with a minimum inhibitory concentration of 0.625 mg protein/ml. The heat treatment of the fluid at 80°C for 2 min eliminated the inhibitory activity against *P. aeruginosa* and *S. aureus* (the species selected as target cells). Similarly, after acid treatment at pH 2.0 the inhibitory action against the two species was completely lost. Nevertheless, after alkaline treatment (pH 12.0) the inhibitory action against *P. aeruginosa* was lost while that against *S. aureus* was only reduced (the inhibition zone of 16.8 ± 4.6 mm was reduced to 9.0 ± 0.9 mm). Taken together, these data suggest that the active factor(s) probably is(are) protein. In fact, Yamazaki et al. (29) have reported that a glycoprotein is responsible for the antibacterial activity of the purple fluid of *Aplysia kurodai*. Likewise, other antibacterial glycoproteins have been reported to be present in different secretions of sea hares (14,30,31).

The fluid was not active against the yeasts *Candida albicans* or *Saccharomyces cerevisiae* nor against the filamentous fungi *Aspergillus niger*, *Penicillium herguei* and *Trichophytum mentagrophytes*.

The results of the hemagglutination assays are shown in Table 2. The dialyzed fluid preferentially agglutinated rabbit erythrocytes and, to a lesser extent, human erythrocytes

(ABO). Treatment of the cells with trypsin revealed the agglutinating activity of the fluid against chicken erythrocytes and increased the sensitivity of human cells. Nevertheless, no activity was detected when the fluid was tested against cow, pig and horse erythrocytes, even when using enzyme-treated cells. This selective agglutination may be due to the different nature of the glycoproteins protruding on the cell surface of the erythrocytes tested. The activity of the fluid varied from 0.4 to 11.4 µg/ml depending on the cell used, thus being comparable in potency to the agglutinin purified from *Aplysia kurodai* eggs, which was shown to react with B cells and rabbit blood cells at concentrations as low as 0.06 µg/ml (32). These authors also reported agglutinins in the serum of *Aplysia dactylomela* which strongly agglutinated human erythrocytes, but reacted weakly with rabbit blood cells, contrary to that observed in the present study. These findings suggest that the hemagglutinating activity may be due to different proteins or that other constituents of these fluids may interfere with this activity. Various simple sugars were reported to be potent hemagglutinin inhibitors (33). In the present study, the agglutination of rabbit erythrocytes by the fluid was inhibited by the glycoprotein fetuin, but not by glucose, mannose, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine or sialic acid (Table 3).

The purple fluid was shown to be toxic to the brine shrimp nauplii, with a calculated LD_{50} of 141.25 µg protein/ml. This effect was dose dependent and despite its unknown mechanism, may well involve the fluid lectin as observed for lectins of plant origin, such as those of *Dioclea guianensis* (23) and *Cratylia floribunda* (34).

The fluid was also highly toxic to mice when injected intraperitoneally (*ip*), within 1 to 12 h, depending on the dose used. The LD_{50} found was 201.8 ± 8.6 mg protein/kg body weight (20 ml of fluid/kg body weight). The typical effects observed invariably included dyspnea and convulsions preceding the death of the animals. These acute effects were very similar to those produced by soyatoxin (SYTX), a seed protein purified from mature commercial soybean sold in Brazil, which is a mixture of undefined cultivars (35). The toxic activity present in the fluid was susceptible to inactivation by heating at 92°C for 5 min.

The electrophoretic profile of the crude and dialyzed fluids (Figure 1) showed a simi-

Table 2 - Agglutination of erythrocytes from various species by the purple fluid of *Aplysia dactylomela.*

[a]One hemagglutination unit (HU) was defined as the minimum protein concentration required to produce visible agglutination. [b]Trypsin treated. n.d., Not detected.

Erythrocytes	Hemagglutinating activity (HU/mg of fluid protein)[a]	
	Untreated	Treated[b]
Human		
A	344	1,428
B	87	357
O	87	357
Rabbit	2,500	2,500
Chicken	n.d.	2,500
Cow	n.d.	n.d.
Pig	n.d.	n.d.
Horse	n.d.	n.d.

Table 3 - Inhibition of hemagglutinating activity.

Assays were done using untreated rabbit cells. [a]Minimal concentration of sugar or glycoprotein required to inhibit 1 HU. n.d., Not detected with the initial concentration of 1 mg/ml.

Sugar or glycoprotein	Minimal concentration (mg/ml)[a]
Galactose	n.d.
Glucose	n.d.
Mannose	n.d.
N-acetyl-galactosamine	n.d.
N-acetyl-glucosamine	n.d.
Sialic acid	n.d.
Fetuin	0.2

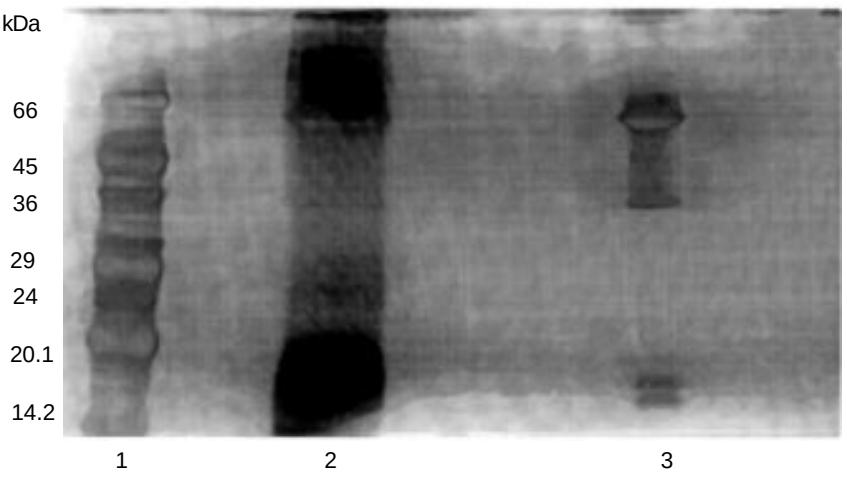

Figure 1 - SDS-polyacrylamide gel electrophoresis of the crude and dialyzed purple fluid of the sea hare *Aplysia dactylomela*. Lane 1, Standard protein markers; lane 2, crude fluid; lane 3, fluid dialyzed against water.

lar distribution of the protein bands. In both fluid samples there was predominance of proteins with apparent molecular mass of 66.0 kDa and below 20.1 kDa. Nevertheless, in the dialyzed fluid proteins between 18.0 kDa and 36 kDa were not observed. This presumably could be due to the different amounts of protein applied in the electrophoresis, since it was our intention to maintain the same volume (30 µl) used in the antibacterial assay.

These preliminary data do not allow us to establish that all of these interesting activities presented by the purple fluid of *Aplysia dactylomela* are due to the same component (s). Further studies are needed on this point. Nevertheless, the protein nature of the active component(s) is clear, as also is the presence of a lectin whose hemagglutinating activity is inhibitable by fetuin, a specific glycoprotein. This is the first time that lectins are reported to be present in the purple fluid of sea hares and also that the purple fluid is toxic to living systems such as brine shrimp nauplii and mice. This study supports the role of the fluid as part of a chemical defense mechanism since it inhibited the growth of many Gram-positive and Gram-negative bacteria and showed toxicity to other living systems.

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