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Evidence for the presence of protease inhibitors in eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters

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Abstract

The plasma of eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters were compared for levels of inhibitory activities against a variety of proteases. Representatives of the serine, cysteine, metallo and aspartic protease mechanistic classes were analyzed, including extracellular proteases produced by two oyster-associated pathogens; *Perkinsus marinus* and *Vibrio vulnificus*. In comparison to *C. virginica*, *C. gigas* plasma exhibited significantly higher specific inhibition levels (ng protease inhibited/μg plasma protein) for papain ($P < 0.001$), pepsin ($P < 0.001$), *P. marinus* protease ($P < 0.001$), trypsin ($P = 0.015$), and *V. vulnificus* protease ($P < 0.001$). Plasma of *C. gigas* did not inhibit the metalloprotease thermolysin. Instead, a significant increase in substrate hydrolysis was seen in wells containing plasma and thermolysin in comparison to wells containing thermolysin only. A similar trend was noted for thermolysin with the eastern oyster samples. These studies indicate the presence of protease inhibitors in the plasma of *Crassostrea* spp., which may have an impact upon host defense mechanisms, in addition to other physiological roles.

Keywords: Oyster; *Perkinsus marinus*; Protease inhibitors; *Vibrio vulnificus*

1. Introduction

Proteins with protease inhibitory activity have been detected in the tissue fluids of several animal phyla [28]. There is mounting evidence that these natural protease inhibitors (PI) are essential to various physiological processes in the body, including the ability to counteract proteases produced by invading microorganisms [8,12,18]. Similar protease inhibitory activities have been identified in the hemolymph of a number of mollusks such as the snail *Biomphalaria glabrata*, the whelk *Busycon canaliculatum*, the squid *Loligo solidissima*, the octopus *Octopus vulgaris*, and the bivalve *Spisula solidissima* [2,5,37]. The contribution of molluscan PI to the host defense mechanisms, however, has not been investigated.

Populations of the eastern oysters (*Crassostrea virginica*), found along the Atlantic and Gulf coasts of the United States, have been devastated by infection with the protozoan *Perkinsus marinus* [7,17,35]. Our studies have demonstrated that *P. marinus* produces extracellular serine proteases [25,26]. Moreover, we have shown that *P. marinus* extracellular proteases compromise several host defense parameters of the oyster [19], and enhance the protozoan's ability to propagate within the host [27]. On the contrary, the Pacific oyster (*Cras-*

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sostrea gigas) is more tolerant to *P. marinus* infection and mortalities seldom occur, even following experimental infection with a large number of protozoal cells [30]. The mechanism(s) leading to this remarkable difference in susceptibility between two closely related oyster species is currently unknown.

Vibrio vulnificus is a halophilic, Gram-negative, opportunistic human pathogen. It is an autochthonous member of bacterial communities associated with estuarine water, sediment, and various marine species, including *C. virginica* populations [21,32]. The organism has been implicated as a cause of human gastroenteritis, wound infection, and septicemia, primarily in immunocompromised individuals, with mortality rates sometimes exceeding 50% [6,36,38]. Virulence mechanisms of *V. vulnificus* include expression of an extracellular metalloprotease that has both elastolytic and caseinolytic activities [22,23]. *V. vulnificus* has also been isolated from *C. gigas* populations on the Pacific coast of the United States and Canada at much lower levels than those seen in *C. virginica* from the Atlantic and Gulf coasts [20].

The present study was therefore initiated to determine whether a difference exists between *C. virginica* and *C. gigas* in the levels of plasma inhibitory activities against a variety of proteases (including those of *P. marinus* and *V. vulnificus*) representative of the serine, cysteine, metallo and aspartic protease mechanistic classes.

2. Materials and methods

2.1. Oysters

Eastern oysters (*Crassostrea virginica*), 9–12 cm in shell length, were obtained from Pemaquid Oyster Farm (Waldoboro, ME). Pacific oysters (*Crassostrea gigas*), averaging 12 cm in shell length, were obtained from Lookout Point Oyster Company (Tillamook, OR). Both groups of oysters were obtained in April of 1997. The oysters were determined to be free from *P. marinus* by Ray's fluid thioglycollate test [33]. The oysters were maintained in separate 400-l tanks supplied with 1 μm filtered York River water (20°C). Salinity was adjusted to mimic that of their native sites, 30 ppt, using Marine Salt Mixture (Marine Enterprises, Baltimore, MD). Oysters were fed with an aqueous suspension of algae paste, *Thalassosira weissflogii* obtained from the Virginia Institute of Marine Science Oyster Hatchery (0.1 g paste/oyster day^{-1}).

2.2. Hemolymph

A small notch was carved in the dorsal side of the oyster shell, adjacent to the adductor muscle, and approximately 1 ml of hemolymph/oyster was collected from the adductor muscle sinus with a 27-gauge needle. Samples were placed on ice immediately following collection, and then centrifuged at $400 \times g$, at 4°C, for 10 min. The cell-free supernatant (plasma) was then collected, filtered through a 0.22-μm filter, aliquoted in 100-μl fractions, and stored at -20°C until use.

2.3. Proteases

Samples from 30 individual oysters of both species were analyzed for inhibitory activities against the following proteases; papain (cysteine protease) from *Papaya latex*, pepsin (aspartic protease) from porcine stomach mucosa, thermolysin (metalloprotease) from *Bacillus thermoproteolyticus rokko*, and trypsin (serine protease) from bovine pancreas, all purchased from Sigma (St. Louis, MO). Samples were also tested for inhibitory capacity against a crude preparation of *P. marinus* extracellular proteins (ECP) containing serine protease(s), prepared according to methods described by La Peyre et al. [26]. Briefly, culture supernatants from 8-week old cultures of *P. marinus* (seeded at 10^6 cell ml^{-1} in bovine albumin-free JL-ODRP-1 medium) were collected, and filtered (0.22 μm). The supernatant was then washed and centrifuged $2000 \times g$, at 4°C, three times, using Centriprep concentrators (Amicon, Beverly, MA) with a molecular weight cut-off of 10000, with 3 vol of ice cold artificial seawater (ASW) 22 g l^{-1} prepared with Forty Fathoms Marine Salt Mixture. Washes were performed to remove phenol red from the culture media. Samples were aliquoted in 1-ml fractions and stored at -20°C until use.

The metalloprotease of *V. vulnificus* was purified from culture supernatants according to the methods previously described [22,23]. Briefly, culture supernatant from *V. vulnificus* (A-9) grown at 37°C for 16 h in 2% protease peptone (Difco, Detroit, MI) with 1.5% NaCl were obtained by centrifugation ($16000 \times g$, 4°C, 20 min). Enzyme grade ammonium sulfate was dissolved in the culture supernatant fluids to a final concentration of 60% saturation (420 g l^{-1}). The precipitate was collected by centrifugation ($23000 \times g$, 20 min) and dissolved in 10 ml of cold 0.1 M ammonium bicarbonate (pH 7.8). The sample was then applied to a refrigerated hydrophobic interaction chromatographic column with phenyl–Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ). The fractions ($\sim$5 ml) were assayed for protein concentration (A_{280}), and for caseinolytic activity.

2.4. Chemicals and stock solutions

Stock solutions of proteases were prepared on ice

as follows; papain, thermolysin, and trypsin were prepared 1 mg ml^{-1} in distilled deionized water. Pepsin was prepared 1 mg ml^{-1} in 10 mM HCl. Subsequent dilutions of protease stock solutions were made in ASW 22 g/l, with the exception of papain, which was diluted in 5 mM cysteine, 300 mM NaCl, 150 mM Tris base, 0.05% Brij 35, 20% sucrose, 2 mM EDTA, pH 6.2. Proteolytic activities were quantified using the substrate hide powder azure (Sigma) as described by Bender et al. [5]. The substrate was prepared as 2.7% (w/v) in assay buffer consisting of 150 mM Tris base, 30 mM $CaCl_2$, 0.05% Brij 35, 20% sucrose, pH 7.5, for all proteases other than pepsin and papain. Pepsin assay buffer pH was adjusted to 2.0, and papain assay buffer consisted of 150 mM Tris base, 300 mM NaCl, 0.05% Brij 35, 2.0 mM EDTA, 5.0 mM cysteine, 20% sucrose, pH 6.2.

2.5. Protein concentration

Protein concentrations of oyster plasma samples, *V. vulnificus* (125 μg ml^{-1}) and *P. marinus* (7 μg ml^{-1}), protease preparations were determined with a BCA protein assay kit (Pierce, Rockford, IL), using the bicinchoninic acid protein assay technique.

2.6. Assessment of proteolytic activitiy and inhibition in oyster plasma

To determine the level of protease inhibitory activities, 20 μl of plasma sample were pre-incubated with 20 μl of protease in a 1.75-ml microcentrifuge tube with shaking, for 20 min at room temperature. A standard curve consisting of serial dilutions of protease was prepared, for each assay. In order to identify background proteolytic levels in the oyster plasma, and/or the assay system, controls containing 0 or 20 μl of plasma, and no protease, were also prepared for each oyster sample. Standard curve and control samples were pre-incubated for 20 min at room temperature prior to addition of the substrate. All samples, standards, and controls were analyzed in triplicate. The protease concentrations used for co-incubation with plasma were as follows; 2500 ng papain, 10.0 μg pepsin, 17.5 ng *P. marinus* ECP protease, 25.0 ng trypsin, 5.0 μg thermolysin, and 31.3 ng *V. vulnificus* protease. The range of concentrations for each protease standard curve were as follows; papain 156.0–5000.0 ng, pepsin 0.1–20.0 μg, *P. marinus* ECP protease 1.1–70.0 ng, trypsin 1.6–50.0 ng, thermolysin 0.2–20.0 μg, and *V. vulnificus* protease 3.9–1250.0 ng protein.

Following the 20-min pre-incubation, a mixture of 500 μl of ASW (22 g/l) and 500 μl of hide powder azure substrate was added to all samples, and the resulting mixtures were shaken vigorously for 3 h at 37°C. All samples were then centrifuged at $13000 \times g$, 4°C, for 5 min to remove the insoluble substrate. Absorbance of the supernatants was read spectrophotometrically (A_{570}) (Dynatech Plate Reader MR 5000, Dynatech, Chantilly, VA). Standard curves were prepared for each assay, of absorbance versus protease protein concentration, after averaging triplicate absorbance values of the standard curve samples. The triplicate A_{570} values for each oyster plasma sample were then averaged, and the level of protease activity determined by comparison to the standard curve, giving an activity value equivalent to protease enzyme ng/sample.

The activity value for total protease ng inhibited/20 μl plasma was then determined for each co-incubation of plasma plus protease, using the following formula:

$$A_{570}\ \text{Protease Inhibited} = \text{Avg. } A_{570}\ \text{Protease} - [\text{Avg. } A_{570}\ \text{Plasma co-incubated with Protease}] - [\text{Avg. } A_{570}\ \text{Plasma}]$$

The level of protease inhibition was then determined by comparison of this A_{570} value to the standard curve, giving an activity value equivalent to total ng inhibited/20 μl plasma.

Data were also represented as percent inhibition (i.e. inhibition caused by 20 μl of plasma). The average A_{570} values for protease alone (at the concentration used for co-incubation with plasma), and for plasma samples co-incubated with protease were determined. Percent inhibition (%In.) was then calculated from the average of each triplicate sample using the following formula:

$$\%\text{In.} = \frac{[\text{Avg. Protease } A_{570}] - [\text{Avg. } A_{570}\ \text{Sample co-incubated with Protease}]}{[\text{Avg. Protease } A_{570}]} \times 100$$

2.7. Statistical analysis

The data were first tested for normality and then analyzed with either Mann–Whitney rank sum or Student *t*-tests. Statistical analysis was performed using SigmaStat computer software (Jandel Scientific, San Rafael, CA).

3. Results

3.1. Protein concentration of oyster plasmas

There was an obvious difference between the eastern and Pacific oysters in plasma protein concentrations ($P < 0.001$). Plasma protein concentrations were approximately 3-fold higher in the eastern oyster, averaging 5.96 mg ml^{-1}, while those of the Pacific oysters averaged 1.61 mg ml^{-1} (Fig. 1).

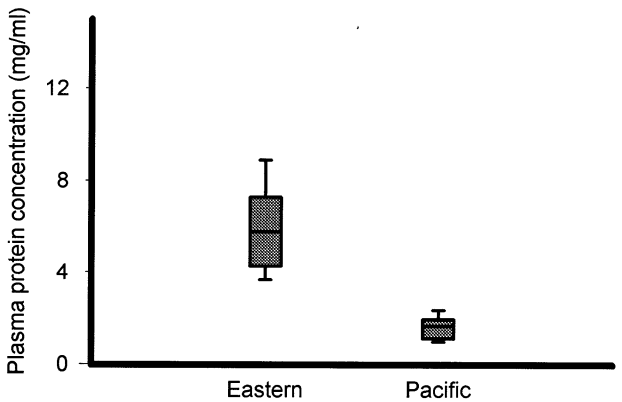

Fig. 1. Protein concentrations in cell-free plasma of oysters. The data are represented as box plots indicating the 25th and 75th percentile, the median is indicated as a transverse line in the box, and the 5th and 95th percentiles are indicated by error bars ($n = 30$ oysters/species).

3.2. Protease inhibitory specific activities in oyster plasma

3.2.1. Papain

Plasma of the Pacific oyster had significantly higher levels of specific inhibition against papain than those in the eastern oyster ($P < 0.001$, Fig. 2). The average inhibitory values were 39.02 ± 21.83 ng, and 137.95 47.89 ng protease inhibited/μg plasma protein for the eastern and Pacific oysters, respectively.

3.2.2. Pepsin

Similar to papain, Pacific oysters possessed a higher inhibitory capacity against pepsin, with an average of 163.55 ± 80.18 ng pepsin inhibited/μg plasma protein, as compared to eastern oysters, which averaged

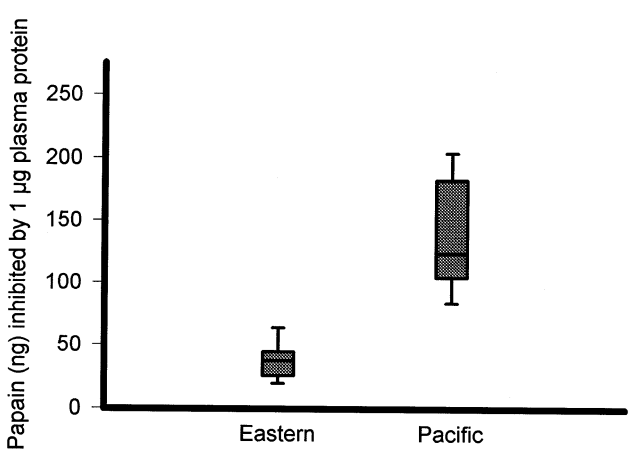

Fig. 2. Inhibition of papain activity by oyster plasma using the substrate hide powder azure. Papain proteolytic activity in the absence of oyster plasma was determined using a standard curve (156–5000 ng). Papain concentration used for incubation with plasma was 2500 ng/well. The data are represented by box plots as explained in the legend of Fig. 1.

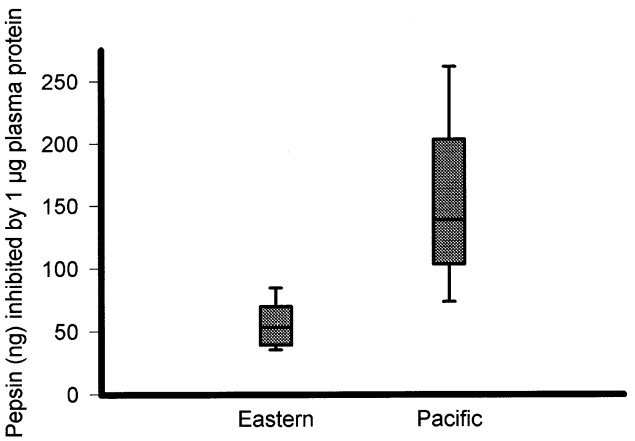

Fig. 3. Inhibition of pepsin by oyster plasma using the substrate hide powder azure. Pepsin proteolytic activity in the absence of oyster plasma was determined using a standard curve (0.1–20.0 μg) of pepsin. Ten μg of pepsin/well were used in the assay. The data are represented by box plots as explained in the legend of Fig. 1.

60.79 ± 28.27 ng pepsin inhibited/μg plasma protein. This difference was statistically significant ($P < 0.001$, Fig. 3).

3.2.3. Serine proteases

The Pacific oyster was 6-fold higher in inhibitory capacity against *P. marinus* ECP as compared to the eastern oyster ($P < 0.001$). The mean value for eastern oysters was 0.05 ± 0.06 ng *P. marinus* ECP inhibited by 1 μg plasma protein, while that of the Pacific oyster was 0.32 ± 0.20 ng/μg plasma protein (Fig. 4A).

In the case of trypsin, the Pacific oysters exhibited a 3-fold higher average level of plasma inhibitory activity in comparison to levels of the eastern oysters. Of the plasma protein, 1 μg inhibited an average of 0.02 ± 0.19 ng trypsin in the eastern oysters and 0.18 ± 0.42 ng in the case of the Pacific oysters (Fig. 4B). Further examination of the data revealed that nearly half ($n = 14$) of the eastern oysters did not possess anti-trypsin activities. Plasma of these oysters actually increased the proteolytic activity by an average equivalent to the activity seen in 0.16 ± 0.08 ng trypsin.

3.2.4. Metalloproteases

Pacific oysters displayed remarkable inhibitory activities against *V. vulnificus* protease (0.98 ± 0.56 ng enzyme inhibited/μg plasma) and no, or extremely low levels of inhibitory activity were detected in the plasma of eastern oysters (0.13 ± 0.33 ng enzyme inhibited/μg plasma, Fig. 5A). The difference between these two groups was found to be statistically different ($P < 0.001$). Plasma of 17 out of 30 eastern oysters heightened hydrolysis of the substrate by an average of 0.10 ± 0.54 ng enzyme/μg plasma. This statistically significant increase was not noted with plasma samples of *C. gigas*.

A similar, but reversed trend was observed with thermolysin. Pacific oyster samples showed no inhibition of thermolysin (Fig. 5B). Instead, a significant increase in substrate hydrolysis was seen (average 2.29 ± 1.14 ng thermolysin inhibited/μg plasma protein) in wells containing plasma and thermolysin, in comparison to wells which contained thermolysin only. A similar trend was noted with the eastern oysters. The plasma of 15 oysters showed an increase (average 0.18 ± 0.14 ng thermolysin/μg plasma protein) in substrate hydrolysis (Table 1). The remaining eastern oysters exhibited an average of 0.23 ± 0.31 ng thermolysin inhibited/μg plasma protein (Fig. 5B). The difference between these two groups was found to be statistically different using the Mann–Whitney rank sum test ($P < 0.001$).

3.3. Percent inhibition/modulation

The concentration of protein in the plasma of *C. gigas* was much lower than levels observed in *C. virginica*. Despite this fact, *C. gigas* plasma exhibited significantly higher percent inhibition values than *C. virginica* in the case of papain ($P < 0.001$), pepsin ($P < 0.001$), and *V. vulnificus* metalloprotease ($P < 0.001$) (Table 1). Plasma of *C. virginica* displayed a higher inhibitory capacity against *P. marinus* ECP per unit volume in comparison to *C. gigas* plasma ($P = 0.004$). However, this trend was reversed when the data were analyzed by specific inhibition per μg/plasma proteins, as shown above (Fig. 4A).

The elevation in levels of proteolysis noted for trypsin and the metalloproteases upon coincubation with oyster plasma, followed the same pattern observed with the data presented in terms of specific activity.

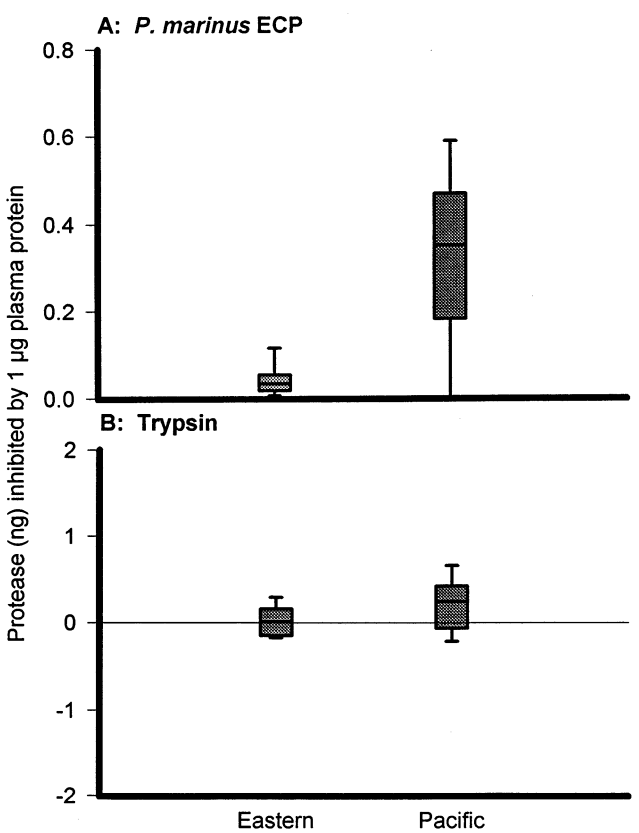

Fig. 4. Inhibition of serine proteases. (A) Proteolytic activity of *P. marinus* extracellular protein (ECP) supernatant in the absence of oyster plasma was determined using a standard curve range of 1.1–70.0 ng ECP protein. A concentration of 17.5 ng ECP/well was selected for incubation with oyster plasma. (B) Trypsin proteolytic activity in the absence of oyster plasma was determined using standards (1.6–50.0 ng). Twenty-five ng of trypsin/well were used for incubation with plasma in the assay. The data are represented by box plots as explained in the legend of Fig. 1.

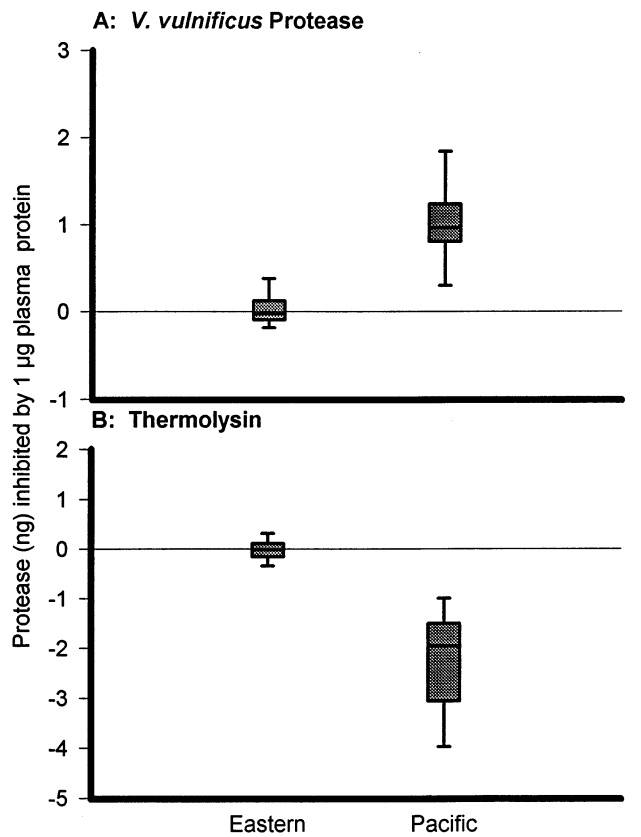

Fig. 5. (A) Inhibition of *Vibrio vulnificus* metalloprotease by oyster plasma using the substrate hide powder azure, ng protease inhibited/μg oyster plasma protein. *V. vulnificus* proteolytic activity in the absence of oyster plasma was determined using standards (3.9–1250 ng). In the assay, 31.3 ng of protease/well were used. (B) Inhibition of thermolysin by oyster plasma using the substrate hide powder azure, ng thermolysin inhibited/μg oyster plasma protein. Thermolysin proteolytic activity in the absence of oyster plasma was determined using standards (0.2–20.0 μg). Five μg of thermolysin/well were used in the assay. The data are represented by box plots as explained in the legend of Fig. 1.

Table 1
Percent inhibition per unit volume of oyster plasma

Protease	Papain	Pepsin	*P. marinus* ECP	Trypsin	*V. vulnificus* protease	Thermolysin
C. virginica	57.4 ± 24.7	37.5 ± 9.16	26.8 ± 1.8	2.8 ± 36.0	-13.1 ± 31.6[a]	-5.4 ± 44.1
C. gigas	75.1 ± 15.3	54.1 ± 18.5	17.8 ± 11.5	12.8 ± 37.5	75.2 ± 24.3	-65.7 ± 38.1
P value[b]	<0.001	<0.001	$=0.004$	NS	<0.001	<0.001

Percent inhibition was calculated as described in Section 2. The data are presented as mean $\pm$ standard deviation ($n = 30$/group and enzyme). NS, non-significant difference between the two groups.
[a] Negative values indicate augmentation in substrate hydrolysis.
[b] Statistical analysis for the enzymes papain, pepsin and *V. vulnificus* protease were performed using the Mann–Whitney rank sum test. *P. marinus*, thermolysin and trypsin enzyme data were analyzed using *t*-test.

4. Discussion

The data presented in this study demonstrate that plasma of both *C. virginica* and *C. gigas* possess factors capable of inhibiting the proteolytic activity of proteases selected to represent the four known mechanistic classes. Individual variability in levels of protease inhibitory (PI) activities was noted within the samples in each species. This degree of variability is consistent with the variability of other physiological and pathological parameters reported in *Crassostrea* spp. [3,9,10,15,16]. It should be emphasized that PI levels reported in this study are those of available PIs, and are subject to the dynamics of interaction and association with various proteases and/or proteins (endogenous or exogenous) that were present in the plasma at the time of hemolymph collection. In this study, values of protease inhibition have been presented primarily as specific activity (ng protease inhibited/μg plasma protein), rather than total PI activity per μl plasma. This is particularly important in the case of *Crassostrea* spp. since information regarding compartmentalization of body fluids, the dynamics of PI synthesis and release, and hemolymph regulation are currently unknown [11].

Despite the limitations mentioned above, it is apparent that the plasma of *C. gigas* has significantly higher levels of specific protease inhibitory activity than *C. virginica*. Inhibitory values observed for thermolysin of *Bacillus thermoproteolyticus rokko* was the only exception to this observation. There was not a definite trend among individual oysters in each population with respect to a general inhibitory capacity for the entire group of tested proteases. For example, an oyster that showed an extremely high inhibitory activity against a particular protease may not exhibit the same degree of inhibition against another protease. Our data also suggests that levels of inhibition varied between proteases of the same mechanistic class. For example, an individual *C. gigas* which exhibited the greatest ability to inhibit *P. marinus* serine protease, showed a very weak inhibition level for trypsin, which is also a serine protease. This degree of discrimination between enzymes of the same class, using the same plasma sample, may be indicative of a specificity of the protease inhibitors present in the plasma of *Crassostrea* spp.

Of interest was the observation that *C. gigas* plasma had extremely high levels of specific inhibitory activity against *P. marinus* proteases relative to *C. virginica*. The higher percent inhibition values for *C. virginica* with *P. marinus* protease, appears to be a function of the higher concentration of plasma protein seen in *C. virginica*. Indeed, when *C. virginica* was experimentally infected with *P. marinus*, levels of plasma proteins declined steadily [24]. In contrast, plasma protein concentration rose by 300% in *C. gigas* infected simultaneously with the same number of *P. marinus* cells. The role PI may play in the observed resistance of *C. gigas* to *P. marinus* is currently under investigation. Recent findings indicate that the addition of commercial bacterial or mammalian protease inhibitors to *P. marinus* cells in vitro inhibited their multiplication and was lethal at higher concentrations [13,14].

The obvious difference noted in the capacity of *C. gigas* plasma to inhibit *V. vulnificus* protease, and to stimulate thermolysin metalloprotease is surprising. This discrepancy in reactivity toward two enzymes of the same catalytic class cannot be fully explained at this point. One may speculate, however, that *C. gigas* plasma contains a factor capable of augmenting the proteolytic activity of thermolysin. The presence of a latent protease in the *C. gigas* oyster hemolymph, which increases in activity upon exposure to thermolysin, is another possible explanation for this finding. The specific activity of some microbial and protozoal metalloproteases are known to be influenced by many factors including pH, cation concentration (e.g. Zn^{2+}, Ca^{2+}), or the presence of certain extracellular matrix proteins such collagen and laminin [29,31,34]. Regardless of the nature of this factor(s), it was present in almost all samples of *C. gigas* examined in this study and is a phenomenon that deserves further investigation.

Among the protease inhibitors identified in vertebrates, α_2-macroglobulin-like activities have been identified in a number of mollusks. Homologues of vertebrate α_2-macroglobulin have been purified from

the hemolymph of octopus, *Octopus vulgaris* [37], and the gastropod mollusk, *Biomphalaria glabrata* [4]. Our previous studies suggest the presence of relatively weak α_2-macroglobulin-like activities in the plasma of both *C. gigas* and *C. virginica* [1]. It is currently unknown whether the protease inhibition exhibited by *Crassostrea* spp. plasma in this study was due to the presence of active site protease inhibitors, α_2-macroglobulin-like molecules, or both. The fact that differences were observed in protease inhibition within the species and individual oysters, suggests the presence of relatively potent specific protease inhibitors as opposed to α_2-macroglobulin, which is a weak, but universal protease inhibitor.

The sum of our data suggests the presence of protease inhibitors in *Crassostrea* spp. that may have an impact on host defense mechanisms in addition to other known physiological functions. Current investigations focus on the isolation, purification, biochemical characterization of protease inhibitors in both oyster species.

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