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BD Tall

JF La Peyre
Virginia Institute of Marine Science

et al

M Faisal
Virginia Institute of Marine Science

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Perkinsus marinus Extracellular Protease Modulates Survival of *Vibrio vulnificus* in Eastern Oyster (*Crassostrea virginica*) Hemocytes†

B. D. TALL,[1]* J. F. LA PEYRE,[2]‡ J. W. BIER,[3] M. D. MILIOTIS,[4] D. E. HANES,[4] M. H. KOTHARY,[4] D. B. SHAH,[1] AND M. FAISAL[2]

Divisions of Microbiological Studies,[1] Science and Applied Technology,[3] and Virulence Assessment,[4] Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, D.C. 20204 and School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, Virginia 23062[2]

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The in vitro effects of the *Perkinsus marinus* serine protease on the intracellular survival of *Vibrio vulnificus* in oyster hemocytes were examined by using a time-course gentamicin internalization assay. Results showed that protease-treated hemocytes were initially slower to internalize *V. vulnificus* than untreated hemocytes. After 1 h, the elimination of *V. vulnificus* by treated hemocytes was significantly suppressed compared with hemocytes infected with invasive and noninvasive controls. Our data suggest that the serine protease produced by *P. marinus* suppresses the vibriocidal activity of oyster hemocytes to effectively eliminate *V. vulnificus*, potentially leading to conditions favoring higher numbers of vibrios in oyster tissues.

Vibrio vulnificus is associated with estuarine environments and with various marine organisms (2, 7, 14) and has been implicated as a cause of gastroenteritis, wound infections, and primary septicemia in humans, with a mortality rate exceeding 50% (8, 19). In humans, serious illness most commonly occurs when raw or undercooked seafood, such as shellfish, is ingested or when open wounds are exposed to seawater carrying *V. vulnificus* (8). *V. vulnificus* has been reported to persist at high levels within *Crassostrea virginica* (eastern oyster) tissues and to reproduce in hemolymph and other tissues during warm weather (17, 18).

Most, if not all, *C. virginica* organisms found in mid-Atlantic and Gulf Coast waters are heavily infected with *Perkinsus marinus*, an oyster pathogen responsible for severe oyster population losses throughout this region (3). All oysters from these waters contain *V. vulnificus* (14). Concern about these two oyster-associated microorganisms is increasing. Whether the prevalence of these organisms in the eastern oyster is correlated cannot yet be determined.

Recently, however, some aspects of oyster mortalities induced by *P. marinus* have been unraveled (10, 11). Studies reported by La Peyre (12) indicate that *P. marinus* produces a serine protease (ECP) as a major virulence factor, which is capable of digesting oyster connective tissues by degrading extracellular matrix proteins. Moreover, Garreis et al. (4) provided evidence that the serine protease is also a potent immunosuppressant which can reduce oyster hemocyte motility and lysosomal activity in oyster hemolymph. Other researchers have found that the ability of oysters to resist infection with *P. marinus* depends on the numbers and activities of hemocytes at the time of infection (3). Reports by La Peyre et al. (9) of declining levels of in vivo lysosomal activity and hemagglutination activity in heavily infected eastern oysters also support these findings.

In our study, we examined the in vitro effects of *P. marinus* serine protease treatment on the uptake and intracellular survival of *V. vulnificus* within oyster hemocytes by using a gentamicin internalization assay. The results showed that protease-treated hemocytes were initially slower to internalize *V. vulnificus* than were untreated hemocytes and that the elimination of *V. vulnificus* by treated hemocytes was significantly suppressed compared with that by similarly treated hemocytes infected with a highly invasive strain of *Salmonella enterica* serotype Enteritidis and a noninvasive *Escherichia coli* strain. Our data suggest that *P. marinus* serine protease suppresses the vibriocidal activity of oyster hemocytes to effectively eliminate *V. vulnificus*, potentially leading to conditions favoring higher numbers of vibrios in oyster tissues.

An unencapsulated biotype 1 *V. vulnificus* strain, 4965T-1, grown on thioproline-NaCl-glutamate agar (pH 8) for 18 h at 30°C, as described by Hanes et al. (5), was used in our experiments. *Salmonella enterica* serotype Enteritidis strain SE-3 and *E. coli* HB101 were used, respectively, as pathogenic, invasive and nonpathogenic, noninvasive control strains. Each of these strains was grown on Trypticase soy agar (TSA) containing 1% NaCl for 18 h at 37°C. Although the mechanism(s) whereby hemocytes kill *V. vulnificus* is unknown, we chose the unencapsulated-phase variant of *V. vulnificus* in these experiments to maximize the killing effects. This decision is supported by evidence recently described by Harris-Young et al. (6).

P. marinus-free oysters were obtained from Mook Sea Farm (Damariscotta, Maine) and were maintained in flumes supplied with 1-μm-pore-size-filtered York River water, 20 ppt salinity. Oyster hemolymph was collected and viable hemocyte counts were performed as described by La Peyre et al. (9). Hemocytes (5×10^5 cells/ml) suspended in 1 ml of JL-ODRP-1 culture medium (9) were added to each well of four 24-well tissue culture plates, allowed to adhere for 30 min at 28°C, and then washed three times with artificial seawater (ASW; 20 ppt salinity). Hemocytes in 12 wells of each 24-well plate were coincubated with 1 ml of cell-free *P. marinus* ECP in JL-ODRP medium containing 100 μg of protein/ml (~3 U of protease activity) for 1 h at room temperature. The ECP was prepared and isolated according to the procedure described by

* Corresponding author. Mailing address: Division of Microbiological Studies (HFS-517), Food and Drug Administration, 200 C St. S.W., Washington, D.C. 20204. Phone: (202) 205-4648. Fax: (202) 401-7740. E-mail: BTall@bangate.fda.gov.

† Virginia Institute of Marine Science contribution number 2240.

‡ Present address: Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803.

TABLE 1. Recovery of *V. vulnificus* 4965T-1, *S. enterica* serotype Enteritidis strain SE-3, and *E. coli* HB01 from primary oyster hemocytes treated with *P. marinus* ECP or left untreated

Strain	CFU/ml recovered from hemocytes (% recovery ± SD) at indicated no. of min					
	Treated			Untreated		
	0	60	120	0	60	120
4965T-1	790 (0.02 ± 0.1)	1,720 (0.04 ± 0.04)[a]	934 (0.02 ± 0.03)	3,700 (0.06 ± 0.03)[a]	345 (0.008 ± 0.005)	355 (0.007 ± 0.007)
SE-3	364 (0.016 ± 0.02)	976.1 (0.38 ± 0.7)	9,950 (0.44 ± 0.2)	3,120 (0.14 ± 0.2)	399.2 (0.17 ± 0.04)	7,786 (0.34 ± 0.01)
HB01	35 (0.005 ± 0.003)[a]	218.6 (0.08 ± 0.09)	5 (0.0006 ± 0.002)	3.3 (0.0005 ± 0.001)	76 (0.033 ± 0.02)	0.00

[a] Statistically significant compared to analogous treated or untreated group, as determined by ANOVA.

La Peyre et al. (10). The remaining 12 wells received 1 ml of fresh medium and were considered untreated controls.

Bacterial cell suspensions (10^8 CFU/ml) harvested in 0.9% saline were diluted in the JL-ODRP-1 medium to give a multiplicity of infection of approximately five bacterial cells per hemocyte. Inocula used to infect ECP-treated hemocytes were prepared in fresh JL-ODRP-1 medium containing ECP. After the bacterial inocula were added to the settled hemocytes, the plates were incubated at 28°C for 0, 60, and 120 min. After each incubation period, the hemocytes were washed with ASW and incubated with 2 ml of JL-ODRP-01 medium containing 100 μg of gentamicin/ml for 1 h at 28°C. The hemocytes were again washed three times with ASW and lysed with 0.5 ml of ice-cold sterile 0.05% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) for 15 min at 28°C. Lysates were diluted 10-fold with ASW, and 100-μl aliquots of each dilution were spread onto TSA plates containing 1% NaCl as the recovery medium. After incubation at 28°C for 24 h, colonies were counted. Recovery data percentages were calculated and analyzed as described previously by using Student's *t* test and one-factor analysis of variance (ANOVA), followed by Dunns' or Student-Newman-Keuls multiple comparisons of means when significant differences ($P < 0.05$) were found (5).

The percent recovery results of the experiments are summarized in Table 1. At 0 min of interaction, significantly more *V. vulnificus* 4965-T1 cells were recovered in hemocytes not treated with ECP than in hemocytes treated with ECP (~4.5 times, 3,700 CFU/ml versus 790 CFU/ml, $P < 0.05$). By 60 min postchallenge, the average number of *V. vulnificus* cells recovered from untreated hemocytes decreased to 345 CFU/ml, while the number of cells recovered from treated hemocytes increased to 1,720 CFU/ml, a greater-than-fivefold difference in recovered cells. The percent recovery for *V. vulnificus* internalized by treated hemocytes was significantly greater than that recovered from untreated hemocytes ($P < 0.05$). Finally, by 120 min postchallenge, the average number of *V. vulnificus* cells recovered from untreated hemocytes was 355 CFU/ml, whereas the number of cells recovered from treated hemocytes decreased from 1,720 to 934 CFU/ml. Even though the percent recovery for *V. vulnificus*-internalized cells by treated hemocytes was approximately 2.5 times greater than that recovered from untreated hemocytes, these numbers were not statistically significant (Table 1).

Conversely, recovery of *E. coli* HB101 at 0 min of interaction showed that significantly more (approximately 10-fold) *E. coli* cells were recovered from treated hemocytes than from untreated hemocytes (35 versus 3.3 CFU/ml, $P < 0.05$). However, the recovery of Enteritidis strain SE-3 from untreated hemocytes at this time was not significantly greater than that recovered from treated hemocytes, even though the number of CFU/milliliter from the untreated hemocytes (3,120 versus 364 CFU/ml) was approximately 8 times greater. On average, by 60 min postchallenge, twice as many cells of Enteritidis and *E. coli* were recovered from the treated hemocytes than from the untreated hemocytes (976.1 strain SE-3 versus 218.6 *E. coli* CFU/ml for the treated hemocytes and 399.2 strain SE-3 versus 76 *E. coli* CFU/ml for the untreated hemocytes). The percentage of recovery of both Enteritidis- and *E. coli*-internalized cells from treated hemocytes, however, was not significantly greater than the percentage recovered from untreated hemocytes. Finally, by 120 min postchallenge, 1.5 times as many cells of Enteritidis were recovered from treated hemocytes as from untreated hemocytes (9,950 versus 7,786 CFU/ml) for treated and untreated hemocytes, respectively. The percent recovery of Enteritidis-internalized cells from treated hemocytes was not significantly greater than that recovered from untreated hemocytes. No *E. coli* cells were recovered from the untreated hemocyte group compared with 5 CFU/ml recovered from treated hemocytes. This trend was not significant.

Severe *P. marinus* infections in the eastern oyster are accompanied by an overwhelming infectious dose, a high rate of proliferation in host tissues, and a depressed oyster immune response, all of which occur by as-yet-undefined mechanisms (3, 12). It has been postulated that the protease may be responsible for the overwhelming immunosuppression culminating in the inability of oyster hemocytes to kill and degrade intracellular *P. marinus* cells (4, 10). Taken together, our results demonstrate that the protease produced by *P. marinus* induces a similar hemocytic immunosuppressive reaction against *V. vulnificus*; protease-treated hemocytes were initially slower to internalize *V. vulnificus* than untreated hemocytes, but once *V. vulnificus* was internalized, the vibriocidal activities of the treated hemocytes against it were suppressed compared with those of the untreated hemocytes. These results correlate well with the results reported by Garreis et al. (4), which demonstrated an inhibition and reduction of antiprotozoan activity in protease-treated hemocytes toward *P. marinus*. Similar inhibitory effects on neutrophil motility and neutrophil and monocyte opsonization have been described for the Gp63 protease produced by *Leishmania major* (16). The internalization and recovery of Enteritidis by hemocytes were unaffected by *P. marinus* protease treatment, and the organism survived quite well for over 2 h inside hemocytes. This lack of effect suggests that Enteritidis is not readily killed by these cells. The process of elimination of *E. coli* by the hemocytes was also unaffected by treatment; however, the outcome did differ in that both groups of hemocytes could effectively eliminate the organism. The mechanism by which the *Perkinsus* serine protease immunomodulates oyster defense mechanisms is currently of great interest and is being intensely studied (10).

Recovery of viable *V. vulnificus* was greater from protease-treated hemocytes, affirming our hypothesis that in the feral setting, higher numbers of *V. vulnificus* in oysters harvested from waters warmer than 25°C may also be due to increased

numbers of *P. marinus* (escalation of infection) and its immunosuppressive activities, controlled by the serine protease (5, 10, 11). In support of this hypothesis, Ordas et al. (15) reported that the treatment of hemocytes obtained from both European mussels and carpet clams with ECP isolated from another *Perkinsus* species, *P. atlanticus*, had an inhibitory effect on the phagocytic ability of hemocytes for *Vibrio tapetis*, the etiological agent of brown ring disease in clams (1). Additionally, La Peyre and Volety reported a dose-dependent reduction in vibriocidal activity in eastern oyster hemocytes treated with ECP and infected with *Vibrio parahaemolyticus* (13). Together, these data suggest that the serine protease expressed by these two *Perkinsus* spp. can modulate the vibriocidal hemocytic response against multiple *Vibrio* spp. More importantly, these studies stress the importance of verifying the health status of the oyster (or clam) as a host and as a vehicle of transmission before attempting to assess the levels of vibrios in these economically important marine species.

In conclusion, we describe an observation by which a secreted protease produced by the oyster pathogen *P. marinus* significantly suppressed the innate vibriocidal ability of eastern oyster hemocytes to eliminate *V. vulnificus* in vitro. This observation is significant for public health, especially for understanding how *V. vulnificus* persists in the oyster and can rise to unsafe levels in edible oyster shell stock.

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