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PROTEASE ACTIVITY IN THE PLASMA OF AMERICAN OYSTERS, *CRASSOSTREA VIRGINICA*, EXPERIMENTALLY INFECTED WITH THE PROTOZOAN PARASITE *PERKINSUS MARINUS*

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ABSTRACT: *Perkinsus marinus* is responsible for disease and mortality of the American oyster, *Crassostrea virginica*. To investigate the interactions between *P. marinus* and oyster hemocytes, protease activity was measured in plasma of oysters collected 4 hr, 24 hr, 4 days, and 2 mo after experimental infection with *P. marinus*. A significant increase in protease activity was observed in oyster plasma 4 hr after injection with *P. marinus*, followed by a sharp decrease within 24 hr. Gelatin-impregnated gel electrophoresis showed the presence of 2 major bands (60 and 112 kDa) and 3 less prevalent bands (35, 92, and 200 kDa) with metalloproteinaselike activity in the plasma of noninfected oysters. Additional bands in the 40- to 60-kDa range, corresponding to *P. marinus* serine proteases, were observed in oyster plasma at early time points after infection. A transient, but significant, decrease in the activity of oyster metalloproteinases was observed at early time points after infection. Coincubation of oyster plasma with *P. marinus* extracellular products resulted in a decrease in oyster metalloproteinases and several *P. marinus* proteases. This study provides insights into the role of proteases in the pathogenesis of Dermo disease.

Dermo disease caused by the endoparasitic protozoan *Perkinsus marinus* (Perkinsoa, Noren et al., 1999) is responsible for widespread disease and mortality of the American cupped oyster, *Crassostrea virginica*, throughout the Gulf of Mexico and the east coast of North America, with correspondingly widespread economic and ecological impact (Ford and Tripp, 1996; Ray, 1996). Infections seem to occur when the parasite, present in the water and encountered as oysters feed, is ingested and crosses the external epithelia of the oyster's mantle, palps, gills, and gut (Dungan et al., 1996; Chintala et al., 2002). This process is probably aided by the release of lytic molecules by *P. marinus* or by being transported in hemocytes that fail to kill the parasite (Mackin, 1951; Mackin and Boswell, 1955; Chintala et al., 2002). The parasite causes a slow-progressing disease, characterized during the final stages by active parasite proliferation and extensive tissue damage, eventually leading to emaciation and death of the oyster (Mackin, 1951; Perkins, 1976).

Proteases have long been recognized as critical factors in disease pathogenesis, immunity, and host–pathogen interactions (Modha et al., 1996; Rhoads and Fetterer, 1997; Nagase and Woessner, 1999; Meza, 2000; Klemba and Goldberg, 2002). Proteases produced by pathogens are involved in the process of infection by inducing parasite differentiation (Rosenthal et al., 1987; Braun-Breton and Pereira, 1988), by facilitating penetration through the host tissues and the release of nutrients (McKerrow et al., 1993), and by stimulating and degrading host proteins involved in immune defenses (McKerrow et al., 1993; Corradin et al., 1999, 2002). Protozoan parasites from species of *Perkinsus* have been previously shown to secrete extracellular serine proteases in vitro (La Peyre et al., 1995; Faisal, Schafhauser et al., 1999; McLaughlin et al., 2000; Ordás et al., 2001), and extracellular proteins (ECP) from *P. marinus* cells in a culture rich in these serine proteases have been shown to affect oyster defenses in vitro and in vivo (Garreis et al., 1996; Oliver et al., 1999; Tall et al., 1999) and to exacerbate disease in oysters (La Peyre et al., 1996).

Host immune cells are also able to produce a large array of extracellular and intracellular proteases involved in a variety of physiological and pathological processes, including wound healing, signaling, cell migration, cytotoxicity, apoptosis, and necrosis (Fineschi and Miller, 1997; Darmon and Bleackley, 1998; Villadangos et al., 1999; Sim and Laich, 2000). Among host proteases, matrix metalloproteinases (MMPs) are responsible for the maintenance and remodeling of the extracellular matrix, playing an important role in immune-related processes like cell migration and inflammation (Massova et al., 1998; Shapiro, 1998; Nagase and Woessner, 1999). Although relatively few MMPs have been isolated from invertebrates (Wada et al., 1998; Llano et al., 2000), metalloproteinaselike activities have been recently observed in oyster and mussel hemocytes (Mannello et al., 2001; Ziegler et al., 2002). A tissue inhibitor of metalloproteinases (TIMP), which tightly regulates the activity of MMPs, has been recently cloned from the hemocytes of the Pacific oyster, *Crassostrea gigas* (Montagnani et al., 2001). *Cg*-TIMP expression is limited to the hemocytes and is upregulated in response to bacterial infection and shell damage, which suggests its involvement in oyster immunity and restructuring of tissues to prevent invasion or repair damage.

The aim of this work was to investigate the profiles and time course of protease activity in oyster plasma at early time points after experimental infection with *P. marinus*, when the host response is vital to the establishment of the parasite. This work may lead to an increase in our understanding of the potential role of proteases in the pathogenesis of Dermo disease.

MATERIALS AND METHODS

Oysters

American oysters, *C. virginica*, 7.6 ± 1.0 cm in shell length (n = 175 per experiment), were purchased from a commercial source (Taylor Shellfish Company, Samish Bay, Washington). These oysters belong to a line resulting from a cross of naturalized *C. virginica* brought from the east coast of the United States to Willapa Bay (Washington) in the early- to mid-1900s and *C. virginica* brought from the east coast during 1980–1990s (J. Davis, pers. comm.). Oysters were placed in groups of 10–15 oysters in 20-L tanks placed in a recirculating system supplied with 1-μm–filtered and ultraviolet-irradiated seawater at a temperature

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of 15 C, which was slowly increased to 20 C during a period of 2 wk. Water salinity and temperature ranged from 25 to 30 ppt and 20 to 25 C during the course of the experiments. Oysters were fed daily with a mixture of *Tetraselmis* and *Isochrysis* (2.5×10^8 cells/day per oyster, Instant Algae™, Reed Mariculture Inc., San Jose, California). Before experimental infections, a subsample of 25 oysters was tested to assess the presence of *P. marinus* by Ray fluid thiogylocollate medium (RFTM) tissue assay (Ray, 1952, 1966) and polymerase chain reaction (PCR; Marsh et al., 1995) and other infectious conditions by histology (Thoessen, 1994). The intensity of *Perkinsus* infections was scored according to the method described by Mackin (1962) (Mackin index = 0–5; e.g., 0 = no infection and 5 = heavy infection). The presence of the crustacean parasite *Mytilicola intestinalis* was observed in the digestive tissue and mantle of 11.3 and 2.4% of the oysters in the first and second experimental infections, respectively. Noninfected and naturally Dermo-infected American oysters were collected from local sources (Wickford Cove and Dutch Harbor, Rhode Island) (Mareiro, 2002).

Perkinsus marinus cultures

Perkinsus marinus ATCC 50510 (originally isolated from oysters from Mobjack Bay, Virginia) cultures were maintained as previously described (Gauthier and Vasta, 1993). In brief, Dulbecco modified Eagle medium–Ham F12 (1:1) was dissolved in 23 ppt artificial seawater (ASW) buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfoninc acid, 7 mM sodium bicarbonate, and supplemented with 100 U/ml each of penicillin G and streptomycin sulfate and 2% fetal bovine serum (Life Technologies Inc., Rockville, Maryland) before filtration through a 0.22-μm filter. Cell cultures were maintained at 28 C with weekly changes of media. Cultures in logarithmic growth phase were used in the experimental infections.

Experimental infections

Two independent experimental infections of oysters with *P. marinus* were carried out in October–December 2000 and July–October 2001. Oyster shells were notched in the edge to make a small hole before parasite inoculation. In the first experiment, oysters were injected with 5×10^5 parasites/g oyster weight diluted in 100 μl of filtered ASW (20 ppt) through the hole into the shell cavity using a syringe fitted with a 27-gauge needle. After injection, notches were covered with rubber bands, and inoculated oysters were left out of the water overnight to promote retention of the parasites. Bands were then removed, and oysters were placed in tanks with running seawater until sampling. In the second experiment, 5×10^6 parasites/g oyster weight diluted in 100 μl of ASW were injected into the adductor muscle, and the oysters were returned to the tanks. Control oysters were injected with 100 μl of ASW and placed in separate 20-L containers. Mortalities were recorded daily for up to 2 mo.

Plasma sample collection and processing

Twenty oysters from each experimental group (ASW and *P. marinus*) were shucked and oyster tissues were grossly examined to determine and record the presence of macroscopic parasites or signs of bacterial infection (brown ring or conchiolin deposits; Bricelj et al., 1992), 4 hr, 24 hr, 4 days, and 2 mo after injection. Hemolymph and fluid contained in the shell cavity (which also contains hemocytes) were collected by aspiration from the pericardial space and from the internal shell volume, respectively, using a 27-gauge needle fitted on a 3-ml syringe. Unless otherwise stated, hemolymph and fluid in the shell cavity were pooled and referred to as hemolymph. Hemolymph samples were placed on ice and centrifuged at 14,000 *g*, 4 C, for 10 min. The cell-free supernatant (plasma) was filtered through a 0.2-μm filter (Millipore, Bedford, Massachusetts) and stored at −20 C until determination of protease activity. Samples of rectal tissue were rinsed in ASW to wash off parasites loosely associated with the tissue and used to determine the level of *Perkinsus* sp. infection by RFTM.

Measurement of total protease activity in plasma

Proteolytic activity was assayed by the increase of trichloroacetic-soluble azopeptides produced upon incubation of hemolymph with azocasein (Windle and Kelleher, 1997). Azocasein (ICN Biomedicals Inc., Costa Mesa, California; 5 mg/ml) was dissolved in Tris–HCl (50 mM, pH 8.0) containing 0.04% sodium azide. One hundred microliters of plasma were incubated with 100 μl of azocasein for 30 min at 37 C. The reaction was terminated by the addition of 400 μl of 10% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation (28,000 *g* for 4 min), and the resulting supernatant was transferred to a clean tube containing 700 μl of sodium hydroxide (525 mM; Fisher Scientific, Swanee, Georgia). Absorbance was measured at 630 nm on a SpectraMax® 340 microplate spectrophotometer system (Molecular Devices, Sunnyvale, California). Negative controls consisted of heat-inactivated plasma samples (10 min at 90 C). Results are expressed as absorbance at 450 nm or as units of protease activity per milligram of plasma protein. One unit of protease activity is defined as the amount of enzyme required to produce an increase of 1.0 absorbance at 450 nm. Total plasma proteins were determined using the BioRad Protein assay (BioRad, Hercules, California) based on the Bradford dye-binding procedure (Bradford, 1976) using bovine serum albumin as standard.

Gelatin-impregnated polyacrylamide gel electrophoresis

Plasma samples collected from oysters in the second experiment (injection into the adductor muscle) were analyzed for profiles of gelatinolytic activity using gelatin-impregnated polyacrylamide gel electrophoresis. Plasma samples (protein concentration, 3–10 μg protein/lane) were mixed 1:1 in nonreducing sodium dodecyl sulfate (SDS) sample buffer (62 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.005% bromophenol blue), loaded on either SDS gels containing 8% acrylamide polymerized in the presence of gelatin (1 mg/ml) (Heussen and Dowdle, 1980) or precast 10% zymogram (gelatin) gels (Novex, Invitrogen, Carlsbad, California), and run on a Xcell II™ mini cell unit (Novex) on constant current. After electrophoresis, gels were incubated in 2.5% Triton X-100 or zymogram renaturing buffer (Novex) for 1 hr to restore proteolytic activity, rinsed twice in phosphate-buffered solution (PBS, pH 7.4), and incubated overnight in PBS or zymogram developing buffer (Novex) at 37 C to allow proteolysis. The gels were stained in a 0.1% solution of amido black in methanol–acetic acid–water (40:10:50%) for 1 hr and destained in the same solvent. Proteases were observed as clear bands in a blue background wherever digestion of copolymerized gelatin occurred. Pictures were taken and analyzed using Kodak electrophoresis documentation and analysis system 120 (Eastman Kodak, Rochester, New York). Results are expressed as ratio between the band intensity (relative object intensity) as determined by densitometry and concentration of protein loaded in the gels in micrograms. Selected samples were concentrated using microfuge concentrators with a membrane cutoff of 10 kDa, according to manufacturer's instructions (Microcon-10, Amicon Inc.; Millipore). Approximate molecular weights of denatured nonreduced proteases in gelatin-impregnated gels were estimated by comparison of clear bands of activity with prestained molecular weight markers (Broad range, BioRad) and by visual comparison with band patterns observed in previously published research (Faisal, Oliver et al., 1999; Faisal, Schafhauser et al., 1999; Ziegler et al., 2002).

The effect of protease inhibitors on gelatinolytic activity was determined by incubating gels overnight at 37 C in PBS containing 1 of the following inhibitors: 10 mM *o*-phenantroline dissolved in dimethyl sulfoxide, 10 mM phenylmethylsulphonyl fluoride (PMSF) dissolved in ethanol, or 10 or 100 mM ethylenediaminetetraacetic acid (EDTA) dissolved in water.

Effect of coincubation with *Perkinsus* ECP on oyster plasma gelatinolytic activity

Plasma samples of 4 oysters from Wickford Cove, Rhode Island (average Mackin index 2.3 ± 1.5), were pooled, mixed 1:1 with 20 μl of ECP (protein concentration of 0.5 mg/ml and protease activity of 10 U/mg of protein) from cultures of *Perkinsus*-1 (P-1; Oliver et al., 1999), and incubated for 1 hr at room temperature with gentle agitation. Twenty-microliter samples from a pool of plasma (protein concentration of 0.6 mg/ml, 4 U/mg protein) from an additional 4 oysters from Dutch Harbor (Rhode Island; average Mackin index of 0) were incubated with 20-μl serial dilutions (1:2, starting concentration of 0.04 mg/ml, 1 U/mg protein) in 9 salts solution (NSS) (Marden et al., 1985) of a fraction of ECP containing low–molecular weight (LMW) proteins. Samples were then run in a gelatin-impregnated 8% SDS–polyacrylamide gel

TABLE I. Prevalence (%) and average intensity (Mackin index, MI) of *Perkinsus* infection in oysters injected with artificial seawater (ASW) or *P. marinus* cells.

	Time after experimental infection							
	4 hr		24 hr		4 days		2 mo	
Experiment no.	Prevalence (%)	MI (SD)*	Prevalence (%)	MI (SD)	Prevalence (%)	MI (SD)	Prevalence (%)	MI (SD)
1								
ASW	0	0	0	0	0	0	0	0
P. marinus	55	0.7 (0.1)	50	0.7 (0.2)	35	0.6 (0.1)	20	0.2 (0.1)
2								
ASW	0	0	0	0	0	0	0	0
P. marinus	100	2.3 (0.2)	100	3.5 (0.3)	100	3.3 (0.2)	100	0.3 (0.6)†

* Infections were evaluated using Ray fluid thiogylocollate medium tissue assay, and the intensity of infection in each oyster was scored using MI, which ranges from 0 (no infection) to 5 (heavy infection). n = 20 oysters per group and time point.
† n = 14 oysters.

electrophoresis (PAGE) and processed as described above. LMW ECP was prepared by passing 100 µl of ECP through Microcon-50 concentrators (Amicon). The filtrate containing proteins less than 50 kDa was concentrated using Microcon-10 concentrators.

Effect of method of hemolymph sample collection on pattern of gelatinolytic activity

Naturally infected oysters from Wickford Cove (average Mackin index 2.3 ± 1.5) were divided into 2 groups of 5 oysters each and notched in the shell. Hemolymph and shell cavity fluid were collected either through the notch in the shell or after shucking by aspiration from the pericardial space and from the internal shell volume, respectively, using a 27-gauge needle fitted on a 1-ml syringe. Hemolymph samples were placed on ice and centrifuged at 14,000 *g*, 4 C, for 10 min. The cell-free supernatant (plasma) from 5 oysters was pooled, filtered through a 0.2-µm filter, and stored at −80 C until determination of protease activity. Hemocytes were resuspended in 100 µl of NSS, homogenized, centrifuged at 14,000 *g*, 4 C, for 10 min, and lysate supernatants were pooled and stored at −80 C. For determination of the pattern of gelatinolytic activity, samples were concentrated using Microcon-10 concentrators, run in a gelatin-impregnated 10% SDS-PAGE, and processed as described above.

Statistics

Statistical analysis was performed using Sigma Stat® software (SPSS Science, Chicago, Illinois). Total protease activity data were analyzed using 2-way analysis of variance (ANOVA). Post-hoc Tukey tests were conducted where significance ($P < 0.05$) was detected. Densitometric levels of gelatinolytic activity were analyzed using a 3-way ANOVA with infection (noninfected and infected), time, and band size (molecular weight) as variables. Multiple comparison procedures (Mann–Whitney rank sum tests or Student's *t*-test) were used to isolate significant groups. Linear regression was used to analyze potential relationships between protease activity and infection intensity and relationships between different bands.

RESULTS

Experimental infections with *Perkinsus marinus*

Prevalence of *P. marinus* ranged from 20% (first experiment) to 100% (second experiment) (Table I). Whereas no mortality was observed in the first experiment, oysters in the second experimental infection reached a cumulative mortality of 30% by 2 mo after injection. No *P. marinus* infection was observed in control oysters by either PCR or RFTM. Although the intensity of Dermo infection was similar in both experiments 2 mo after injection (Table I), there was a significant difference between levels of infection between the 2 experiments at earlier time points after injection. Moreover, quantification of *P. marinus* in the hemolymph of selected oysters collected 6 wk after injection of the parasite in the adductor muscle (second experiment) showed a Mackin index of 2.1 ± 1.2 (indicative of moderate infections), suggesting that oyster mortalities occurring between 15 and 60 days after injection of the parasite were due to heavy *P. marinus* infections.

Total protease activity in plasma of experimentally infected oysters

Total protease (caseinolytic) activity was observed in the plasma of both control and experimentally infected oysters (Fig. 1). Significantly higher levels of protease activity were observed in the plasma of oysters injected with *P. marinus* 4 hr after experimental infection (Fig. 1). There was no significant difference in plasma protease activity 24 hr or 4 days after injection into the shell cavity of either ASW or *P. marinus* (first experiment, Fig. 1a). However, significantly lower levels of protease activity ($P < 0.05$) were observed in the plasma of oysters collected 24 hr after injection of *P. marinus* into the adductor muscle (second experiment, Fig. 1b).

Gelatinolytic activity in plasma of experimentally infected oysters

The patterns of bands with gelatinolytic activity were highly variable between individual oysters. However, differences could be observed between experimental groups at the different time points (Figs. 2, 3; Table II). Bands with gelatinolytic activity of approximate molecular weights of 35, 60, 92, 112, and 200 kDa were observed in the plasma of oysters injected in the adductor muscle with filtered ASW (Fig. 2). The most prevalent bands were an intense and broad band of approximately 60 kDa and a band of 112 kDa (Table II). A 35-kDa band, a sharp 92-kDa band, and a faint band of 200 kDa were observed in the plasma of a small proportion of oysters (Fig. 2, lane 1; Table II). Injection of *P. marinus* elicited additional LMW bands (44- to 53-kDa range) in oyster plasma as early as 4 hr after injection (Table II). The LMW bands were most prevalent 24 hr and 4 days after injection with *P. marinus* (Fig. 3). A slight upward

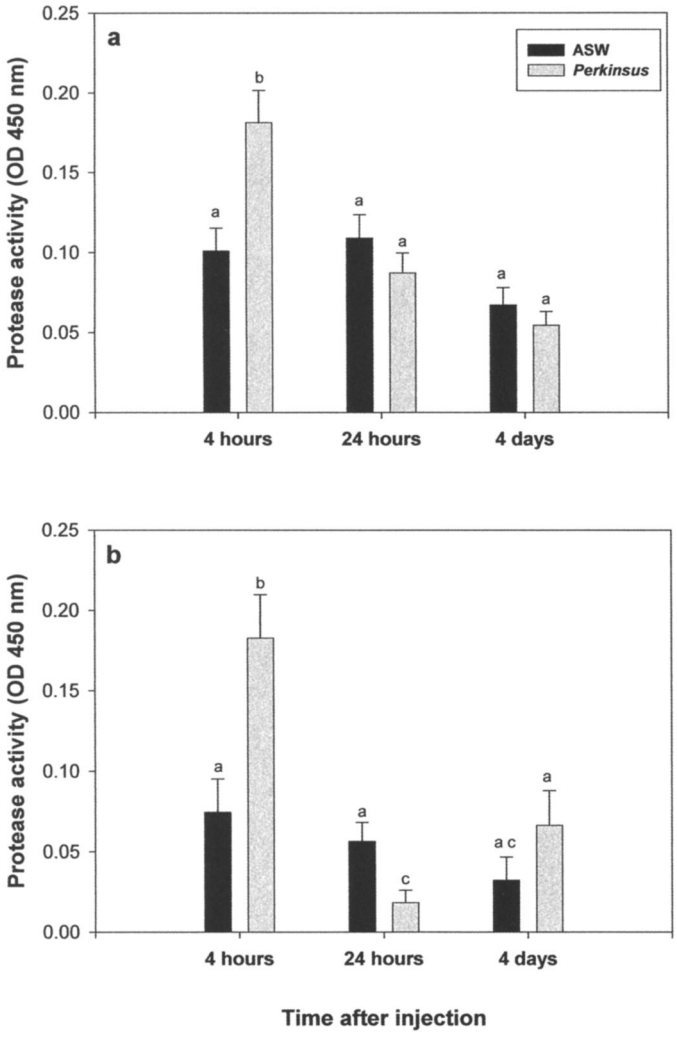

FIGURE 1. Total protease activity in plasma of oysters injected with filtered artificial seawater (ASW) or *Perkinsus marinus* (*Perkinsus*). **a.** Protease activity in plasma of oysters that were injected in the shell cavity (first experiment). **b.** Protease activity in plasma of oysters that were injected in the adductor muscle (second experiment). Protease activity in the plasma of at least 5 oysters per group and per experiment was determined using the azocasein assay. Results are expressed as average ± standard error of mean OD_{450nm}. Same letter indicates no statistical significance.

shift in the molecular weight of the 60-kDa band (Fig. 3b, lanes 1, 2) and the presence of 1 or 2 additional bands in the 57- to 62-kDa range were also observed in the plasma of several oysters injected with *P. marinus* (Fig. 3b, lanes 3–8). An overall increase in the prevalence of the 35-, 112-, and 200-kDa bands was observed in plasma of oysters injected with *P. marinus* (Table II). Oysters collected 2 mo after injection of *P. marinus* showed patterns of protease activity similar to oysters injected with ASW (Table II).

The 112-kDa band was not inhibited with the serine protease inhibitor PMSF (10 mM) or the metalloprotease inhibitors EDTA (10 mM) and *o*-phenantroline (10 mM). The 60-kDa band was partially inhibited with the metalloprotease inhibitors *o*-phenantroline (10 mM) and EDTA (10 mM). Total inhibition of the 35-kDa band, as well as partial inhibition of the 60- and 112-kDa bands, was detected after incubation of the gels with 100 mM EDTA. LMW proteases (40–50 kDa) were inhibited with 10 mM PMSF (data not shown).

Densitometric analysis of the intensities of the 60- and 112-kDa bands with gelatinolytic activity in plasma of oysters injected with ASW showed significantly higher levels of these proteases 4 and 24 hr after injection, as compared with levels measured at later time points, suggesting that the stress of injection or shell damage could induce the activation of oyster proteases (Fig. 4a). However, significantly lower levels of activity of the 60- and 112-kDa bands were observed in the plasma of oysters collected 4 hr after injection *P. marinus* (Fig. 4). Low levels of the 60-kDa protease persisted 24 hr after injection of *P. marinus*. No difference in the intensity of the 60- and 112-kDa bands was observed in the plasma of oysters injected with either ASW or *P. marinus* 4 days and 2 mo after injection. No significant correlation was observed between levels of protease activity for the 60- and 112-kDa bands or between band intensity and intensity of *P. marinus* infection (data not shown).

Effect of *Perkinsus* ECP on the activity of oyster plasma proteases

Coincubation of pools of plasma from noninfected or naturally infected *C. virginica* with *P. marinus* ECP or a LMW (less than 50 kDa) fraction of *P. marinus* ECP resulted in a dose-dependent decrease in the intensity of the 60- and 112-kDa oyster proteases (Fig. 5, lanes 6–8). The incubation of oyster plasma with *P. marinus* ECP also resulted in a change in the gelatinolytic profile of the ECP, causing a decrease in the intensity of bands in the 80- to 100-kDa range (Fig. 5, lane 3).

Effect of method of hemolymph sample collection on pattern of gelatinolytic activity

The patterns of gelatinolytic activity in pools of plasma from noninfected (Dutch Harbor; Fig. 5) and plasma and lysates of hemocytes from naturally infected (Wickford Cove, Mackin index 2.3 ± 1.5; Figs. 5, 6) *C. virginica* from Rhode Island were similar to the patterns observed in the plasma of *C. virginica* used in the experimental infections (Samish Bay, Washington; Figs. 2, 3). Bands of 35, 60, and 112 kDa were observed in noninfected oysters, whereas additional bands in the 40- to 53-kDa range were observed in the plasma and hemocyte lysates of naturally infected oysters (Fig. 6). Method of hemolymph sampling (aspiration through notch vs. shucking) did not appear to affect the profile of protease activity (Fig. 6).

DISCUSSION

Although the drastic conditions of experimental infections do not reproduce the natural route of exposure to pathogens, these experiments have proven useful in the study of host–pathogen interactions and mechanisms of disease resistance (Druilhe et al., 2002). In this study, the availability of in vitro cultures of *P. marinus*– and *Perkinsus* sp.–free susceptible oysters has allowed us to investigate the role of proteases in early infection, when parasite and host come in contact for the first time. Although the virulence of *P. marinus* is severely diminished when the parasite is cultured (Ford et al., 2002), we were able to establish successful infections in 1 of the 2 experiments per-

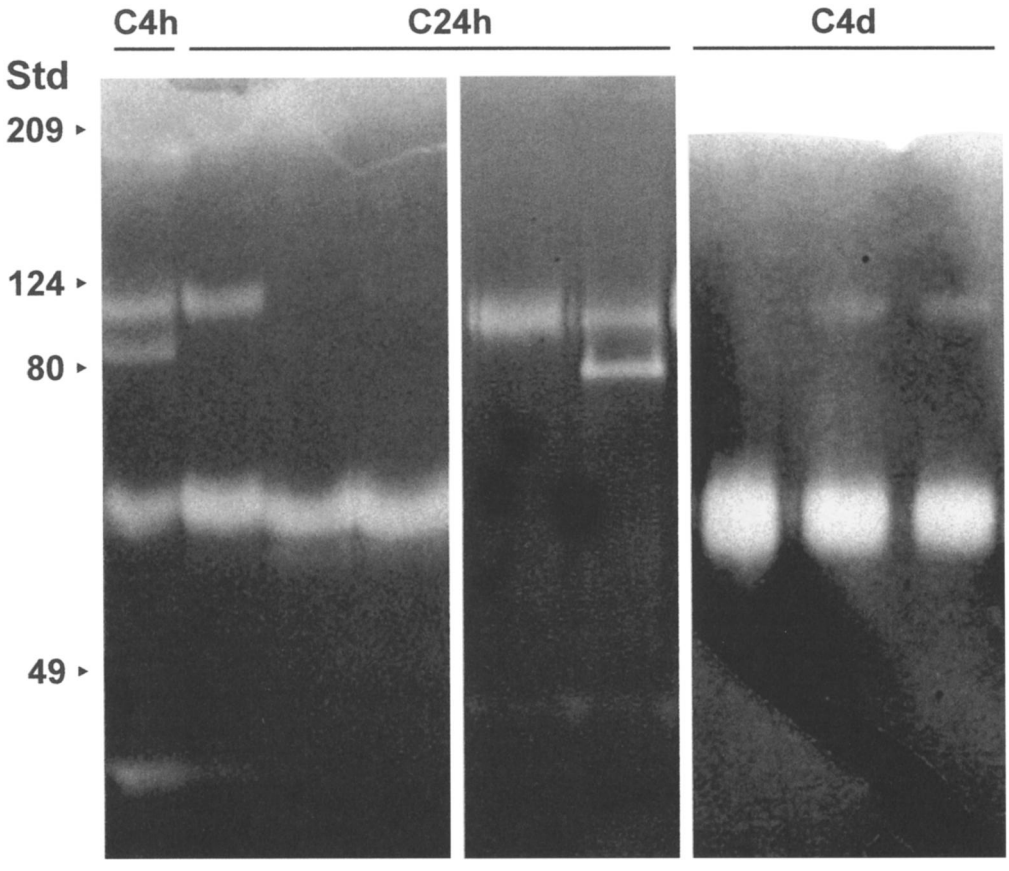

FIGURE 2. Representative patterns of gelatinolytic activity in the plasma (5–10 μg protein/lane) in control oysters (C) collected 4 hr, 24 hr, and 4 days after injection in the adductor muscle with filtered artificial seawater (ASW). Bands of activity are shown as clear bands over a dark background.

formed in this study. The different outcomes from the 2 experimental infections may have been due to differences in the method of challenge (injection into the shell cavity vs. injection in the adductor muscle), the dose of the parasite, or the metabolic condition of oysters at the time of challenge (Chintala et al., 2002). Nevertheless, differences in the profile of protease production between the successful and unsuccessful experimental infections provide clues to the role of proteases in the pathogenesis of Dermo disease.

Successful experimental infection with *P. marinus* resulted not only in an increase in protease activity in oyster plasma as soon as 4 hr after injection but also in the appearance of LMW bands of gelatinolytic activity. We have also observed the presence of LMW protease bands in the plasma of naturally infected oysters. The fact that these bands of protease activity are seen only in infected oysters, the similarity to band patterns observed in the analysis of supernatants of *P. marinus* in culture (La Peyre et al., 1995; Faisal, Schafhauser et al., 1999), and the inhibition pattern consistent with their nature as serine proteases indicate that these LMW proteases are most probably produced by the parasite. This is, to our knowledge, the first report of the detection of *P. marinus* serine proteases in the plasma of either experimentally or naturally infected oysters. The expression of *P. marinus* antigens in vivo has been studied using polyclonal antibodies to *P. marinus* ECP, but the nature of these parasitic antigens is unknown (Ottinger et al., 2001). An increase with time in the intensity of bands with metalloproteaselike activity has been previously observed in the plasma of oysters collected biweekly after experimental infection with *P. marinus* (Faisal, Oliver et al., 1999), but this study failed to detect the presence of LMW serine proteases. Lack of detection could be attributed to limits in the sensitivity of substrate-impregnated gel electrophoresis, which, in our experience, does not detect protease activity in oyster plasma unless at least 10 μg of plasma protein is loaded in the gels. Our success in detecting LMW serine proteases in the plasma of experimentally and naturally infected oysters could be because of the large amounts of parasites that were injected into the adductor muscle and the high concentrations of sample loaded in the gels. Interestingly, we failed to detect any of the high–molecular weight (HMW, more than 60 kDa) bands characteristic of *P. marinus* ECP (Faisal, Schafhauser et al., 1999; MacIntyre et al., in press). Differences in the patterns of parasitic protease activity in vivo and in vitro could be due to (1) differences in the profiles of protease activity between different *P. marinus* isolates, (2) proteolytic degradation of HMW parasitic proteases by oyster proteases, resulting in the appearance of bands of lower molecular weight, (3) induction of changes in the profiles of parasitic protease activity in the presence of oyster plasma, resulting in activation of LMW parasitic proteases and downregulation of HMW parasitic proteases, or (4) selective inhibition of the activity of HMW parasitic proteases by factors present in oyster plasma.

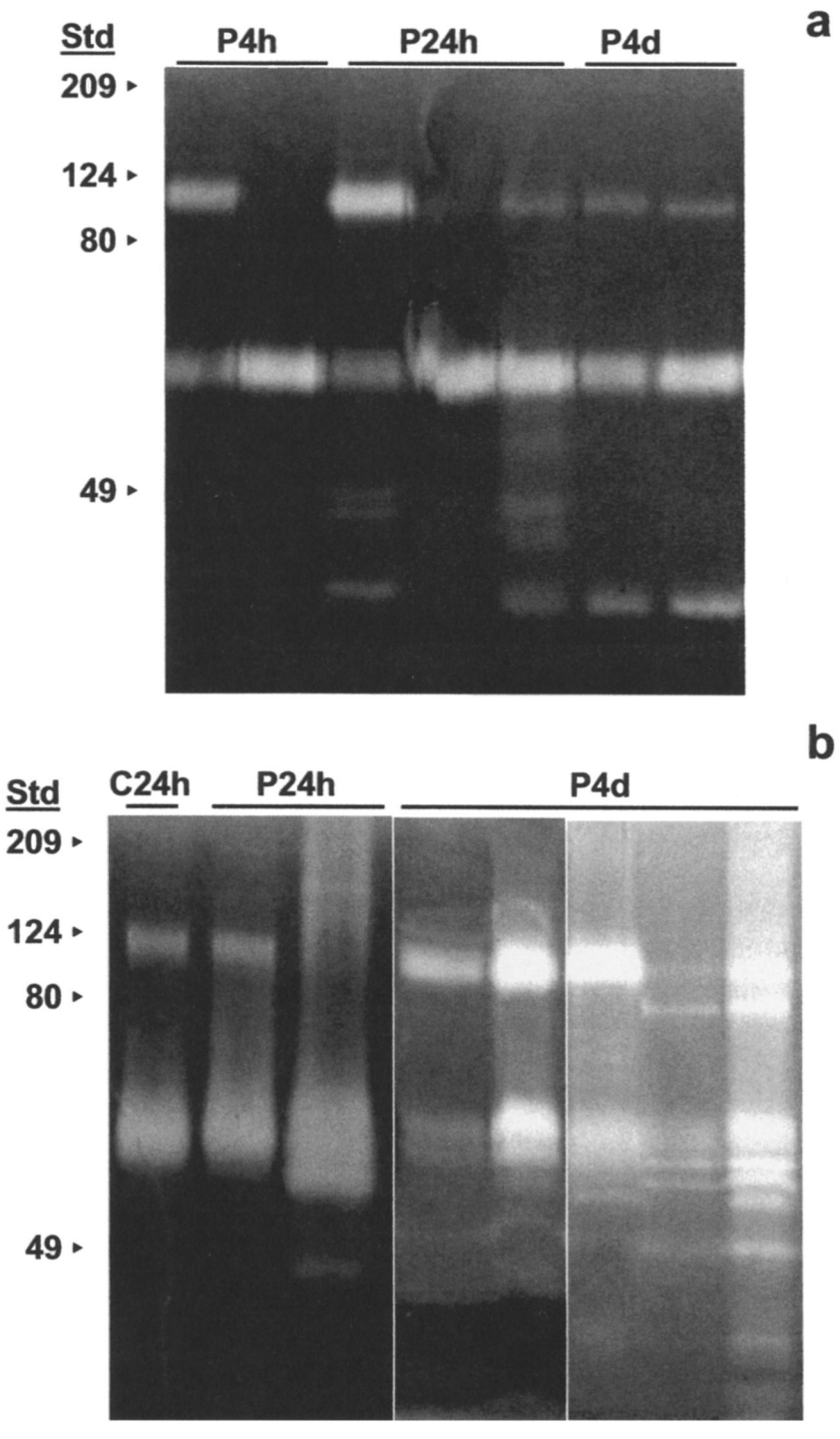

FIGURE 3. Representative patterns of gelatinolytic activity in the plasma (5–10 μg protein/lane) of oysters collected 4 hr, 24 hr, and 4 days after injection in the adductor muscle with *Perkinsus marinus* (P) or ASW (C). Bands of activity are shown as clear bands over a dark background. Lanes 7 and 8 correspond to the same sample run at 2 different concentrations (3 and 10 μg protein/lane, respectively).

Profiles of proteolytic activity have been shown to be dependent on the *Perkinsus* species and isolate and vary significantly depending on culture conditions (McLaughlin et al., 2000; MacIntyre et al., 2003). For example, whereas *P. marinus* isolate P-1 produces an array of LMW and HMW proteases, other *P. marinus* isolates have been shown to predominantly produce LMW proteases in vitro (C. Earnhart, pers. comm.). However, our in vitro experiments also showed that coincubation of oyster plasma with the ECP of *P. marinus* (P-1) results in a selective decrease in the intensity of several parasitic HMW proteases (80–100 kDa), suggesting the presence of factors in the plasma of *C. virginica* that could selectively inhibit or cause the degradation of parasitic proteases. Our results are also consistent with the recent findings of MacIntyre et al. (2003), which

TABLE II. Percentage of oyster plasma samples in which each band (kDa) of gelatinolytic activity was observed.

Experimental group	Percentage of band occurrence‡							
	200	112	92	60	53,51	46	44	35
ASW*								
4 hr (5)†	17	100	40	100	—	—	—	17
24 hr (6)	—	83	17	100	—	—	—	—
4 days (6)	—	67	17	100	—	—	—	33
2 mo (3)	—	100	—	100	—	—	—	—
*Perkinsus marinus**								
4 hr (7)	—	57	—	100	14	29	—	29
24 hr (11)	18	100	45	100	27	73	18	64
4 days (9)	22	100	22	100	11	22	11	44
2 mo (3)	—	100	—	100	—	—	—	33

* Oysters were injected in the adductor muscle with either artificial seawater (ASW) or *P. marinus* cells.
† Time after injection (number of oysters tested).
‡ — Indicates 0%.

showed that supplementation of *P. marinus* cell cultures with fresh plasma from *C. virginica* induces a change in the profile of protease activity in *P. marinus* P-1 ECP, resulting in the appearance of LMW proteases and a decrease in the activity of several HMW proteases. The appearance of LMW proteases in *P. marinus* ECP supplemented with *C. virginica* plasma was not because of activation of oyster proenzymes or modification of HMW proteases or inactive proteases, suggesting the selective induction of de novo LMW parasitic proteases by factors present in oyster plasma (MacIntyre et al., 2003).

This research also describes the effect of experimental infection with *P. marinus* on the activity and profile of oyster plasma proteases. Little is known about the role of host proteases in the response of oyster hemocytes against infection. Ziegler et al. (2002) recently characterized a 68-kDa MMP-like enzyme, as well as the presence of 2 additional bands of proteolytic activity of approximately 40 and 100 kDa, in the lysate of hemocytes from *C. virginica*. Bands of metalloproteaselike activity of 51, 60, 77, and 119 kDa have been reported in the plasma of noninfected *C. virginica* (Faisal, Oliver et al., 1999; Faisal, Schafhauser et al., 1999). The presence of 2 additional bands of about 138 and 220 kDa was observed in plasma of oysters collected from 2 to 10 wk after experimental infection with *P. marinus* (Faisal, Oliver et al., 1999). Our comprehensive evaluation of the profiles of gelatinolytic activity in the plasma of a large number of noninfected and infected *C. virginica* showed the presence of bands of metalloproteinaselike activity of approximately 35, 60, 92, 112, and 200 kDa in the plasma of *C. virginica*. Considering differences in methodology between the different studies, as well as the inaccuracy inherent to the estimation of molecular weights in gelatin-impregnated SDS-PAGE gels (Martinez and Cazzulo, 1992; Faisal, Schafhauser et al., 1999), we hypothesize that the 60-, 112-, and 200-kDa proteases detected in our study correspond to the 60-, 119- or 138-, and 220-kDa proteases from Faisal, Oliver et al. (1999). The bands of gelatinolytic activity at about 40, 68, and 100 kDa observed by Ziegler et al. (2002) could correspond to the 35-, 60-, and 112-kDa bands detected in this study. The additional sharp 92-kDa band detected for the first time in our study is

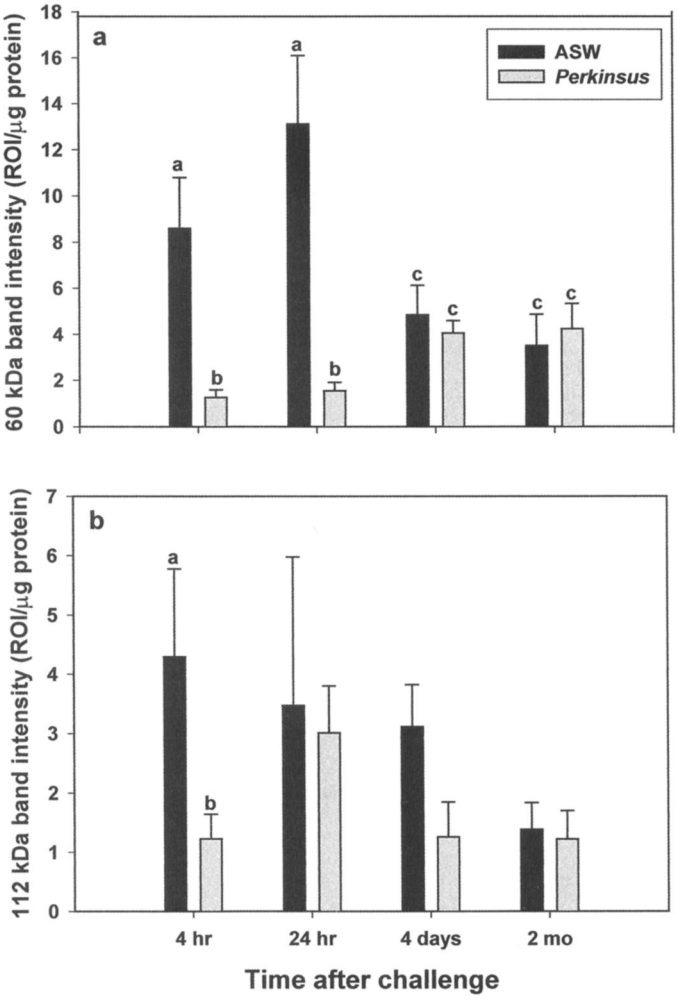

FIGURE 4. Densitometric analysis of the intensity of selected bands with gelatinolytic activity in the plasma of oysters collected at different time points after injection in the adductor muscle with filtered artificial seawater (ASW) or *Perkinsus marinus* (*Perkinsus*). **a.** Intensity of the 60-kDa band. **b.** Intensity of the 112-kDa band. Results are expressed as average ± standard error of mean relative object intensity units per microgram of plasma protein loaded in the gel of at least 5 oysters per experimental group.

detected in only 22% of the oysters, which may explain why it has not been previously observed in other research (Faisal, Oliver et al., 1999; Faisal, Schafhauser et al., 1999; Ziegler et al., 2002). Whereas 2 distinct bands of 51 and 60 kDa were observed in both noninfected and infected oysters in previous research (Faisal, Oliver et al., 1999), we observed only 2 distinct bands in the 57- to 60-kDa range in infected oysters. Although incomplete inhibition of the broad 57- to 60-kDa band by metalloprotease inhibitors suggests the presence of several proteases of differing nature, incomplete inhibition could be also due to technical difficulties in inhibiting large amounts of proteases in substrate-impregnated gels.

It is also unclear if any of the bands with metalloproteinaselike activity detected in *C. virginica* plasma correspond to products of proteolytic degradation. Although the increase in the prevalence of the 35-kDa band in oysters infected with *P. marinus* (47% vs. 20% in control oysters) is concurrent with a decrease in the intensity of the 60- and 112-kDa bands, no

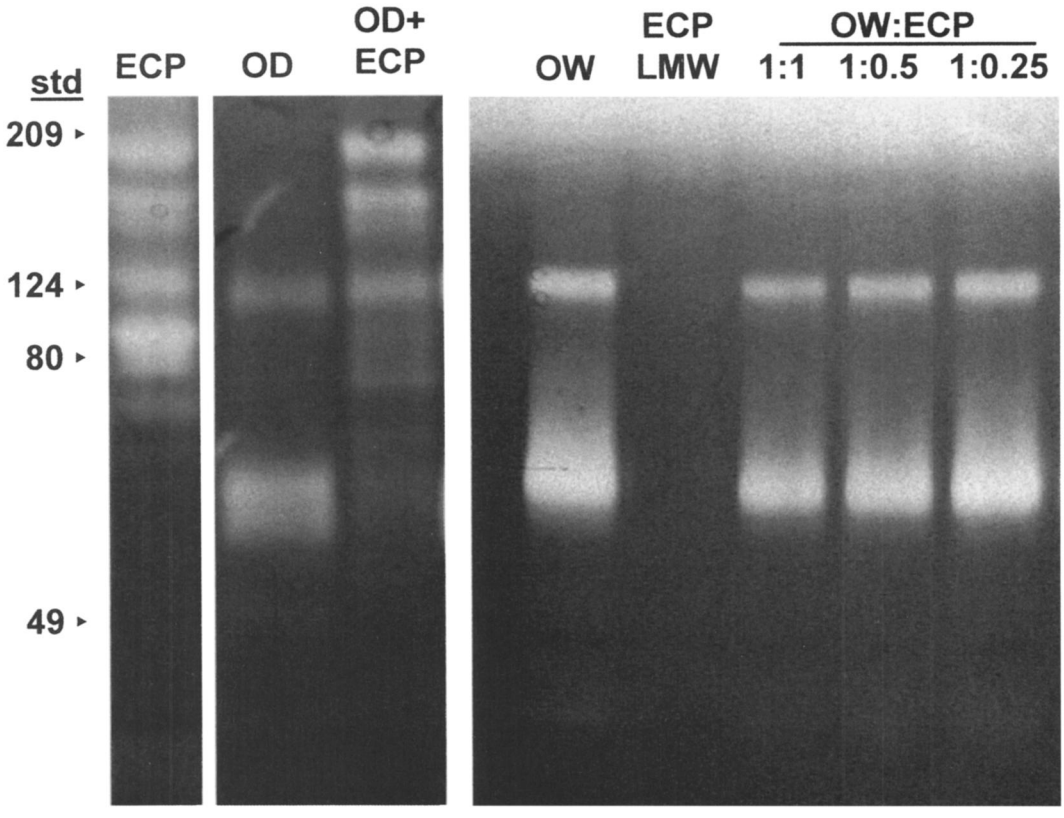

FIGURE 5. Effect of coincubation of oyster plasma with *Perkinsus* ECP on the pattern of gelatinolytic activity. ECP = P-1 extracellular proteins; OW = plasma from naturally infected oysters (Mackin index 2.3 ± 1.5); OD = plasma from noninfected oysters; ECP LMW = LMW (less than 50 kDa) fraction of the ECP.

significant correlation was observed in the intensities of the different bands in our study. Proteolytic band profiles in the plasma of *C. virginica* are similar to profiles of MMPs seen in the plasma of other molluscs (Mannello et al., 2001), marine invertebrates (Iida et al., 1991; Quigley et al., 1993; Robinson, 1997), and even vertebrate species (Massova et al., 1998; Shapiro, 1998; Nagase and Woessner, 1999), indicating that these bands may well correspond to several individual MMPs. Further isolation and characterization of both oyster MMPs and parasitic proteases would provide great insights into their functions, mechanism of regulation, and roles in immune defenses and pathology.

One of the most significant findings of this study is the significant decrease in total protease activity observed in the plasma of oysters collected 24 hr after injection of *P. marinus* into the adductor muscle. This decrease was not observed in the first experimental infection, which did not progress to clinical Dermo disease. This was an unexpected finding because we had also observed a significant increase in total protease activity 4 hr after injection of the parasite, as well as the appearance of bands of protease activity from parasitic origin, most prevalent 24 hr after injection of the parasite. Interestingly, a significant and transient decrease in the intensity of the oyster's metalloproteinases was observed in the plasma of experimentally infected oysters at early time points after injection of the parasite. One possible explanation to these findings is that *P. marinus* proteases are selectively degrading oyster proteases. Selective degradation of proteins in the plasma of *C. virginica* by *Perkinsus* proteases has been reported previously (Oliver et al., 1999). Furthermore, our in vitro experiments show that coincubation of oyster plasma with culture supernatants of *P. marinus* containing parasitic proteases results in the degradation of oyster proteases. Alternatively, the transient decrease in oyster proteases after experimental infection with *P. marinus* could be due to the production of metalloproteinase inhibitors by oyster hemocytes in response to *P. marinus* infection. Although we have not evaluated in this study the levels of metalloproteinase inhibitors in the plasma of experimentally infected oysters, changes in the levels of protease inhibitors in the plasma of bivalves under disease conditions have been observed in the soft-shell clam, *Mya arenaria* (Elsayed et al., 1999), the Atlantic surfclam, *Spisula solidissima* (Armstrong and Quigley, 1992), and the oysters *C. virginica* (Faisal et al., 1998; Oliver et al., 2000) and *C. gigas* (Faisal et al., 1998; Montagnani et al., 2001; Romestand et al., 2002). It has been hypothesized that protease inhibitors have a role in the ability of the Dermo-resistant oyster *C. gigas* to eliminate *P. marinus* (Faisal et al., 1998; Romestand et al., 2002).

It is unclear as to what role MMPs play in the defenses of *C. virginica* against the parasite or how the transient dysregulation of oyster MMPs observed in this study could affect parasitic infection. In molluscs, migration of hemocytes to the site of injury is one of the initial responses to infection or shell damage (Mackin, 1951; Perkins, 1976; Cheng, 1996). MMPs are likely involved in hemocyte migration and repair of the tissue at the site of injury. Dysregulation of MMPs by the par-

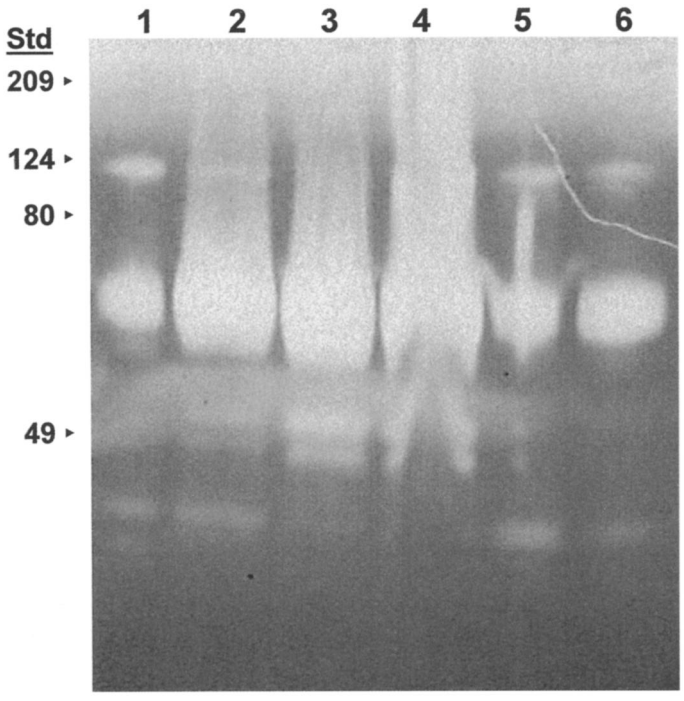

FIGURE 6. Effect of sampling method and source of hemolymph on the patterns of gelatinolytic activity in pools of plasma and hemocyte lysates from naturally infected oysters (Mackin index = 2.3 ± 1.5). Lane 1, plasma of hemolymph collected from pericardial sinuses by aspiration through notch; lane 2, lysate of hemocytes from hemolymph collected from pericardial sinus by aspiration through notch; lane 3, lysate of hemocytes from hemolymph collected from the shell cavity of shucked oysters; lane 4, plasma of hemolymph collected from the pericardial sinuses of shucked oysters; lane 5, plasma of hemolymph collected from the shell cavity of shucked oysters; lane 6, hemocyte lysate of hemolymph collected from the pericardial sinuses of shucked oysters. About 10–15 μg protein was loaded per lane.

asite may constitute a virulence mechanism by decreasing the ability of hemocytes to reach the site of injury (Perkins, 1969, 1976; Cheng, 1988; Volety and Fisher, 2000), preventing elimination of the parasite and tissue repair (Mackin, 1951; La Peyre et al., 1995; Bushek et al., 1997; Chintala et al., 2002).

This characterization of the time course of protease activity in the plasma of oysters during the early stages of experimental infection with *P. marinus* highlights the complex interactions occurring between this protozoan parasite and its host, the American oyster *C. virginica*. This research showed that in early stages of experimental infection, there is an increase in the activity of *P. marinus* LMW proteases as well as a transient but significant and rapid (within 24 hr) decrease in the activity of oyster metalloproteinases. It has been suggested previously based on the observed inability of plasma from oyster species resistant to Dermo disease (*C. gigas* and *Crassostrea arakiensis*) to induce *P. marinus* LMW proteases in vitro that the induction of LMW parasitic proteases may be a good indicator of *P. marinus* susceptibility (MacIntyre et al., 2003). A thorough evaluation of the mechanisms of MMP activity and regulation in response to parasitic infection in both resistant and susceptible species of oysters could determine if the activity of LMW parasitic proteases or MMPs could be used as early indicators of disease and provide insights into the role of proteases and protease inhibitors in the mechanisms of resistance to *P. marinus*.

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