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Cytotoxic and antibacterial properties of *Mytilus galloprovincialis*, *Ostrea edulis* and *Crassostrea gigas* (bivalve molluscs) hemolymph

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Abstract

Mussel (*Mytilus galloprovincialis*) plasma contains cytotoxic activity against both vertebrate (erythrocytes and mouse tumour) and protozoan cells. Procaryotes (*Escherichia coli* and *Vibrio alginolyticus*) were not sensitive to the cytotoxicity. The activity was still present in dialyzed samples but was inhibited by heating at 45°C. Large individual variability which was not correlated with protein concentration and an increasing number of reactive specimens following injection, suggested that naive mussels were in various stages of immune response. Purification by anion exchange chromatography followed by gel filtration revealed a 320 kDa cytotoxic polymeric protein that acts through a polymerization process after binding onto target cell membranes as revealed by ultrastructural observation.

European and Pacific oysters (*Ostrea edulis* and *Crassostrea gigas*) expressed antibacterial activity against both Gram negative and Gram positive bacteria which was most probably due to small proteins. When tested against the marine pathogenic *Vibrio alginolyticus*, hemocyte lysates of both species were more active than cell-free plasma. Antibacterial activity showed significant individual variability that was dramatically reduced by stimulation through mechanical stress or injection. The number of spontaneously active Pacific oysters increased from 50 to 100% following a single injection of bacteria.

These results strongly support the view that bivalve molluscs possess sensitive immuno-defense mechanisms that will greatly aid the development of aquaculture systems by employing refined techniques of transgenesis.

Keywords: Cytotoxicity, antibacterial, hemolymph, hemocyte lysate, mussel, oyster, *Mytilus galloprovincialis*, *Crassostrea gigas*, *Ostrea edulis*.

Activités cytotoxique et antibactérienne présentes dans l'hémolymphe de Mytilus galloprovincialis, Ostrea edulis *et* Crassostrea gigas *(mollusques bivalves).*

Résumé

Le plasma de la moule *Mytilus galloprovincialis* possède une activité cytotoxique dirigée à la fois contre des cellules de vertébrés (hématies et cellules tumorales de souris) et contre des protozoaires. Les cellules procaryotes (*Escherichia coli* et *Vibrio alginolyticus*) ne sont pas sensibles à l'activité cytotoxique. L'activité est toujours présente après dialyse des échantillons mais est inhibée par chauffage à 45 °C. L'existence d'une importante variabilité individuelle, non corrélée à la concentration protéique, ainsi que l'augmentation du nombre des animaux présentant une activité à la suite d'injection, suggèrent que les moules prélevées dans la nature sont déjà plus ou moins engagées dans la réponse immunitaire. La purification par chromatographie échangeur d'anions suivie de filtration sur gel, révèle une protéine cytotoxique multimérique de 320 kDa. Des observations en microscopie électronique montrent que cette protéine agit par polymérisation après fixation sur la membrane des cellules cibles.

L'huître plate (*Ostrea edulis*) et l'huître creuse (*Crassostrea gigas*) possèdent une activité antibactérienne dirigée à la fois contre les bactéries Gram négatif et Gram positif. Cette activité est très probablement due à de petites protéines. Chez les deux espèces, le lysat des hémocytes est plus actif que le plasma envers la bactérie marine pathogène *Vibrio alginolyticus*. L'activité antibactérienne présente une variation

individuelle importante mais cette variation est très réduite chez les animaux stimulés par stress mécanique ou injection. Le pourcentage d'huîtres creuses qui sont actives passe de 50 à 100 % à la suite d'une seule injection de bactéries.

Ces résultats suggèrent fortement que les mollusques bivalves possèdent des mécanismes de défense immunitaire qui, grâce à l'application de techniques de transformation génétique, pourront être d'une grande utilité pour le développement de l'aquaculture.

Mots-clés : Cytotoxicité, antibactérien, hémolymphe, lysat d'hémocytes, moule, huître, *Mytilus galloprovincialis*, *Crassostrea gigas*, *Ostrea edulis*.

INTRODUCTION

In bivalve molluscs, cellular immune reactions are largely mediated through circulating hemocytes which are morphologically heterogeneous (Cheng, 1981). Based upon light and electron microscopy, hemocytes of the mussel *Mytilus edulis* have been classified into two main types: granular and agranular or hyalin cells (Moore and Lowe, 1977; Rasmussen *et al.*, 1985; Adema *et al.*, 1991). Despite the remaining controversy over classification, it is possible to recognize different subtypes of granulocytes based on light microscopic observation: the type I granulocytes or basophilic cells or macrophages and the type II or eosinophilic granulocytes (*see* Sima and Vetvicka, 1990 for review). Ultrastructural techniques associated with analysis of lectin binding properties and the study of hydrolytic enzymes contained in the granules have also led to the description of two granulocyte types with different granule size (Pipe, 1990*a*, *b*; Noël *et al.*, 1994).

Granulocytes play an important role in internal defence reactions through their phagocytic and cytotoxic capacities. An oxidative killing mechanism has been investigated extensively using, among others, the technique of luminol-dependent chemiluminescence. Thus, respiratory burst and generation of reactive oxygen intermediates have been demonstrated in the hemocytes of oysters (Larson *et al.*, 1989; Bachère *et al.*, 1991), scallops (Le Gall *et al.*, 1991) and mussels (Pipe, 1992; Noël *et al.*, 1993). Moreover, hemocyte-mediated cytolytic activity, due to the presence of lipase, has been described in the clam, *Corbicula fluminea* (Yoshino and Tuan, 1985). In mussels, functional studies using mammalian cytokines and anti-cytokine antibodies, indicate that *Mytilus* hemocytes respond to interleukin 1 (IL-1) and tumour necrosis factor (TNF) suggesting that cell activation pathways utilizing similar molecules have been conserved during evolution (Hughes *et al.*, 1990, 1991).

In many invertebrate species, several kinds of immune-related humoral activities have been reported. Bivalves possess several types of so-called non-specific humoral defence molecules including agglutinins and glycoproteins that have opsonizing properties (Johnson, 1964; Arimoto and Tripp, 1977; Renwrantz and Stahmer, 1983) that can be related to the lectin group (Pipe, 1990*a*), bactericidins (Mori *et al.*, 1980) and lysosomal enzymes (Cheng, 1983). Moreover, lysozyme-like activity has been found in numerous bivalve species (Cheng and Rodrick, 1974; Cheng *et al.*, 1975; McHenery *et al.*, 1986; Takahashi *et al.*, 1986). Factors also related to serine proteases have been demonstrated in oyster and mussel hemolymph (Bachère *et al.*, 1990; Pipe, 1990*b*) as the cytolytic activity in *C. fluminea* (Yoshino and Tuan, 1985) which can be regarded as humoral components of immune defence.

In this paper, the results of studies on hemocyte-derived humoral defence activities in marine bivalves are described. In the mussel, *Mytilus galloprovincialis*, cytolytic/cytotoxic activity has been analyzed. Vertebrate erythrocytes have been used extensively to demonstrate the presence of cytotoxic molecules in invertebrate extracts because of rapidity, simplicity and unequivocal results obtained with the hemolytic assay. In *M. edulis*, an adaptation of the *in vitro* plaque assay has been employed to demonstrate the presence of agglutinins and cytolytic molecules in plasma (Wittke and Renwrantz, 1984; Leippe and Renwrantz, 1985) and the secretory role of hemocytes (Leippe and Renwrantz, 1988).

In oysters, interactions with parasites have only been recently investigated, both in terms of mortality (Burreson *et al.*, 1994) and host immune responses that involve lysozyme (Chu and LaPeyre, 1989), circulating and tissue-infiltrating hemocytes (Ford *et al.*, 1993), plasma hemagglutinin (LaPeyre *et al.*, 1995), iron-binding proteins (Gauthier and Vasta, 1994) and the role of stress proteins (Tirard *et al.*, 1995). Marine bacteria, particularly those that belong to the Vibrionaceae family, cause mass morbidities and mortalities in commercially-cultured marine bivalves (*see* Bachère *et al.*, 1995 for review); we have therefore investigated natural and inducible antibacterial activity in the two oyster species, *Ostrea edulis* and *Crassostrea gigas*.

MATERIALS AND METHODS

Mussel and oyster hemolymph sampling

In order to perform *in vitro* cytotoxicity assay, hemolymph of *Mytilus galloprovincialis* was withdrawn with a sterile 5 ml syringe and a 0.6 × 25 hypodermic needle, introduced directly into the sinuses

of the posterior adductor muscle through a small artificial opening performed at the edges of the two shell valves. Routinely, about 1.5 ml per mussel was collected and centrifuged immediately at 800 g for 15 min at 4°C to remove all cells and debris. The supernatant, referred to as plasma, was extensively dialyzed against vertebrate isotonic 50 mM TRIS-buffered saline (pH 8.5) before immediate use or storage at −80°C.

Adult specimens of two oyster species were used, the European flat oyster, *Ostrea edulis*, and the Pacific oyster, *Crassostrea gigas*. Hemolymph (about 1 ml for *O. edulis*, 2 ml for *C. gigas*) was withdrawn from the pericardial sinus by using a sterile hypodermic needle (0.6 × 25) attached to a sterile 5 ml syringe. Hemocytes were spun down by 15 min centrifugation at 800 g, 4°C and the pellets were washed with 2% NaCl. After resuspending the cells in 2% NaCl, the suspensions were sonicated to prepare hemocyte lysates. Debris were removed by 10 min centrifugation at 11,000 g, 4°C and the plasma and the lysate were stored at −80°C until use.

Protein concentrations were determined by Bradford's method using bovine serum albumin (BSA) as standard.

Cytotoxic and antibacterial assays

Mammalian erythrocytes were used as target cells to determine the cytotoxic activity of mussel plasma according to the technique described by Roch *et al.* (1989) except that incubations took place at 37°C. Mouse myeloma 653 cells were maintained in RPMI 1640 (GIBCO) culture medium supplemented with 10% foetal calf serum. The viability of 3×10^4 cells in 50 µl culture medium was determined by trypan blue dye exclusion after 1 h of contact at 37°C with an equal volume of mussel plasma. Protozoan oyster parasites, *Bonamia ostreae*, were purified from infected *O. edulis* according to the protocol of Mialhe *et al.* (1988) and suspended in sterile sea water. The viability of 6×10^5 protozoans in 50 µl sea water was determined by acridin orange-ethidium bromide staining, observed with a UV microscope after 1 h of contact at 20°C with an equal volume of mussel plasma. Assays were done in triplicate and results were expressed as arithmetical mean ± standard error on the mean.

Marine bacteria (*Vibrio alginolyticus*, *V. tubiashii*) were grown in Bacto Marine Agar 2216 (Difco). *Escherichia coli* and *Micrococcus luteus* were grown in liquid nutrient broth made of 10 g Bacto Tryptone (Difco), 5 g Bacto Yeast Extract (Difco) and 10 g NaCl per litre, pH 7.2. Twenty µl of bacterial suspension containing 10^6 bacteria in tests with samples from *M. galloprovincialis* and *O. edulis*, and 200 bacteria in tests with *C. gigas* samples, were added to 80 µl of bivalve sample and incubated for 1 h at 20°C. Bacterial killing was determined using the colony forming unit (CFU) technique, after serial dilutions were appropriate, and overnight incubation at 24°C (Vibrios) or 37°C (*E. coli*, *M. luteus*) according to the technique of Boman *et al.* (1974). Antibacterial activity was determined by calculating differences between numbers of CFU in tests which had been incubated in the presence of bivalve samples and those in controls that consisted of bacteria incubated without bivalve sample according to the formula:

$$\% \text{ of inhibition} = \frac{\text{Number of colonies in control} - \text{Number of colonies in test}}{\text{Number of colonies in control}} \times 100$$

Assays were performed in triplicate.

Chromatographic separation of mussel plasma

Plasma from 30 mussels was pooled, then dialyzed overnight at 4°C against 50 mM TRIS-HCl buffer, pH 7.5 (*fig.* 1). Flocculated proteins were removed by 10 min centrifugation at 11,000 g, 4°C. Aliquots of 6 ml were loaded onto a Resource Q 1 ml anion exchange column (Pharmacia) previously equilibrated with 50 mM TRIS-HCl, pH 7.5. Elution was at 1 ml/min with 0-400 mM NaCl in a linear gradient. Absorbence was recorded at 280 nm (Beckman Gold System). Hemolytic activity was tested along the entire elution profile after 1 h dialysis against 200 mM NaCl, 10 mM $CaCl_2$, 50 mM TRIS-HCl, pH 8.5. Active fractions of 7 identical anion exchange chromatography runs were pooled and partially freeze-dried in order to reduce the volume. After 1 h dialysis against 200 mM NaCl, 10 mM $CaCl_2$, 50 mM TRIS-HCl, pH 8.5, aliquots of 2 ml were separated in gel filtration through a Sec 3,000 (Beckman) HPLC column eluted at 1 ml/min with the same buffer. Activity was tested directly with the fractions leaving the column.

Electrophoresis

The protein profiles of the various active fractions separated in chromatography were visualized by native or SDS-PAGE on 4-15% acrylamide gel (Pharmacia Phast-System). Pure protein was reduced by 3 min boiling in presence of 5% β-mercaptoethanol. After migration, gels were silver stained according to the manufacturer's instructions. High molecular weight and low molecular weight protein calibration kits (Pharmacia) were used as molecular mass markers.

Electron microscopic examination

Human group O red blood cell (HORBC) membranes were prepared by incubating 10 µl of packed erythrocytes with 50 µl of purified cytotoxic gel filtration fraction for 30 min at 37°C. After 2 min centrifugation at 7,500 g, the pellet of treated HORBC

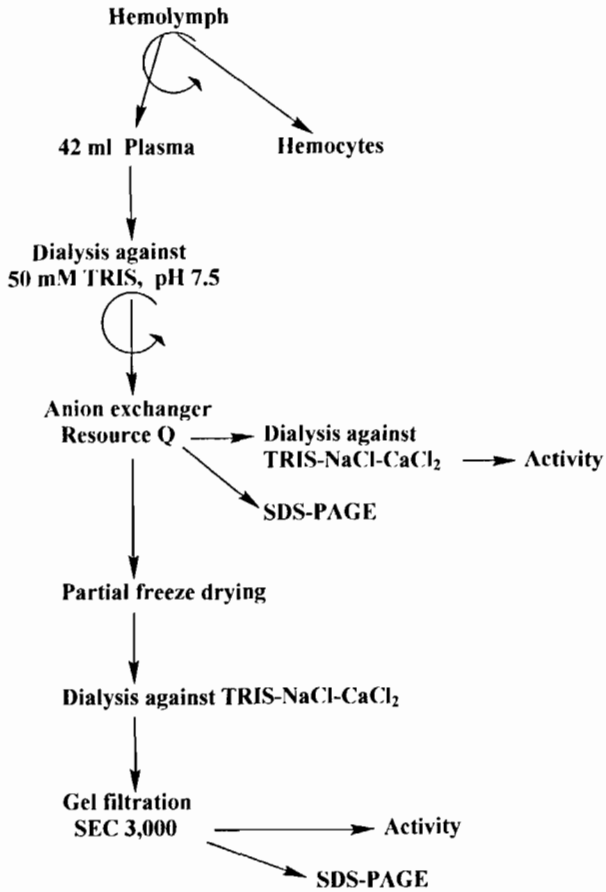

Figure 1. – Purification procedure of mussel cytotoxic complex by two-step chromatography.

was resuspended in TRIS-saline, and a droplet of this suspension was layered on collodion-carbonated grids covered with poly(L)-lysine. Controls consisted of HORBC lyzed in distilled water. After drying, the grids were negatively stained with 2% disodium phosphotungstate, pH 7.0. Observations were made at 60 mA on a Hitashi HU11C.

RESULTS

Cytotoxic activity in mussel plasma

Target specificity

Differences in percentages of cell death for vertebrate erythrocytes used as target cells were noted (*table* 1). The percentages ranged from 46% for monkey to 72% for rabbit erythrocytes. Mouse tumour cell line was also sensitive to mussel plasma with 45% of the cells being killed after 1 h of contact. *Bonamia ostreae* was also killed by the mussel plasma. By contrast, the viability of two Gram negative bacteria, *Vibrio alginolyticus* and *Escherichia coli*, was not affected by incubation in the presence of dialyzed mussel plasma. Proper controls including vertebrate erythrocytes, mouse tumour cells, *B. ostreae* and bacteria demonstrated that mortality is not due to osmotic differences.

Table 1. – Specificity of cytotoxic activity in mussel plasma. Percentage cell death was determined for the different targets with the appropriate biological assay as mentioned in the text. Results expressed are arithmetic means of triplicates ± SE.

Targets	Percentages of cell death
Vertebrate erythrocytes (2×10^7)	
Rabbit	72.3 ± 5.2
Human blood group A	65.7 ± 1.8
blood group B	63.0 ± 1.5
blood group O	63.7 ± 2.4
Mouse	61.3 ± 0.7
Horse	52.7 ± 0.3
Sheep	52.3 ± 0.7
Monkey	46.7 ± 0.9
Mouse myeloma 653 (3×10^4)	45.5 ± 2.5
Protozoan parasite *Bonamia ostreae* (6×10^5)	51.0 ± 9.8
Bacteria (10^6)	
Vibrio alginolyticus	0
Escherichia coli	0

Characteristics of the activity

Diluting mussel plasma resulted in less activity and the shape of the dose-response curve clearly indicated dose-dependent activity. Initial dilutions of plasma dramatically decreased activity which was finally no longer detectable after a 32-fold dilution (*fig.* 2). The time-course of cytotoxic activity was evaluated by incubating human O group red blood cells (HORBC) for various periods of time at 20°C. The lytic reaction occurred rapidly and was detectable after only 5 min of contact. The percentage of killed cells increased rapidly up to 30 min with maximum activity (73%) observed after 60 min of contact.

Heat stability of cytotoxic activity was determined by testing the activity in plasma that had been heated previously for 1 h at various temperatures. This treatment demonstrated that the activity was stable from 4°C to 25°C, and then rapidly decreased to be completely inactivated at 45°C.

Change of pH was effected by overnight dialysis in the same medium adjusted to various pH. For pH 4, no lysis was detectable after 1 h incubation at 37°C. In acidic medium ranging from pH 5 to 7, the percentage of cell death was about 20%. For basic pH of 7.5, 8 or 9, around 50% of the erythrocytes were lysed. Maximum efficiency of lytic activity was obtained for pH 8.5 with 70% of lysis. More basic pH, such as pH 10 resulted in less activity with only 40% of lysis.

Plasma previously incubated with increasing concentrations of chymotrypsin, then dialyzed overnight, was no longer active. Complete inhibition was

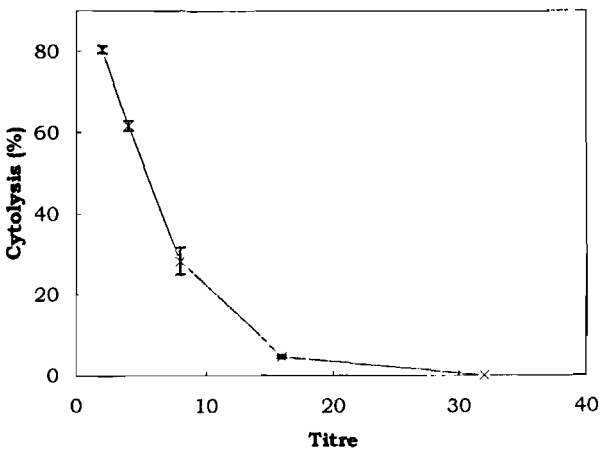

Figure 2. – Dose-response curve of cytotoxic activity of dialyzed mussel plasma against human O red blood cells (HORBC). Haemoglobin content of supernatant was measured by absorption at 541 nm after 1 h incubation at 37°C. Each point represents the arithmetic mean of triplicates ± SE.

obtained using 40 U/mg of plasma protein (specific activity was 49 U/mg of chymotrypsin). Similar results were induced by incubation with trypsin, except that complete inhibition was obtained with 10,600 U/mg of plasma protein (specific activity was 12,900 U/mg of trypsin). Addition of 2 mM of phenyl methyl sulfonyl fluoride (PMSF) to the reaction medium resulted in 25% inhibition of the cytotoxic activity.

Individual variability

The seventeen plasma samples collected from individual mussels revealed enormous differences in cytotoxic activity towards HORBC, ranging from 8 to 81% (*table* 2). Most mussels exhibited an activity of between 50 and 70%. Less extended variability was observed with the total protein content of the various plasma samples with values ranging from 550 μg/ml to 990 μg/ml.

Isolation and identification of cytotoxic protein

Anion exchange

Elution at 1 ml/min with a linear gradient of NaCl resulted in 3 major peaks (*fig.* 3*A*). Cytotoxic activity against HORBC was clearly located within the first peak that was eluted by 200 mM NaCl and it was not associated with the major peak leaving the column. The protein contents of the active fractions was analyzed in 4-15% SDS-PAGE. Under non-reducing conditions, the presence of strong labelling on top of the separating gel suggested the existence of a high molecular mass (HMM) protein (*fig.* 4). The same HMM band was present in dialyzed plasma, but not after reduction of the active peak. A clear band of about 320 kDa was also observed consistently, the labelling of which seemed to correlate with the degree of activity. Other bands, particularly one at 25 kDa, were also present. Plasma samples inactivated

Table 2. – Individual variability of mussel plasma cytolytic activity towards HORBC and corresponding total protein content. Results are arithmetic means ± SE ($n = 3$).

Individual	Degree of cytolysis	Plasma protein concentration μg/ml
1	8.8 ± 0.3	620
2	20.3 ± 0.9	900
3	22.7 ± 0.3	740
4	30.3 ± 0.9	720
5	35.0 ± 1.2	980
6	40.0 ± 0.6	550
7	40.3 ± 0.7	640
8	50.3 ± 4.0	660
9	51.3 ± 1.4	630
10	55.0 ± 1.5	670
11	57.7 ± 1.8	690
12	62.0 ± 1.2	990
13	64.0 ± 1.7	650
14	67.3 ± 1.7	630
15	68.0 ± 0.6	550
16	81.3 ± 0.3	830
17	81.7 ± 0.7	710

by heating at 45°C followed by centrifugation never demonstrated the presence of the first peak of anion exchange chromatography, nor the HMM or the 320 kDa protein bands.

Gel filtration

After being pooled, partially concentrated by freeze-drying and dialyzed against NaCl-TRIS buffer, aliquots of 2 ml were loaded onto a Sec 3,000 column (Beckman). Elution at 1 ml/min resulted in 5 peaks. Cytotoxic activity, against HORBC, was located only in the first one (*fig.* 3*B*). According to the molecular calibration of the column, this peak contained proteins of molecular mass higher than 700 kDa. Analyzed in native PAGE, the active peak appeared to consist of a single protein of high molecular mass (not illustrated). In the presence of SDS, but under non-reducing conditions, only the HMM, 320 and 25 kDa bands were clearly visible (*fig.* 4). Under reducing conditions, the HMM and the 320 kDa bands were dramatically decreased and some other bands, of 100 and 43 kDa, appeared.

Mechanism of action

Incubation of dialyzed plasma or active fractions with ghost membranes of HORBC obtained by osmotic lysis resulted in total inhibition of supernatants. Analyzed in SDS-PAGE, these inactive supernatants did not contain the 320 kDa protein.

Transmission electron microscopy of negatively stained HORBC lysed by purified active fraction showed them to be like flat ghosts covered with numerous small, dense round-shaped deposits of phosphotungstate (*fig.* 5). Detailed observations revealed that the deposits corresponded to clear rings

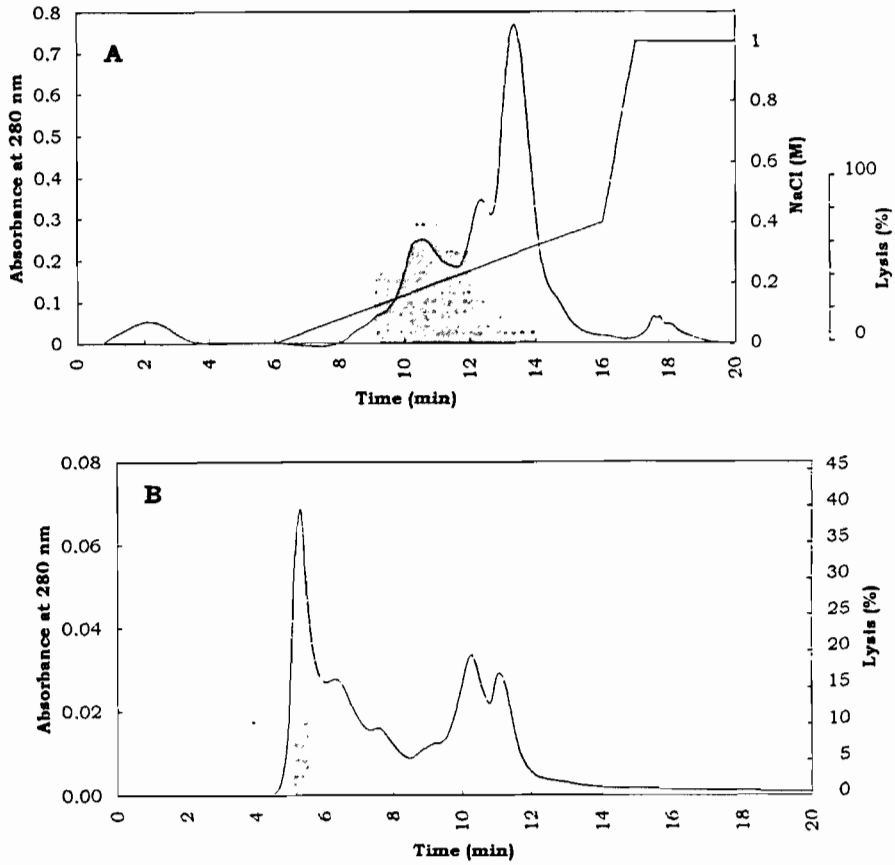

Figure 3. – HPLC profiles of mussel plasma separated by ion exchange chromatography (A) and gel filtration (B). Absorption was at 280 nm. Cytotoxic activity was located by testing the various fractions against HORBC (histograms).

of 5-10 nm in diameter with a dense nucleus. Such particular structures were never present in erythrocytes lysed by incubation in distilled water.

Antibacterial activity in oyster hemolymph

Specificity of antibacterial activity in hemocyte lysates

After establishing that hemocyte lysates from *Ostrea edulis* contained antibacterial activity, lysates were tested against a panel of bacterial species. These comprised Gram negative bacteria: *Vibrio alginolyticus*, *V. tubiashii* (both pathogenic for marine invertebrates), *Escherichia coli*, and one Gram positive, *Micrococcus luteus*. Antibacterial activity appeared to be directed towards all species tested, but was least effective against *E. coli* (average inhibition about 25%) and most effective against *V. alginolyticus* (average inhibition about 50%). Inhibition of *V. tubiashii* was very variable with percentages that ranged from 25 to 95% depending upon the oyster specimen.

With *Crassostrea gigas*, activity of lysates was tested against *V. alginolyticus* and three as yet unidentified enterobacteriaceae, isolated from *C. gigas*. Against *V. alginolyticus*, activity was not as high as in the flat oyster since average values did not exceed 30% inhibition. The oyster-derived enterobacteriaceae were less sensitive with the lysate giving about 20% inhibition.

Characteristics of antibacterial activity

The antibacterial activity in both oyster species is most probably due to peptides. Treatment of lysate with chymotrypsin or trypsin dramatically diminished the activity. Repeated cycles of freezing-thawing-heating (30 min, 60°C) did not destroy the activity. The first step in a chromatographic separation procedure was performed using lysate from *O. edulis*. From a Resource Q anion exchanger, the bulk of the protein was recovered in one large peak eluted after 4 min, followed by three small peaks in the next 15 min. None of this material contained any detectable activity. The cluster of five small peaks eluted between 20 and 30 min did contain activity. By far the highest activity was found in peak 6, eluted after 23 min (*fig.* 6).

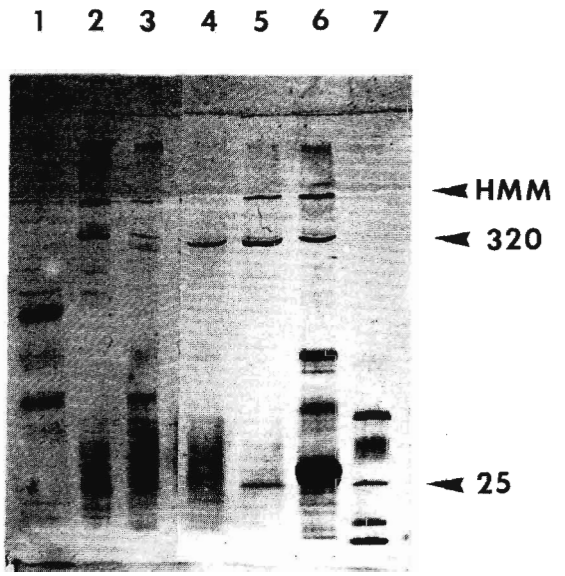

Figure 4. – SDS-PAGE analysis of cytotoxic fractions obtained from chromatography of mussel plasma. Active gel filtration fraction under non-reducing (2) and reducing (3) conditions. Note the presence of a high molecular mass (HMM) band and of 320 and 25 kDa bands in track 2 and the decrease of both HMM and 320 kDa bands and the presence of smaller MM bands in track 3. Active anion exchange chromatography fraction under non-reducing (5) and reducing (4) conditions. Note the identity between tracks 5 and 2 and the absence of HMM band in track 4. Dialyzed plasma before separation by chromatography (6). Note the presence of HMM, 320 and 25 kDa bands among with many others. Kit of HMM (1) and LMM (7) markers.

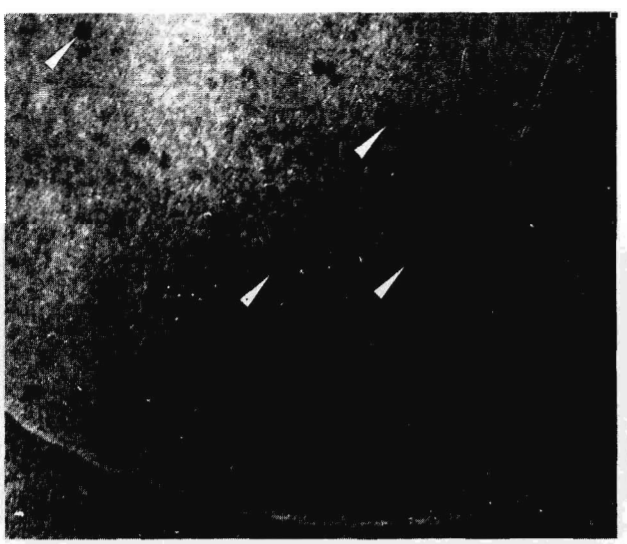

Figure 5. – Transmission electron microscopy (TEM) of erythrocyte membranes lysed by mussel cytotoxic molecules and negatively stained. Note the presence of dark spots (arrowheads). Bar represents 50 nm.

Localization of activity, individual variability

Activity was detected in both hemolymph plasma and hemocyte lysate. In the two species, *O. edulis* and *C. gigas*, activity (measured against *V. alginolyticus*) was stronger in lysate (average values 50 and 30% inhibition respectively for the two species) than in plasma (5 and 10% respectively). In *C. gigas*, levels of activity against oyster-derived enterobacteriaceae also differed among lysates and plasma. Whereas plasma now showed higher activity, often 100% but on average about 55% inhibition, the average value of activity in lysates reached only 20% inhibition.

Testing individually of more than 50 oysters revealed that the antibacterial activity, like mussel cytotoxic activity, showed considerable individual variability. For example, hemocyte lysate from purchased (wild) specimens of *O. edulis* tested against *V. alginolyticus*, showed activity varying between 10 and 70% (average values about 45%). In native *C. gigas*, 50% of the specimens tested had no detectable activity against *V. alginolyticus*. In the remaining 50%, activity varied between 25 and 55% inhibition. Average activity of all the tested *C. gigas* was about 30% whereas average inhibition of active samples was about 40% inhibition.

First attempts of stimulation

Different protocols for stimulation, or induction, of antibacterial activity were chosen for the two species. In *O. edulis*, a protocol of applying physical and mechanical stress was used, based upon empirical findings in our laboratory. The protocol consisted of removing oysters from their tanks, leaving them to dry at 18°C for 4 h. At regular intervals the oysters were vigorously shaken in a box for 1-2 min. Oysters were then returned to the tanks and allowed to recover for 20 h before collecting hemolymph. Control oysters were maintained in sea water throughout the experiment without shaking. Pools of lysate from unstimulated, control *O. edulis* showed about 20% inhibition of *V. alginolyticus*. The stress-treatment resulted in an increase in activity to about 60% inhibition.

In *C. gigas*, induction was attempted by injecting bacteria. A test-group of ten oysters was anaesthetized by immersion in a solution of 0.3 M $MgCl_2$ in sea water and then injected, into the pericardial sinus, with 4×10^8 heat-killed *V. alginolyticus* in 100 μl sterile sea water. Three control groups received, respectively, no treatment, anaesthesia alone, and anesthesia plus injection of saline. Hemolymph was sampled 48 h after treatment. Hemocyte lysates from individual oysters were tested against *V. alginolyticus*. Of the ten controls receiving no treatment, six had significant activity whereas in the anaesthetized controls, only five out of ten samples were active. In saline-injected controls, the number of positive samples had increased to nine, and all ten bacteria-injected oysters showed activity at levels of 42%. The mean percentage of inhibition in active samples from untreated controls was 35% whereas in all three anaesthetized groups, 45% inhibition was observed.

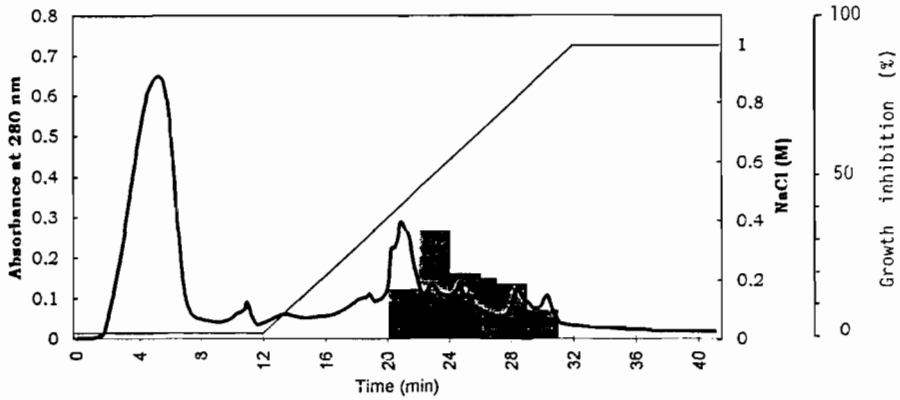

Figure 6. – HPLC profile of pooled oyster, *Ostrea edulis*, hemocyte lysate separated by anion exchange chromatography on Resource Q column. Anti bacterial activity was located by testing fractions against *Vibrio alginolyticus* (histograms).

DISCUSSION

Commercially mass-cultured marine invertebrates are continuously at risk from infections by pathogens, with substantial losses as the consequence (*see* Bower *et al.*, 1994, for synopsis). In order to decrease such risks, there have been attempts to increase their immuno-defence capabilities by stimulating their defence system, by selecting strains with enforced resistance, or by transgenic manipulations (Mialhe *et al.*, 1995).

According to the high individual variability, it seems that no relationship exists between cytotoxic activity and protein concentration. The differences in sensitivity of the vertebrate erythrocytes tested probably reflected differences in cell membrane composition rather than a specificity of the effector molecules. A mouse tumour cell line was also found to be sensitive to cytolysis by mussel plasma, despite the capacity to repair the cell membrane, a capacity lacking in the erythrocytes. Parasites may be expected to be particularly resistant to environmental attacks and be capable of escaping or, at least, repairing severe membrane damage. Meanwhile, *Bonamia ostreae* was also lysed by mussel plasma, revealing how powerful this activity is.

Inhibition and time-course of the cytotoxic reaction evidenced that the lysis depends on the concentration of active molecules and on the duration of contact between effector molecules and target cells. Cytotoxic activity is mediated by proteins and strictly depends on the pH of the incubation medium.

In both mussels and oysters, individual variability of cytotoxicity or antibacterial capabilities, may be interpreted as reflecting the various degrees of immune reactivity in animals under natural conditions where they are continuously confronted with environmental assaults. This hypothesis is supported by the fact that stimulating field-collected mussels, by injecting erythrocytes or bacteria, resulted in an increased number of strongly reacting individuals (unpublished results). Similarly in oysters, injections of either saline or bacteria induced previously non-existing activity. As no total counts of hemocytes were done, it cannot be excluded that lysates from stimulated oysters had been prepared from higher numbers of hemocytes than lysates from unstimulated animals, e.g. due to the mobilization of hemocytes from tissues into circulation (van der Knaap *et al.*, 1987). In either case, – higher activity per cell or recruitment of hemocytes – injected animals were capable of a reaction.

Chromatographic purification of cytotoxic activity from mussel plasma, together with electrophoretic analysis, revealed that active molecules are of molecular mass superior to 700 kDa. Inactive 320 kDa protein seems to represent a basic unit, the polymerization of which induces lysis. Under reducing conditions, the 320 kDa unit was split into several proteins, suggesting a complex assembly of several different polypeptidic chains.

The pictures observed on the erythrocyte membranes lysed by mussel plasma strongly suggest that the mechanism of action involves the formation of transmembrane channels. These structures were generally very numerous giving the appearance of a membrane perforated with transmembrane pores. Similar pictures were previously observed on vertebrate erythrocytes lysed by annelid (Roch *et al.*, 1989) or echinoderm (Canicatti, 1987) hemolysins. Transmembrane pore formation is also the mode of action of complement (Humphrey and Dourmashkin, 1969), perforins (Dourmashkin *et al.*, 1980; Tschopp *et al.*, 1986) and many bacterial toxins (Freer *et al.*, 1968).

In oysters, the mode of action of the antibacterial activity remains to be investigated. However, two distinct activities are involved: the low percentage of inhibition of *V. alginolyticus* found in samples from *C. gigas* suggests a bacteriostatic activity, whereas the same samples may be bactericidal against *C. gigas*-derived enterobacteriaceae. Moreover, a sample may contain one, both or neither of these putative different activities.

In the present paper, we have demonstrated a number of defence activities in marine bivalves that can be easily stimulated to increase the levels of activity. As all the activities can be quite easily quantified, they lend themselves to experimental selection of specimens with high activity. The antibacterial activity, which probably results from one or more peptides, is a likely candidate for use in attempts to increase resistance, for instance by genetic manipulation.

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