Aquat. Living Resour. 14 (2001) 343−349

S0990744001011433/FLA

Antibacterial activities of oyster (*Crassostrea virginica*) and mussel (*Mytilus edulis* and *Geukensia demissa*) plasma

Robert S. Anderson*, Amy E. Beaven

Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, P.O. Box 38, Solomons, Maryland 20688, USA

Received 19 June 2001; accepted 5 October 2001

Abstract − Anti-*Bacillus megaterium* activity was measured in unfractionated plasma withdrawn from three common US East Coast bivalve molluscs: an oyster *Crassostrea virginica* and the mussels *Geukensia demissa* and *Mytilus edulis*. The activities of the plasma samples from these bivalves were also measured against a *C. virginica* pathogen *Perkinsus marinus*. Strong anti-*B. megaterium* activity was measured in plasma from *C. virginica* and *M. edulis*, but was not detected in *G. demissa*. Bactericidal activity was found in hemocyte extracts from all bivalves in this study, suggesting a cellular origin of cytotoxic humoral factors. Peptides (< 10 kDa) were separated from the plasma samples by ultrafiltration; weak antibacterial peptide activity was quantified in *C. virginica* peptides, but not in peptides from the mussels. In the case of *P. marinus*, plasma from *M. edulis* or *G. demissa* was strongly cidal as compared to plasma from *C. virginica*. This difference in activity probably reflects the low pathogenicity of this oyster parasite for the mussel species tested. In summary, the bactericidal activity of plasma proteins from these bivalves showed considerable interspecies variation and did not necessarily correlate directly with antiprotistan activity. When present, antibacterial and antiprotistan activities seemed to be associated with plasma proteins rather than < 10-kDa plasma peptides, with the possible exception of *C. virginica* anti-*B. megaterium* activity and the occasionally expressed anti-*P. marinus* activity of *M. edulis* peptides. The precise identity of the plasma protein(s) responsible for the antimicrobial activities measured have yet to be determined, but it is likely that agents other than, or in addition to, lysozyme play significant roles in the process.

antibacterial factors / antimicrobial factors / antimicrobial peptides / plasma proteins / *Crassostrea virginica* / *Mytilus edulis* / *Geukensia demissa* / *Perkinsus marinus* / *Bacillus megaterium*

Résumé − Activités antibactériennes du plasma de l'huître (*Crassostrea virginica*) et des moules (*Mytilus edulis* et *Geukensia demissa*). Nous avons mesuré l'activité anti-*Bacillus megaterium* du plasma entier de trois mollusques bivalves communs sur la côte est des États-Unis : l'huître *Crassostrea virginica* et les moules *Geukensia demissa* et *Mytilus edulis*. L'activité des échantillons de plasma de ces bivalves a aussi été mesurée vis-à-vis du protiste *Perkinsus marinus*, pathogène pour *C. virginica*. Une forte activité anti-*B. megaterium* est mesurée dans le plasma de *C. virginica* et *M. edulis*, mais n'est pas décelée chez *G. demissa*. L'activité bactéricide est observée dans des extraits d'hémocytes chez ces trois bivalves, ce qui supposerait une origine cellulaire des facteurs humoraux cytotoxiques. Des peptides (< 10 kDa) ont été séparés des échantillons de plasma par ultrafiltration ; une faible activité antibactérienne de cette fraction peptidique a été mesurée chez *C. virginica*, mais pas chez les moules. Dans le cas de *P. marinus*, le plasma de *M. edulis* ou de *G. demissa* montre une forte activité antiprotiste, comparé au plasma de *C. virginica*. Cette différence au niveau de l'activité reflète probablement la faible pathogénicité de ce parasite de l'huître pour les espèces de moules testées. En résumé, l'activité bactéricide des protéines du plasma de ces bivalves montre une variation considérable suivant les espèces et n'est pas nécessairement et directement corrélée avec l'activité antiprotiste. Lorsque ces activités antiprotistes et antibactériennes sont présentes, elles semblent être associées aux protéines du plasma, plutôt qu'aux peptides (< 10 kDa), à l'exception peut-être de l'activité anti-*B. megaterium* de *C. virginica* et de l'activité anti-*P. marinus* occasionnelle de *M. edulis*. L'identité précise de la protéine ou des protéines du plasma responsable(s) des activités antimicrobiennes mesurées reste encore à déterminer, mais il est probable que des agents autres que le lysozyme, ou supplémentaires au lysozyme, jouent un rôle significatif dans le processus.

facteurs antibactériens / facteurs antimicrobiens / peptides antimicrobiens / protéines du plasma / *Crassostrea virginica* / *Mytilus edulis* / *Geukensia demissa* / *Perkinsus marinus* / *Bacillus megaterium*

**Correspondence and reprints:* fax: +1 410 326 7210.
E-mail address: anderson@cbl.umces.edu. (R.S. Anderson).

1. INTRODUCTION

The hemocytes of bivalve molluscs are thought to play important roles in host defenses against microorganisms. The cells may destroy microbes intracellularly, or may exert cidal effects by virtue of agents secreted into the plasma. Many cytotoxic molecules have been described in the hemocytes and plasma of bivalves including lysosomal hydrolases (Pipe, 1990), reactive oxygen species (Adema et al., 1991; Anderson, 1996), reactive nitrogen species (Arumugan et al., 2000), and antimicrobial proteins and peptides (Roch et al., 1996). These activities may be found as naturally-occurring components of the hemolymph, or their activities may be enhanced by infections or various experimental treatments. Furthermore, the expression and/or specificity of cytotoxic agents may vary between bivalve species. For example, hemocytes from mussel and oyster species can be stimulated to produce greater levels of reactive oxygen species than clam hemocytes (Anderson, 1994). There is also evidence that reactive oxygen species may play a lesser role in bivalves than in host defenses of vertebrates (Bramble and Anderson, 1999). Among the prominent oxygen-independent antimicrobial defense molecules of bivalves are bacteriolytic enzymes such as lysozyme (McDade and Tripp, 1967; Hardy et al., 1976).

In a previous study (Anderson and Beaven, in press), the activities of unseparated plasma, lysozyme, < 10-kDa peptides, and hemocyte lysate from several bivalve species were measured against *Perkinsus marinus*, the parasite responsible for a lethal disease (Dermo) of the eastern oyster *Crassostrea virginica*. Dermo disease is responsible for the significant destruction of oyster stocks along the Atlantic coast (Burreson and Ragone Calvo, 1996). The study of anti-*P. marinus* activity in plasma fractions from *C. virginica*, *Crassostrea gigas*, *Geukensia demissa* and *Mytilus edulis* showed unique activity profiles among species. Those results prompted this study in which antibacterial (anti-*B. megaterium*) activities were quantified in comparable plasma fractions. A comparative study of anti-*B. megaterium* and anti-*P. marinus* activities was carried out to determine the interspecies distribution of these putative defense mechanisms.

2. MATERIALS AND METHODS

2.1. Bivalves

The bivalves studied co-inhabit US eastern coastal waters and included commercially-obtained oysters (*C. virginica*) from the Wicomico and/or St Mary's rivers, Maryland; ribbed mussels (*G. demissa*) collected at Chincoteague, Virginia; and blue mussels (*M. edulis*) purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts. Bivalves for the study were not collected during the summer months to avoid stress related to disease (especially *P. marinus* infections in *C. virginica*), elevated water temperature, hypoxia or gametogenesis. Light to moderate *P. marinus* infections are not uncommon in eastern oysters from the Wicomico or St Mary's rivers during the summer, but *P. marinus* is rarely detected in hemolymph or tissues at other times. The bivalve species were housed in separate tanks under identical conditions, at 12 °C in aerated, recirculated water systems containing 25 ppt artificial sea salts (Aquarium Systems, Inc., Mentor, Ohio). Levels of nitrate, nitrite and ammonia were monitored and half or full water changes carried out whenever the parameters were outside the acceptable limits (nitrate > 200 ppm, nitrite > 0.1 ppm, ammonia > 0.1 ppm). Routine acclimation periods of ≥ 7 d took place prior to experimentation. All bivalves were fed a reconstituted frozen algal slurry containing *Isochrysis* sp., *Chaetocerus gracilis* and *Tetraselmis* sp. or live cultured algae (*Isochrysis galbana* and *Thalassiosira weissflogii*).

2.2. Anti-*B. megaterium* activity of plasma and < 10-kDa fractions

The following method of sampling is typical of the bulk of this study, exceptions will be noted as required during presentation of the results. Samples were withdrawn from the adductor muscle hemolymph sinus using a syringe equipped with a 1½ in. 22 gauge needle. Samples were pooled from ≥ 6 bivalves, held on ice, and then centrifuged (300 *g*, 10 °C, 10 min) to separate the plasma from the hemocytes. Studies were performed on at least three separate pools; the value for each pool was the average of a number of intrapool replicates, as indicated in the methods for each assay. The plasma was filter-sterilized by passage through a 0.2-μm Whatman Puradisc syringe filter and frozen (–20 °C) until further use; the frozen plasma was thawed and tested within 1 month of collection. The protein content of the plasma, expressed as bovine serum albumin equivalents, was measured (BCA protein assay kit, Pierce Co.), and the desired concentrations reached by dilution with filter-sterilized Instant Ocean sea salts (IO) adjusted to 25 ppt. *Bacillus megaterium* (ATCC #14581) was grown overnight in nutrient broth (NB) with constant shaking at 30 °C. A bacterial suspension in NB was prepared (~10^8 bacteria·mL^{-1}; OD_{600} = 0.549). The bactericidal assay was based on that described by Volety et al. (1999) to measure hemocyte-mediated killing of bacteria. Experimental and control sets from each pooled plasma sample were carried out in four replicate wells in a standard 96-well microtiter plate. In one set (bacteria), 25 μL bacterial suspension was added to 25 μL IO; another set (bacteria + plasma), contained 25 μL bacterial suspension and 25 μL bivalve plasma; a blank set (blank) contained 25 μL NB and 25 μL plasma. The microtiter plate was incubated in a saturated humidity chamber for 3 h at room temperature (~20 °C) to permit interaction of the plasma and the

bacteria. The plate was then centrifuged at 2 000 *g* for 10 min at 21 °C, and 40 μL supernatant removed from all wells, thus removing most of the fluid phase without disturbing the pelleted bacteria. A growout period for the surviving bacteria was initiated by adding 50 μL sterile NB to all wells and incubating 2 h at 26 °C. Finally, 10 μL 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate (MTS-PMS reagent, Promega Corp., Madison, WI) was added to the wells and incubated for 1 h at 26 °C. The numbers of viable bacteria were determined colorimetrically by measuring the production of reduced MTS formazan at 490 nm on an ELISA plate reader. Absorbance values were corrected by subtraction of the blank, as described earlier. A killing index (% killed) was calculated with corrected absorbance values using the following equation where *KI* is killing index, $corrA_{BP} = A_{490}$ of bacteria + plasma corrected by subtraction of blank value, and $corrA_B = A_{490}$ of bacteria alone corrected by subtraction of blank value.

$$KI = \left(1 - \frac{corrA_{BP}}{corrA_B}\right) \times 100$$

Antibacterial activity of < 10-kDa plasma peptides was measured by the MTS-PMS assay described above. About 9 mL filter-sterilized, pooled bivalve plasma was fractionated by ultrafiltration with Millipore Ultrafree Protein Concentrators by passage through 100-kDa and 10-kDa exclusion filters. Molecules passing through the 10-kDa ultrafilter were frozen (–20 °C) and tested for anti-*B. megaterium* activity within 1 month.

2.3. Anti-*B. megaterium* activity of hemocyte extracts

The hemocytes were separated from the hemolymph as previously described and washed with 25 ppt IO. The cells were stored frozen (–20 °C) until further use. The cells were thawed and vigorously disrupted by sonication for 30 s. This was followed by freezing (–20 °C) and thawing three times. The cell debris was removed by centrifugation (300 *g*, 10 min, 10 °C); the supernatant (hemocyte extract) was filter-sterilized (0.2 μm), protein concentration determined, and its bactericidal activity measured using the MTS-PMS assay as used in the plasma and < 10-kDa peptide activity experiments.

2.4. Lysozyme activity

Lysozyme activity was expressed as egg white lysozyme equivalents. Lysozyme dilutions (0–20 $\mu g \cdot mL^{-1}$) were prepared in 0.05 M HEPES buffer, pH 6.8. These lysozyme standard solutions (0.25 mL) were mixed with 2.0 mL *Micrococcus lysodeikticus* (*M. luteus*) cell wall preparations (0.01 g in 50 mL 0.05 M HEPES buffer) in order to produce a lysozyme standard curve. The $\Delta OD_{540} \cdot min^{-1}$ for each lysozyme concentration was measured spectrophotometrically. The resultant linear relationship between activity and lysozyme concentration was used to quantify lysozyme levels in full strength or diluted bivalve plasma, or < 10-kDa peptide fractions.

2.5. Anti-*P. marinus* activity of bivalve plasma

Perkinsus marinus (strain I) cultures were originally provided by Drs M. Faisal and J. La Peyre of the Virginia Institute of Marine Science. The cultures were maintained in DME/HAM F-12 medium (Dulbecco modified Eagle's medium/Ham's nutrient mixture) reconstituted with 10 ppt IO, containing 1 % penicillin-streptomycin solution, 2 % fetal bovine serum, and was HEPES-buffered at pH 6.5. All medium components were purchased from the Sigma Chemical Co. This medium was based on that originally described by Gauthier and Vasta (1993). *P. marinus* cells were centrifuged (300 *g*, 10 min, 10 °C) and resuspended in DME/HAM F-12 medium without phenol red in order to minimize possible interference with spectrophotometer readings.

Flasks containing an initial concentration of *P. marinus* $cells \cdot mL^{-1}$ in the presence (experimentals) or absence (controls) of known concentrations of plasma in the medium were incubated for 170 h at 26 °C; previous studies have shown that *P. marinus* remains in the log phase of growth at this time (Anderson and Beaven, in press). An aliquot from each flask was read in a spectrophotometer at 560 nm vs. the appropriate blank (medium). The OD_{560} readings were converted to *P. marinus* $cells \cdot mL^{-1}$ using a previously-constructed standard curve that showed a linear relationship between absorbance and cell numbers of $\leq 15 \times 10^6$ mL^{-1} ($R^2 = 0.9861$). Finally, the effects of bivalve plasma on *P. marinus* density were calculated using the following equation where *CI* = % inhibition of culture growth, $A_{PP} = A_{560}$ of 170 h *P. marinus* culture in the presence of plasma, and $A_P = A_{560}$ of 170 h *P. marinus* culture in the absence of plasma.

$$CI = \left(1 - \frac{A_{PP}}{A_P}\right) \times 100$$

3. RESULTS

3.1. Antimicrobial activity of bivalve serum proteins and hemocytes

Preliminary studies showed that plasma from all bivalve species tested had measurable microbicidal or -static properties at protein concentrations readily obtained by dilution (*figure 1*). However, the antibacterial (anti-*B. megaterium*) and anti-protistan (anti-*P. marinus*) activities varied from species to species. For example, 1 500 $\mu g \cdot mL^{-1}$ *C. virginica* plasma protein totally inhibited *B. megaterium*, but was only ~15 % effective vs. *P. marinus*. On the other hand, this situation was apparently reversed for *G. demissa*

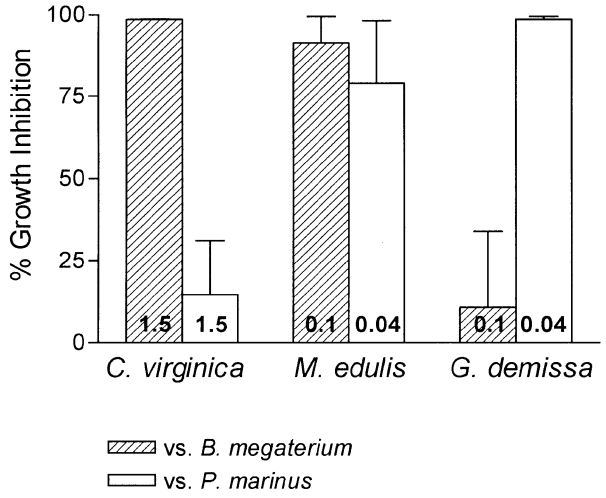

Figure 1. Comparison of mean ± SD (n = 3) anti-*Bacillus megaterium* and anti-*Perkinsus marinus* activities of plasma from three bivalves. For each species, the activities against the two microbes were determined using the same three pooled samples. Plasma protein concentrations in the medium (mg·mL^{-1}) are indicated on each bar. Antibacterial activity was determined by the MTS-PMS assay after 3 h bacteria-plasma contact (four replicates per pool); anti-*P. marinus* activity was measured turbidometrically after 170 h exposure in culture (two replicates per pool).

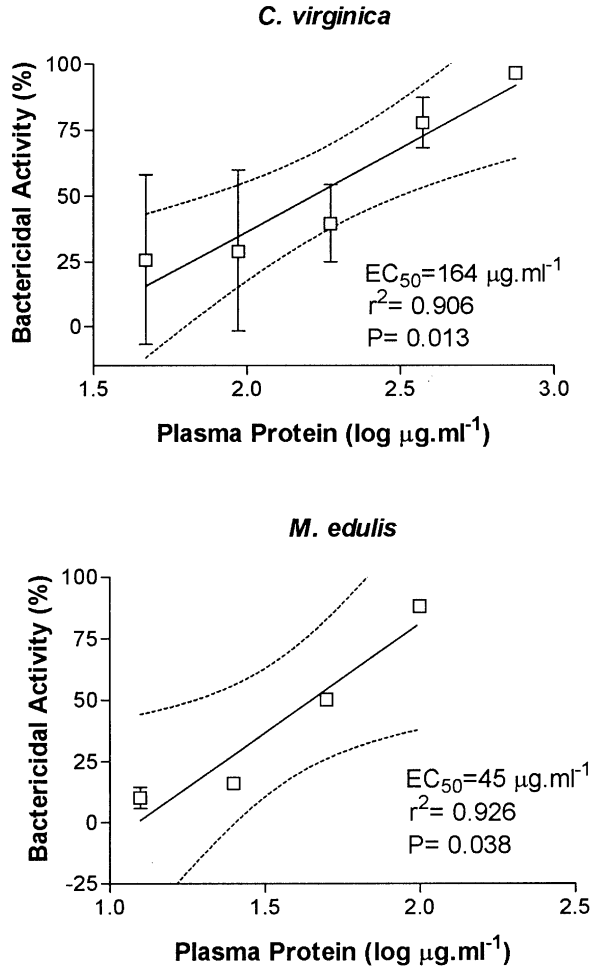

Figure 2. Anti-*Bacillus megaterium* activity in plasma from *Crassostrea virginica* and *Mytilus edulis*. The points represent mean bactericidal activity ± SD, n = 3 for each plasma protein concentration; the dashed lines show the 95 % confidence limits. EC_{50} values were calculated from the regression lines and equal the concentration of plasma proteins (µg·mL^{-1}) that inhibit 50 % of bacterial growth after 3 h exposure.

serum where anti-*P. marinus* activity far exceeded anti-*B. megaterium* activity. In order to address this observation in a quantitative fashion, studies were undertaken to determine the plasma protein concentration required for 50 % inhibition of *B. megaterium* growth (EC_{50}); bivalve plasma anti-*P. marinus* EC_{50} values have already been reported (Anderson and Beaven, in press). By using the EC_{50} values, *M. edulis* plasma proteins were shown to be about 3.5-fold more active than those of *C. virginica* against *B. megaterium* (*figure 2*). EC_{50} data from *G. demissa* are not presented because no activity was recorded at the serum protein concentrations tested (≤ 100 µg·mL^{-1}). Hemocyte lysates from all bivalve species tested effectively inhibited *B. megaterium* (*figure 3*). Hemocyte extracts showed dose-dependent inhibition of *B. megaterium*, but showed considerable variations with regard to species, *C. virginica* extracts were the least active per microgram protein.

3.2. Anti-*B. megaterium* activity of plasma peptides

Plasma constituents that could pass through an ultrafiltration system that retained > 10-kDa molecules were tested for anti-*B. megaterium* activity. These would include antimicrobial peptides similar to those described in other species. *C. virginica* peptides showed antibacterial properties, but this activity was weak (*figure 4*). The regression line suggests increased activity with increasing peptide concentration, but the slope of the line is only slightly different from zero (P = 0.042), therefore no meaningful EC_{50} value can be calculated. Peptides from the two mussel species tested had no apparent antibacterial activity at the levels tested in *figure 4* (data not presented).

3.3. Lysozyme activity in plasma and plasma peptides

Unfractionated plasma from all bivalves in the study had lysozyme activity (*figure 5*); however, *C. virginica* had levels many-fold greater than *G. demissa* or *M. edulis*. The < 10-kDa fractions of the plasma showed little or no lysozyme activity.

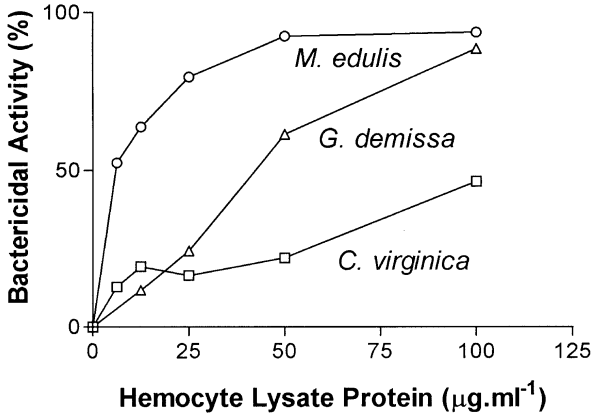

Figure 3. Anti-*Bacillus megaterium* activity of < 100 μg protein·mL^{-1} bivalve hemocyte extracts. Hemocytes were pooled from six to eight individuals of each species; points for each bivalve represent bactericidal activity of dilutions of a lysate of the same pooled sample.

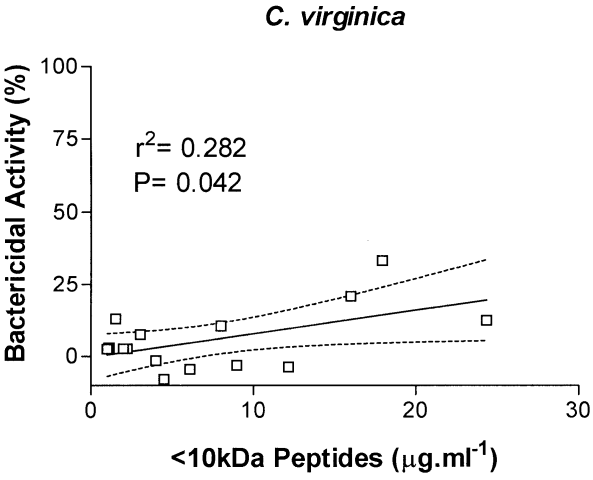

Figure 4. Effect of < 10-kDa serum peptides from *Crassostrea virginica* on *Bacillus megaterium*. Each point represents an individual data point gathered from three separate pools; dashed lines show the 95 % confidence limits.

4. DISCUSSION

The protistan *P. marinus* is a lethal parasite of *C. virginica*, but it is not known to cause lethal infections in *M. edulis* or *G. demissa*; *B. megaterium* causes no known infectious diseases of bivalves, but has been a model bacterium used in other studies of molluscan defense responses (Foley and Cheng, 1975, 1977). In *figure 1*, the anti-*B. megaterium* and anti-*P. marinus* activities of plasma samples from each bivalve species are compared. Estimates of anti-*P. marinus* activities from these species had previously been reported (Anderson and Beaven, in press), but the values

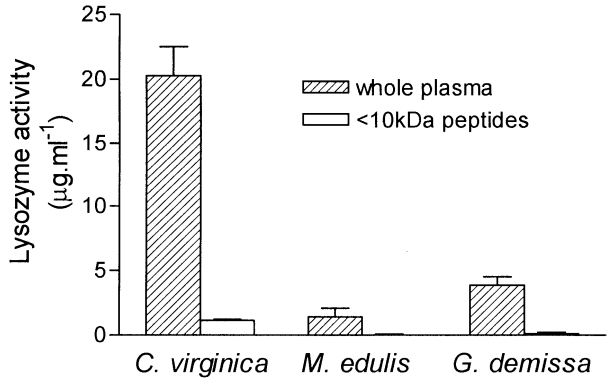

Figure 5. Mean ± SD (n = 3) lysozyme activity in the sera and < 10-kDa serum peptides of *Crassostrea virginica*, *Mytilus edulis* and *Geukensia demissa*.

reported here were from the same plasma samples used to determine the anti-*B. megaterium* activity. On the basis of activity per unit weight plasma protein, it seemed clear that antibacterial and antiprotistan activity profiles vary greatly from each other within the same species and between species. For example, *C. virginica* plasma proteins have greater activity against *B. megaterium* than *P. marinus*; the situation is reversed in the case of *G. demissa*. Also, the anti-*P. marinus* properties of plasma proteins from the two mussel species are significantly greater than *C. virginica* plasma. Nothing is known concerning modulation of antimicrobial activities by conditions the animals might have experienced prior to collection or changes associated with laboratory maintenance. However, the animals were acclimated in recirculated artificial sea water, at the same temperature and salinity, in an attempt to control general metabolic activity and exposure to estuarine pathogens. Since all three bivalves can be found in *P. marinus*-containing aquatic environments, one may speculate that differences in anti-*P. marinus* activity could partially explain their differences in susceptibility to Dermo disease. Of the species tested, only *M. edulis* showed high levels of both anti-*B. megaterium* and anti-*P. marinus* activities. Volety et. al. (1999) reported growth enhancement of *Vibrio parahaemolyticus* by *C. virginica* plasma due to its possible nutrient value. If plasma from *C. virginica*, *M. edulis* or *G. demissa* produced stimulatory effects on *B. megaterium* or *P. marinus*, the effects were masked by inhibitory activity leading to the observed net antimicrobial effects.

Differences in antibacterial potential suggested by the data in *figure 1* were quantified by determination of the plasma protein concentration in the MTS-PMS assay required for 50 % inhibition (EC_{50}) for each species (*figure 2*). *B. megaterium* was more sensitive to *M. edulis* plasma ($EC_{50} \cong 45$ μg·mL^{-1}) than *C. virginica* plasma ($EC_{50} \cong 164$ μg·mL^{-1}); bactericidal effects of *G. demissa* plasma were too variable, and dose-independent, to permit EC_{50} calculations. The

hemocytes may be a source of plasma antibacterial agents because hemocyte lysates were shown to possess concentration-dependent anti-*B. megaterium* activity (*figure 3*). The data in *figure 3* are preliminary, in that they are from dilutions of one sample of extracted, pooled hemocytes from each species; however, it is interesting that anti-*B. megaterium* activity in *G. demissa* hemocytes was comparatively strong, while such activity in its plasma was quite low. This might indicate that antibacterial agents are synthesized and stored in the hemocytes, but not released into the plasma until the cells are stimulated by microbial infection or other challenges, as first described by Cheng et al. (1975) and Cheng (1992). As mentioned previously, the effects of disease and other stressors on levels of humoral antimicrobial activity need to be studied in greater detail.

The exact identities of the plasma components responsible for antimicrobial activities described here remain unknown, but peptides with cytotoxic activities have been reported in *M. edulis* (Charlet et al., 1996; Mitta et al., 2000) and *M. galloprovincialis* (Hubert et al., 1996; Mitta et al., 1999a). In *M. galloprovincialis* these defensin-like peptides were synthesized and stored in hemocytes, to be released upon bacterial challenge (Mitta et al., 1999b). Low molecular weight (< 10 kDa) peptides were separated by ultrafiltration in this study and tested against *B. megaterium* at concentrations of $\leq 25\ \mu g \cdot mL^{-1}$. No activity was detected in peptides from the mussels tested, and very low activity was seen in *C. virginica* plasma peptides (*figure 4*). Therefore, it seems unlikely that < 10-kDa plasma peptides play major roles in anti-*B. megaterium* activity. These peptides were reported to have little anti-*P. marinus* activity in *C. virginica* or *G. demissa*, although activity varied widely in various populations of *M. edulis* (Anderson and Beaven, in press).

Lysozyme is a well-known antimicrobial enzyme in the hemocytes and plasma of bivalve molluscs (McDade and Tripp, 1967; McHenry et al., 1979; Chu and La Peyre, 1989); it will lyse *B. megaterium* and has been suggested to play a role in protecting oysters against *P. marinus* infections (Chu and La Peyre, 1993). Lysozyme activity in bivalves is associated with cysteine-rich polypeptides ranging from 11 (Nilsen et al., 1999) to 18 kDa (McHenry and Birckbeck, 1979). The level of lysozyme activity in *C. virginica* plasma was high as compared to that in *G. demissa* or *M. edulis* (*figure 5*). Lysozyme activity of the plasma peptide fractions was virtually undetectable in *M. edulis* and *G. demissa* and was low in *C. virginica*, probably because it would be excluded by the ultrafilter used to separate the < 10-kDa fractions. It is not known at this time if the slight anti-*B. megaterium* activity seen in the < 10-kDa fraction of *C. virginica* plasma results from the presence of antibacterial peptides or lysozyme. The data suggest no correlation between the plasma levels of lysozyme and antimicrobial activity, e.g. lysozyme activity: *C. virginica* >> *G. demissa* ≥ *M. edulis*; anti-*B. megaterium* activity: *M. edulis* > *C. virginica* (*G. demissa*: none); and anti-*P. marinus* activity: *M. edulis* ≅ *G. demissa* >> *C. virginica*. Based on these observations, it seems unlikely that lysozyme alone can account for the total antimicrobial activity in bivalve plasma. The results also suggest substantial interspecies variation in profiles of particular humoral defense molecules, and/or that involvement of these defense molecules in antibacterial or antiprotistan activities probably differ within and between bivalve species.

Acknowledgements. This study was funded by National Sea Grant College, Oyster Disease Research Program, Grants NA86RG0037 and NA46RG0091.

References

Adema, C.M., van der Knaap, W.P.W., Sminia, T., 1991. Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. Rev. Aquat. Sci. 4, 201–223.

Anderson, R.S., 1994. Hemocyte-derived reactive oxygen intermediate production in four bivalve mollusks. Dev. Comp. Immunol. 18, 89–96.

Anderson, R.S., 1996. Production of reactive oxygen intermediates by invertebrate hemocytes: immunological significance. In: Söderhäll, K., Vasta, G., Iwanaga, S. (Eds.), New Directions in Invertebrate Immunology. SOS Publications, Fair Haven, NJ, pp. 109–129.

Anderson, R.S., Beaven, A.E., in press. A comparative study of anti-*Perkinsus marinus* activity in bivalve sera. J. Shellfish Res.

Arumugan, M., Romestand, B., Torreilles, J., 2000. Nitrite released in haemocytes from *Mytilus galloprovincialis*, *Crassostrea gigas* and *Ruditapes decussatus* upon stimulation with phorbol myristate acetate. Aquat. Living Resour. 13, 173–177.

Bramble, L.H., Anderson, R.S., 1999. Lack of involvement of reactive oxygen species in the bactericidal activity of *Crassostrea virginica* haemocytes in contrast to *Morone saxatilis* phagocytes. Fish Shellfish Immunol. 9, 109–123.

Burreson, E.M., Ragone Calvo, L.M., 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. J. Shellfish Res. 15, 17–34.

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J.A., Bulet, P., 1996. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. J. Biol. Chem. 271, 21808–21813.

Cheng, T.C., 1992. Selective induction of release of hydrolases from *Crassostrea virginica* hemocytes by certain bacteria. J. Invertebr. Pathol. 59, 197–200.

Cheng, T.C., Rodrick, G.E., Foley, D.A., Koehler, S.A., 1975. Release of lysozyme from hemolymph cells of *Mercenaria mercenaria* during phagocytosis. J. Invertebr. Pathol. 25, 261–265.

Chu, F.L.E., La Peyre, J.F., 1989. Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). J. Invertebr. Pathol. 54, 224–232.

Chu, F.L.E, La Peyre, J.F., 1993. *Perkinsus marinus* susceptibility and defense related activities in eastern oysters *Crassostrea virginica*: temperature effects. Dis. Aquat. Org. 16, 223–234.

Foley, D.A., Cheng, T.C., 1975. A quantitative study of phagocytosis by hemolymph cells of the Pelecypods *Crassostrea virginica* and *Mercenaria mercenaria*. J. Invertebr. Pathol. 25, 189–197.

Foley, D.A., Cheng, T.C., 1977. Degranulation and other changes of molluscan granulocytes associated with phagocytosis. J. Invertebr. Pathol. 29, 321–325.

Gauthier, J.D., Vasta, G.R., 1993. Continuous in vitro culture of the eastern oyster parasite *Perkinsus marinus*. J. Invertebr. Pathol. 62, 321–323.

Hardy, S.W., Fletcher, T.C., Gerrie, L.M., 1976. Factors in hemolymph of the mussel, *Mytilus edulis* L., of possible significance as defense mechanisms. Biochem. Soc. Trans. 4, 473–475.

Hubert, F., Noël, T., Roch, P., 1996. A member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincialis*). Eur. J. Biochem. 240, 302–306.

McDade, J.E., Tripp, M.R., 1967. Lysozyme in the hemolymph of the oyster *Crassostrea virginica*. J. Invertebr. Pathol. 9, 531–535.

McHenry, J.G., Birckbeck, T.H., 1979. Lysozyme of the mussel, *Mytilus edulis* (L.). Mar. Biol. Lett. 1, 111–119.

McHenry, J.G., Birkbeck, T.H., Allen, J.A., 1979. The occurrence of lysozyme in marine bivalves. Comp. Biochem. Physiol. B Comp. Biochem. 63, 25–28.

Mitta, G., Hubert, F., Noël, T., Roch, P., 1999a. Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis*. Eur. J. Biochem. 265, 71–78.

Mitta, G., Vandenbulcke, F., Hubert, F., Roch, P., 1999b. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. J. Cell Sci. 112, 4233–4242.

Mitta, G., Hubert, F., Dyrynda, E.A., Boudry, P., Roch, P., 2000. Mytilis B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. Dev. Comp. Immunol. 24, 381–393.

Nilsen, I.W., Overbo, K., Sandsdalen, E., Sandaker, E., Sletten, K., Myrnes, B., 1999. Protein purification and gene isolation of chlamysin, a cold-active lysozyme-like enzyme with antibacterial activity. FEBS Lett. 464, 153–158.

Pipe, R.K., 1990. Hydrolytic enzymes associated with the granular haemocytes of the marine mussel *Mytilus edulis*. Histochem. J. 22, 595–603.

Roch, P., Hubert, F., van der Knaap, W., Noël, T., 1996. Present knowledge on the molecular basis of cytotoxicity, antibacterial activity and stress response in marine bivalves. Ital. J. Zool. 63, 311–316.

Volety, A.K., Oliver, L.M., Genthner, F.J., Fisher, W.S., 1999. A rapid tetrazolium dye reduction assay to assess the bactericidal activity of oyster (*Crassostrea virginica*) hemocytes against *Vibrio parahaemolyticus*. Aquaculture 172, 205–222.