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POTENTIAL ROLE OF PROTEASE-ANTIPROTEASE INTERACTIONS IN PERKINSUS MARINUS INFECTION IN CRASSOSTREA SPP.

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Abstract

Perkinsus marinus causes devastating losses in populations of the eastern oyster (Crassostrea virginica). Our studies have demonstrated that P. marinus secretes extracellular serine proteases which enhance parasite propagation and compromise host defences. Crassostrea virginica, however, possesses several inhibitors of these proteases. The Pacific oyster (C. gigas) is resistant to P. marinus and possesses protease inhibitors with significantly higher specific activities than those found in C. virginica. Interestingly, Crassostrea spp. themselves, elaborate metalloprotease activities which can be detected in their plasma, and are increased during infection by these two Crassostrea species.

Introduction

The eastern oyster (Crassostrea virginica), a monomeryan lamellibranch, is a significant component of the food, culture, and economy of millions of people along the U.S. Atlantic and Gulf coasts. Over the past five decades, the drastic decline of eastern oyster fisheries has ignited an increasing concern over the balance and integrity of the Chesapeake Bay watershed and its rich biodiversity. Deadly infections with the protozoan Perkinsus marinus may be partially responsible for this decline. In contrast, the Pacific oyster (Crassostrea gigas), whose fisheries represent more than 85% of the world oyster production, is less susceptible to this protozoan (Meyers et al., 1991). Identifying the mechanism(s) responsible for this sharp contrast in resistance between these two related oyster species may provide useful insights into host-parasite relationships in oysters.

Immune-Related Processes in Oysters.

To survive and reproduce in the hostile marine environment, oysters need efficient defence mechanisms for protection against micro-organisms. Like other invertebrates, oysters do not exhibit adaptive immunity or memory and little work is available on their defence mechanisms. Oyster defence mechanisms appear to involve both cellular and humoral processes (Cheng, 1996; Ford and Tripp, 1996).

Due to their phagocytic characteristics, oyster haemocytes are vital for nutrition as well as protection against pathogens (Cheng, 1996). Haemocytes are also capable of releasing antimicrobial compounds including lysozymes and oxygen metabolites (Bramble and Anderson, 1998). Humoral factors such as agglutinins, precipitins, haemolysins, and opsonins are found in the haemolymph and on the surface of oyster haemocytes (Fries, 1984; Vasta et al., 1984; Chu 1988). However, lineage, classification, and functions of haemocytes remain controversial (Cheng, 1996). Moreover, there is a limited understanding of oyster defence strategies against parasitic invasion because most of our knowledge of the oyster immune system is based on the responses elicited by oysters inoculated with inanimate and soluble antigens.

It is possible that haemocytes may only have a minimal role in combating P. marinus. The early studies of Mackin (1951) and Perkins (1976) suggested that haemocytes might, in fact, facilitate the spread of the protozoan throughout the oyster. Most P. marinus meronts can survive haemocytic phagocytosis (for up to 12 hrs) in both Crassostrea species. Perkinsus marinus has been found to actively proliferate in haemocytes of infected oysters (La Peyre et al., 1993; 1995a). In contrast to this haemocyte research, little attention has been given to investigating the contribution of humoral factors against P. marinus.

Perkinsus marinus: Potential Role of Extracellular Proteases in Pathogenicity.

The successful propagation of P. marinus in vitro (La Peyre et al., 1993; La Peyre and Faisal, 1995a) has allowed several important studies on the biology and pathogenic mechanisms of this organism. Serum-free and chemically defined culture media were developed that supported the propagation of P. marinus and allowed the identification of its secreted products (La Peyre and Faisal, 1996; 1997).

Analysis of the cell-free supernatant of P. marinus cultures by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining revealed the presence of at least 19 protein bands ranging in molecular weight from 23 to 200 kDa. Studies have also demonstrated that some P. marinus secreted proteins are proteases that digest complex proteins including extracellular matrix proteins, oyster plasma, and tissue homogenates (La Peyre and...
proteases have been purified using bacitracin-sepharose affinity chromatography (Faisal et al., 1999a). The purified protease fractions contained >87% of the protease activity initially loaded onto the column with very high specific activity that resulted in 8-11-fold increase in protease activity (Figure 1). Analysis of the chromatographic fractions demonstrated that only six bands were present in the enriched protease fraction (Figure 2). Gelatine gel electrophoresis revealed two groups of proteases; 3-5 high intensity bands in the 36-50 kDa molecular weight range, and 2-3 faint protease bands in the 90-195 molecular weight range (Figure 3). The isolated proteases hydrolysed a variety of protein substrates including oyster plasma (Figure 4). 

All of the isolated *P. marinus* proteases were deemed to be of the serine protease family, the presence of the serine protease inhibitors such as PMSF, benzamidine and chymostatin blocked their activity, whereas inhibitors of cysteine, aspartic, and metalloproteases showed little or no inhibition. Using serine protease-specific peptide substrates, it was further demonstrated that *P. marinus* proteases belong specifically to the chymotrypsin-like class of serine proteases (proteolysis occurred only to the substrate: N-Succ-Ala-Ala-Ala-Prolyl-Phenyl-p-Nitroanilide). The 41.7 kDa monomeric, N-glycosylated, serine protease (designated Perkinsin, Faisal et al. 1999a) has been identified as the major *P. marinus* extracellular protease (Figure 5).

The role of proteases as parasitic virulence factors is well documented (McKerrow et al., 1993; AbuHatab et al., 1995). Some proteases are instrumental in invading host tissues and cells, counteracting host defence mechanisms, and providing sources of nutrition. Hence, a direct relationship is believed to exist between the expression of proteases and pathogenicity of parasites (Wilson et al., 1989; Keene et al., 1990; Beckage et al., 1993; Michalski et al., 1994). Several studies have demonstrated that *P. marinus* proteases and other secreted proteins suppress oyster defence mechanisms and favour the protozoan's propagation in infected oysters.

Garreis et al. (1996) reported that *P. marinus* proteases reduced random and stimulated haemocyte motility. The percent random migration of haemocytes treated with proteases (6.5±2.4%) was significantly less (p<0.0001) than migration of non-treated haemocytes (14.0±5.9%). Interestingly, *P. marinus* cells and lysates alone stimulate haemocyte mobility. This stimulated mobility, however, was drastically suppressed upon inclusion of *P. marinus* proteases. It was also demonstrated that *P. marinus* proteases significantly reduced the ability of phagocytose and kill *Vibrio vulnificus* (Tall et al., 1999). Further studies demonstrated that *P. marinus*-induced immunosuppression affects oyster plasma factors. Garreis et al. (1996) found a significant reduction in lysozyme and haemagglutination activities in oyster plasma samples co-incubated with *P. marinus* proteases (P<0.05). The role of *P. marinus* proteases in disease pathogenesis was also investigated in vivo. Oysters were fed lipid vesicles containing either fresh culture medium or concentrated proteases preparations (La Peyre et al., 1996). Following 6 weeks of infection, the level of *P. marinus* infection was determined using the total body burden assay. The number of *P. marinus* cells was significantly greater (P<0.015) in oysters fed proteases (755,581±344,938 parasite/g) than in oysters fed fresh medium (101,037±27,769 parasite/g) suggesting that *P. marinus* proteases favour the propag-
performs the pathogen. The observation that *P. marinus* secreted products in vitro, however, did not ensure their expression and use in vivo. Therefore, the detection of these extracellular products in vivo would further support the contentions that they were important components in the virulence of *P. marinus*. In a study by Otteringer et al., 1999a, polyclonal antibodies to ECP derived from *in vitro* propagated *P. marinus* were produced. These antibodies were used in diagnostic enzyme-linked immunosorbent assays (ELISA) to detect and correlate *P. marinus* ECP in oyster tissue homogenates and plasma with the current disease diagnostic standard for Dermo, the fluid thioglycolate medium (FTM) assay as described by Ray, 1952, 1966. The antibodies were successful in detecting *P. marinus* ECP in oysters which had infections ranging from light to heavy as rated by the Mackin Scale (Mackin, 1962). Confirmatory immunoblots of infected oyster plasma using the antibody revealed reactivity against the same soluble antigens that were produced *in vitro*. Oysters that were diagnosed as negative for *P. marinus* infections by FTM were also negative by the diagnostic ELISA, however, there were rare occurrences of false negatives (negative by ELISA, positive by FTM) and false positives (positive by ELISA, negative by FTM). The study by Otteringer et al., 1999a was the first to correlate the expression of *P. marinus* virulence factors and soluble antigens in vivo.

### Novel Immune-Related Molecules in the Eastern and Pacific Oysters

In mammals, cascades of protease inhibitors, host proteases, reactive oxygen species, and antimicrobial peptides have been identified in tissue fluids that are involved in defence mechanisms (Simon, 1993) and inflammatory responses (Niedbala, 1993). These molecules prevent shifts in the microbial protease/host protease or the microbial protease/host antiprotease balance to favour of the invading pathogen (Simon, 1993). Important in this process are host metalloproteases involved in tissue rearrangements during infection (Doenhoff, 1997; Johansson, 1999; Yamamoto and Saito, 1998). It is through the remodelling of the matrix protein that the host is able to encapsulate invading parasitic cells and prevent their spread. Molecules of similar function have been identified in marine crustaceans (Holmblad and Soderhäll, 1999) and molluscs (Roch, 1999).

In bivalve molluscs, *Perkinsus* spp. elicits profound inflammatory responses within the connective tissues in the immediate vicinity of the parasite. This process leads to a tissue rearrangement that encapsulates protozoal cells. This process seems to be successful in the case of venerid (Montes et al., 1995) and softshell clams (McLaughlin and Faisal, 1998). In case of the eastern oyster, an identical host reaction occurs at the early stages of infection, however, *P. marinus* prevails and disseminates systemically (Mackin, 1951). The elegant studies of Montes et al., 1996 described an inducible 225-kDa protein(s) that the authors believe is crucial in the defence against *Perkinsus* spp. infection. Intrigued with these pioneering studies, several investigators explored the presence of similar immune-related molecules in *C. virginica* and *C. gigas* and their possible involvement in resistance/susceptibility to *P. marinus* infection.

1. **Protease Inhibitors:**
   To counteract exogenous and regulate endogenous proteases, many organisms produce a variety of protease inhibitors (PI). This group constitutes the third largest group by...
weight of vertebrate serum proteins (Travis and Salvesen, 1983). The number and diversity of PI specificities is very high but can be classified into two main groups. The first group of PIs, the active-site protease inhibitors, specifically binds to and blocks the active site of proteases from one mechanistic class (serine, metallo, cysteine, or aspartic PIs). Inhibitors of serine protease (serpins) are the best characterised and are comprised of at least 10 families found in fungi, plants, invertebrates, and vertebrates (Las-kowski and Kato, 1980; Eguchi, 1993). Most serpins are approximately 370-390 amino acids in length (Huber and Carrell, 1989). The mechanism of regulation or inhibition of serine protease cascades by serpins involves binding to the serine-histidine-aspartic acid complex in the protease active site. The specificity of serpins for a given serine protease depends on the variable amino acid sequence of its reactive site loop that binds to the active site of a serine protease (Potempa et al., 1994).

The second group encompasses the high molecular weight macroglobulins (e.g., α-macroglobulin, αM) that partially inhibit proteases of all major classes irrespective of their catalytic mechanism. Protease-binding α-macroglobulins (αM) have not only been detected in vertebrates (from fish to mammals) but also in invertebrates (Arms-trong and Quigley, 1992; Eguchi 1993; Eguchi et al., 1993). Protease-αM binding is initiated in the bait region, a particularly well exposed stretch of peptide that is located near the centre of the four identical αM subunits. As a result of this covalent binding with the protease, a conformational change occurs in the αM molecule, whereby the protease is entrapped by αM. Therefore, αM-bound-protease retains its ability to hydrolyse substrates with small but not large molecular weights (Søtrup-Jensen, 1989). Protease inhibitors appear to be vital in host defence because they neutralise proteases of invading pathogens thus preventing their invasiveness (Boucias and Pendland, 1987; Mixter et al., 1999; Sleasman et al., 1999; Alexander and Ingram, 1992). For example, rainbow trout Oncorhynchus mykiss is relatively resistant to furunculosis, a deadly disease caused by Aeromonas salmonicida. Freedman (1991) and Ellis (1991) purified an αM-like molecule from rainbow trout sera that neutralised A. salmonicida protease, the major virulence factor of this bacterium. Brook trout (Sal-velinus fontinalis), a closely related salmonid species, is highly susceptible to furunculosis and its αM-activity against A. salmonicida protease was much weaker (10-15%).

Protease inhibitors have been identified in the plasma of six molluscs, namely; the snail Biomphalaria glabrata, the whelkBusycon canaliculatum, the squid Loligo solidissima, the octopus Octopus vulgaris, the bivalve Spisula solidis-sina, and the softshell clam Mya arenaria (Armstrong and Quigley, 1992; Bender and Bayne, 1992; Thøgersen et al., 1992; Eyres et al., 1999). It is possible that protease inhibitors play an important role in the host defence mechanisms of molluscs. Fryer et al. (1991) demonstrated that resistant strains of the snail Biomphalaria glabrata to the trematode Schistosoma mansoni possesses PI activity at significantly higher levels than susceptible strains. In this study, commercially available proteases (e.g., bovine chymotrypsin) were used to assay the PI activities in molluscan plasma. It is not known, however, whether molluscan protease inhibitors exert a specific action against proteases produced by a particular pathogen.

Evidence for the presence of α-macroglobulin-like molecule in oyster plasma: Protease inhibition by αM results from the entrapment of protease within the molecular cage of αM molecules. This mechanism leaves the active site free to react with low molecular weight (LMW) but not high molecular weight substrates (HMW). Trypsin activity was reduced by C. virginita plasma when the HMW hide powder azure (HPA) was used as a substrate, however, this activity was retained when BAPNA, a LMW substrate, was used (Adham and Faisal, 1997). Eastern oyster plasma protected trypsin from the active site inhibitor (e.g., Soybean Trypsin Inhibitor, SBTI) which provides evidence for the presence of an αM-like molecule in oysters.

**Figure 6:** P. marinus burden (number of parasite/g wet oyster tissue) in oysters infected with 10⁷ parasite and treated with either liposomes containing fresh medium or liposomes containing conditioned medium (ECP) (modified after La Peyre et al., 1996 with publisher’s permission). **Figure 7:** Inhibition of Vibrio vulnificus metalloprotease by oyster plasma using hide powder azure as a substrate. A concentration of 31.1 ng V. vulnificus protease/well was selected for incubation with oyster plasma. **Figure 8:** Inhibition of Perkinsus marinus serine proteases in culture supernatant (ECP) by oyster plasma using hide powder azure as a substrate. A concentration of 17.5 ng P. marinus ECP/well was selected for incubation with oyster plasma (Figs 7 and 8 modified after Faisal et al., 1998 with publisher’s permission).
Evidence for the Presence of Protease Inhibitors in Eastern and Pacific Oysters: Plasma of eastern and Pacific oysters was compared for levels of inhibitory activities against a variety of proteases. Representatives of the serine, cysteine, metallo, and aspartic protease mechanistic classes were analysed, including *P. marinus* and *Vibrio vulnificus* proteases. In comparison to *C. virginica*, *C. gigas* plasma exhibited significantly higher specific inhibition levels for papain (*P*<0.001), pepsin (*P*<0.001), *V. vulnificus* protease (*P*<0.001, Fig 7), Trypsin (*P*<0.015), and *P. marinus* protease (*P*<0.001, Fig 8) (Faisal et al., 1998).

Additionally, Oliver et al. (1999a) observed *P. marinus* protease-specific inhibitory activity in the low molecular weight (<10 kDa) fraction of *C. virginica* plasma. Proteolytic protection of p35 was revealed upon comparison of artificial (PBS) and endogenous (plasma-based) diluents employed during exposures of plasma proteins to *P. marinus* proteases. It was found that p35 was eliminated when a standard buffer (PBS) was added to the *P. marinus* protease-plasma protein exposure; however, p35 was preserved when a low molecular weight (LMW), plasma-based, diluent was used instead. The results suggested that LMW inhibitors of *P. marinus* proteases were present in oyster plasma. A control (non-parasitic) serine protease, α-chymotrypsin, was employed to ascertain the specificity of the protease inhibitors. Although α-chymotrypsin possessed ample proteolytic activity for *C. virginica* plasma proteins, the anti-proteases could only specifically inhibit *P. marinus* proteases.

Such specificity of anti-protease activity is not uncommon among LMW serine proteases. Oliver et al. (1999a) suggested that LMW inhibitors of *C. virginica* might play an important role in defence against *P. marinus* invasion and may have evolved specifically to defend against *P. marinus*. Furthermore, it was hypothesised that LMW inhibitors of *C. virginica* might be important biochemical markers of disease resistance.

**Figure 9.** Plasma of *Crassostrea gigas* (five μg/lane) showing bands of inhibited activities of *Perkinsus marinus* proteases. Plasma proteins were first separated by SDS-PAGE. Plasma samples were mixed with equal volumes of sample buffer, applied to 5-10% acrylamide gel copolymerised with 1% gelatine and electrophoresed at 30 mA constant current for 60 min. The commercial serine protease inhibitor Soybean Trypsin Inhibitor (SBTI) was included as a control for inhibitor function. The gels were incubated with 30 fold-concentrated *P. marinus* protease preparations for 2 hr and then stained with Coomassie blue. Band areas of protease inhibition are darkly stained on a transparent background (complete hydrolysis of gelatine). This protocol is modified after Uriel & Bergen (1968) and Eguchi et al. (1982) with publisher’s permission.

**Figure 10.** Plasma of *Crassostrea virginica* (five μg/lane) showing bands of inhibited activities of *Perkinsus marinus* proteases. Notice the increased intensity of p16 and the appearance of p253 in infected plasma. The assay was performed as described in the legend of Figure 9.

**Figure 11.** Correlation between low molecular weight *P. marinus* protease inhibitory activity (LMW-PMPI) of *C. virginica* and Dermo intensity as rated on the Mackin scale (Mackin, 1962). Individual oysters from selectively bred families were plotted according to their LMW-PMPI and rating on the Mackin scale. The solid line within the box plots denotes the median and the dotted line denotes the mean. The 25th and 75th percentiles are denoted by the box plot. The 10th and 90th percentiles are denoted by the error bars. Filled circles represent individual data points that lie outside of the 10th and 90th percentiles.
C. virginica

C. gigas

\[
\begin{align*}
&60 \text{kDa} \\
&51 \text{kDa}
\end{align*}
\]

< 220 kDa

< 112 kDa

Figure 12. Detection of metalloproteases in oyster plasma by gelatine-impregnated SDS-PAGE. Plasma samples were loaded at 5 µg/lane in the case of Crassostrea virginica and at 1 µg/lane in the case of C. gigas. Arrows and numbers correspond to standard molecular weight standards. Note the progressive appearance of two additional proteases in C. virginica.

C. gigas

C. virginica

\[
\begin{align*}
&200 > \\
&116 < \\
&66 < \\
&31 < \\
&2 \\
&4 \\
&6 \\
&8 \\
&10
\end{align*}
\]

Weeks post-infection

Figure 13. Detection of metalloproteases in the plasma of P. marinus infected oysters. Plasma samples were loaded at 1 µg protein/lane in the case of C. virginica, and at 10 µg protein/lane in the case of C. gigas. Arrows and numbers correspond to standard molecular weight standards. Note the progressive increase of bands' intensities as infection advances and the inverse correlation between disease intensity and PMPI levels (Figure 11).


Using electrophoretic separation and zymography, proteases with gelatine degrading capacities in plasma of both Crassostrea species were detected. In C. gigas, plasma samples loaded at a protein concentration of 1 µg/lane, displayed an obvious band of gelatine degradation with an apparent apparent molecular weight of 112 kDa, and another band of lower intensity at 220 kDa. Proteolytic activity was virtually undetectable in C. virginica plasma samples when loaded at 1 µg protein/lane. However, when the sample concentrations were increased to 5 µg protein/lane, two light bands of gelatine degradation were observed, with apparent molecular weights of 51 and 60 kDa (Figure 12). Characterisation of these proteolytic bands utilising a suite of inhibitors clearly suggests that the gelatine degradation observed in all bands was caused by metalloproteases present in oyster plasma. It seems that zinc is essential for oyster metalloproteases because 1,10-phenanthroline and captopril inhibited all proteolytic bands (ongoing studies).

Following experimental P. marinus infection, two additional protease bands of 138 and 220 kDa were visualised in the plasma of C. virginica analysed during progression of infection, along with the original 60 and 51 kDa bands (Figure 12). Characterisation of these proteolytic bands using a suite of inhibitors clearly suggests that the gelatine degradation observed in all bands was caused by metalloproteases present in oyster plasma. It seems that zinc is essential for oyster metalloproteases because 1,10-phenanthroline and captopril inhibited all proteolytic bands (ongoing studies). No new protease bands were detected in the plasma of infected C. gigas (Figure 13). Concurrent with the appearance of the new bands, other oyster proteases in both species increased significantly in intensity with infection (ongoing experiments). This finding suggests...
involvement of oyster metalloproteases in infection or an associated stress response.

3. Production of Reactive Oxygen Species by Oyster Haemocytes.

Production of ROS by oyster haemocytes stimulated with zymosan or phorbol myristate acetate (PMA) have been associated stress response. Involvement of oyster metalloproteases in infection or an innate immune response has been observed (Adema et al., 1991). Exposure of oysters to P. marinus was associated with an increased production of ROS by haemocytes (Anderson et al., 1995). Surprisingly, several studies provided evidence that ROS activity is ineffective against P. marinus and other bacterial pathogens probably because of the production of antioxidant agents by the pathogens (Bramble and Anderson, 1997; Volety and Chu, 1995).

Conclusions and Future Directions:

There are several gaps in the current understanding of host defence mechanisms and strategies utilised by eastern and Pacific oysters. The availability of cultured P. marinus isolates coupled with the sharp difference in P. marinus susceptibility between these two oysters provides a unique model to study the expression and inducibility of immune-related molecules and cellular functions. There is also a need to isolate and characterise oyster protease inhibitors, proteases, and anti-microbial peptides. Identification of factors controlling expression of these molecules may provide explanation to disease resistance in the absence of adaptive immunity.

Acknowledgements


