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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 in *C. virginica*. Interestingly, *Crassostrea* spp. themselves, elaborate metalloprotease activities which can be detected in their plasma, and are increased during *P. marinus* infections. Together our work suggests that there may be a broad spectrum of humoral host defences that is brought to bear on *P. marinus* infections by these two *Crassostrea* species.

Introduction

The eastern oyster (*Crassostrea virginica),* a monomyarian 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 it's 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 might 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 lysosomal enzymes (Cheng, 1984) and toxic oxygen metabolites (Bramble and Anderson, 1998). Humoral factors such as agglutinins, precipitins, haemolysins, and opsonins are found in the hemolymph 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* merozoites 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 as many as 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

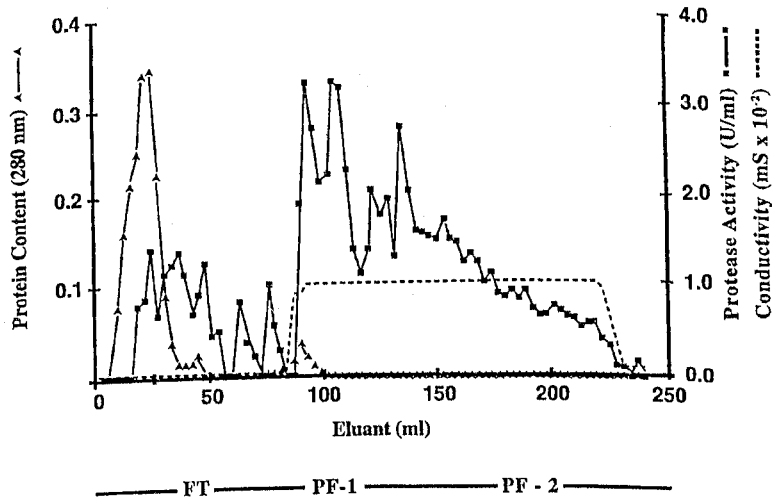
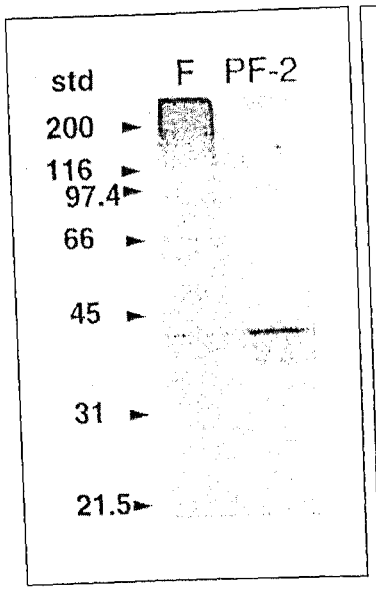
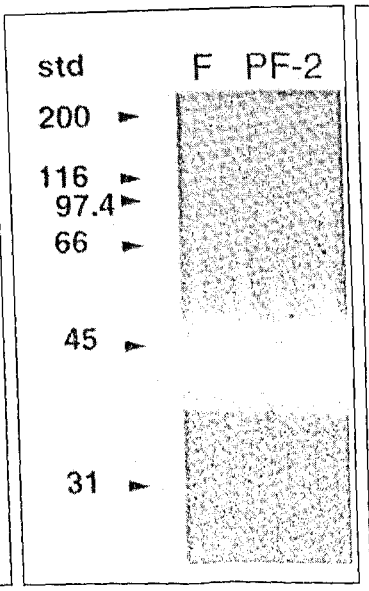
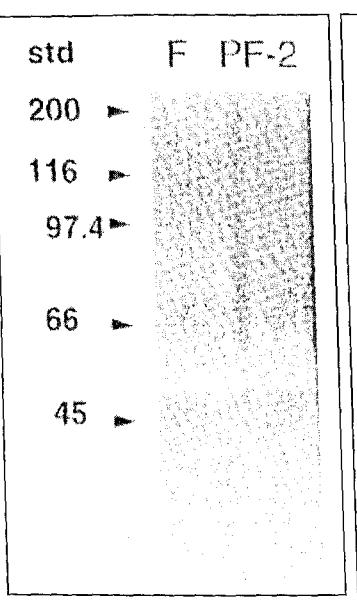


Figure 1: Elution profile of *Perkinsus marinus* extracellular protease(s) from bacitracin-sepharose affinity column. The column (10.5 cm x 1.5 cm) was loaded with 1.9 mg proteins from culture supernatant, washed with 4 volumes of buffer A [50 mM ammonium acetate, pH 6.5, 10 mM $CaCl_2$, 10% (w/v) sucrose), and then eluted with 9 volumes of buffer B [buffer A containing 1M NaCl, 12.5% (v/v) isopropanol]. FT: Flow through, PF1 & PF2: Fractions with enriched protease activities (from Faisal *et al.*, 1999a with publisher's permission).







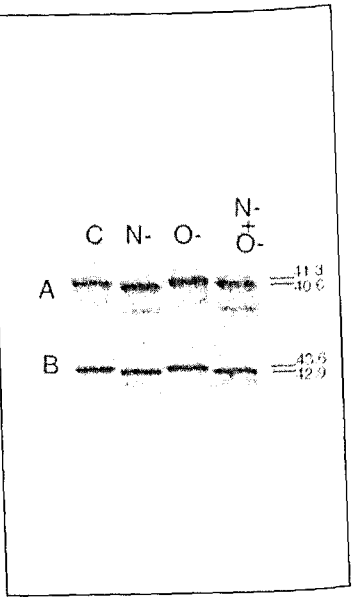


Fig 2 **Fig 3** **Fig 4** **Fig 5**

Figure 2: Native SDS-PAGE and silver staining of affinity-purified samples (1 µg/ml protein/lane) of *P. marinus* culture supernatant isolated by bacitracin-sepharose affinity chromatography. F: culture supernatant, PF2: Protease-enriched fractions.
Figure 3: Gelatine hydrolysing extracellular protease activities of *Perkinsus marinus* resolved by gelatine-SDS-PAG electrophoresis of affinity-purified samples (loaded at 0.5 µg/ml protein/lane). F: feed, PF2: Protease-enriched fractions
Figure 4: *Perkinsus marinus* protease hydrolysing plasma of the eastern oyster resolved by gelatine-SDS-PAG electrophoresis of the chromatographic samples (loaded at 0.5 µg/ml protein/lane). F: culture supernatant, PF2: Protease-enriched fractions
Figure 5: SDS-PAGE and silver staining of the major *P. marinus* protease band (Perkinsin) subjected to O-linked and N-linked deglycosylation reactions. Protease samples underwent a deglycosylation reaction in the absence (C) or presence of 0.5 U of N-Glycosidase F (N), 2mU Neuraminidase/2.5 mU O-Glycosidase (O), or both (N+O). Following incubation for 18 h at 37°C, these samples were mixed with SDS-PAGE sample buffer and analysed by SDS-PAGE under native (A) or reduced (B) conditions (Figs 2 to 5 from Faisal *et al.*, 1999a with publisher's permission).

Faisal, 1995b; La Peyre *et al.*, 1995b). *Perkinsus marinus* 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-Phen-p-Nitroanalide. 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 protozoon'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* protease 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 fervour the propaga-

Table 1 *Perkinsus* burden (Number of hypnospores/g wet oyster tissue) in both experimentally and naturally infected oysters. Experimentally infected oysters were infected with 10^7 parasites and then fed lipid vesicles containing either artificial seawater (ASW), 5mg/ml or 50mg/ml bacitracin dissolved in ASW (N=15 oysters / group) for six weeks. Naturally infected oysters were fed lipid vesicles containing either ASW or 10mg/ml bacitracin dissolved in ASW (N=20 oysters / group) for 10 weeks.

	Means ±SD
Experimental infection	
ASW	$3.2x10^5$ ($4.7x10^5$)
Bacitracin (5mg/ml)	$3.3x10^4$ ($2.5x10^4$)
Bacitracin (50mg/ml	$5.3x10^4$ ($6.4x10^4$)
Natural infection	
Pretreatment	$10.9x10^6$ ($30.7x10^6$)
ASW	$67.4x10^6$ ($144x10^6$)
Bacitracin (10mg/ml	$2.5x10^6$ ($3.0x10^6$)

tion of *P. marinus* and its spread within the host perhaps acting like aggressin (Figure 6).

P. marinus Produces ECP in vivo.

The discovery that *P. marinus* produces extracellular proteins and proteases *in vitro* was an important first step in identifying and characterising potential virulence factors of 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 Ottinger *et al.* (1999), 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 thioglycollate 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 Ottinger *et al.* (1999) was the first to confirm the expression of *P. marinus* virulence factors and soluble antigens *in vivo*.

P. marinus Target and Protection.

Oliver *et al.* (1999a) attempted to determine the potential pathogenic effects of *P. marinus* serine proteases on the plasma proteins of the eastern oyster (*Crassostrea virginica*) and the Pacific oyster (*Crassostrea gigas*) *in vitro*. Specifically, this study sought to characterise oyster plasma protein targets of *P. marinus* proteases. It was found that *C. virginica* possessed a plasma protein of 35 kDa (p35) which appeared to be digested upon exposure to *P. marinus* proteases. Oliver *et al.* (1999a) suggested that elimination of this target might represent an important component in the pathogenesis. *C. gigas* plasma proteins, however, did not appear to be as sensitive to digestion by *P. marinus* proteases as was *C. virginica*.

Proteases are important for P. marinus survival.

Proteases of *P. marinus* seem to be vital for its survival and propagation. *P. marinus* cells lost their ability to divide upon incubation with low concentrations of protease inhibitors such human $\acute{\alpha}_2$-macroglobulin, Phenylmethylsulphonyl fluoride (PMSF), Chymostatin, or Aproteinin. Higher concentrations of these inhibitors were lethal to *P. marinus*. Moreover, Bacitracin^R, a weak protease inhibitor, suppressed the growth and propagation of *P. marinus* growth *in vitro*.

The sensitivity of *P. marinus* to Bacitracin was examined in two clinical trials. In the first trial, individual oysters were injected with 10^7 Perkinsus-1 cells, then fed Bacitracin at a concentration of 5 or 50 mg/ml encapsulated in lipid vesicles daily for six weeks. Parasite body burden was significantly reduced in oysters given bacitracin as compared to control oysters. In the second experiment, naturally infected oysters received encapsulated 10-mg/ml bacitracin for 10 weeks. Treated oysters had significantly lower levels of infection than control oysters given encapsulated seawater (Table 1, Faisal *et al.*, 1999b).

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 Crustacea (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 (Laskowski 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., $\acute{\alpha}_2$-macroglobulin, $\acute{\alpha}_2$M) that partially inhibit proteases of all major classes irrespective of their catalytic mechanism. Protease-binding $\acute{\alpha}$-macroglobulins ($\acute{\alpha}$M) have not only been detected in vertebrates (from fish to mammals) but also in invertebrates (Armstrong and Quigley, 1992; Eguchi 1993; Eguchi *et al.*, 1993). Protease-$\acute{\alpha}_2$M binding is initiated in the bait region; a particularly well exposed stretch of peptide that is located near the centre of the four identical $\acute{\alpha}_2$M subunits. As a result of this covalent binding with the protease, a conformational change occurs in the $\acute{\alpha}_2$M molecule, whereby the protease is entrapped by $\acute{\alpha}_2$M. Therefore, $\acute{\alpha}_2$M-bound-protease retains its ability to hydrolyse substrates with small but not large molecular weights (Sottrup-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 $\acute{\alpha}_2$M-like molecule from rainbow trout sera that neutralised *A. salmonicida* protease, the major virulence factor of this bacterium. Brook trout (*Salvelinus fontinalis*), a closely related salmonid species, is highly susceptible to furunculosis and its $\acute{\alpha}_2$M-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 whelk *Busychon canalicatum*, the squid *Loligo solidissima*, the octopus *Octopus vulgaris*, the bivalve *Spisula solidissima*, and the softshell clam *Mya arenaria* (Armstrong and Quigley, 1992; Bender and Bayne, 1992; Thogersen *et al.*, 1992; Elsayed *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* possess PI activity at significantly higher levels than susceptible strains. In this study, commercially available proteases (e.g., bovine chymotrypsin) were used to assess 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 $\acute{\alpha}$-macroglobulin-like molecule in oyster plasma: Protease inhibition by $\acute{\alpha}$M results from the entrapment of protease within the molecular cage of $\acute{\alpha}$M 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. virginica* 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 $\acute{\alpha}$M-like molecule in oysters.

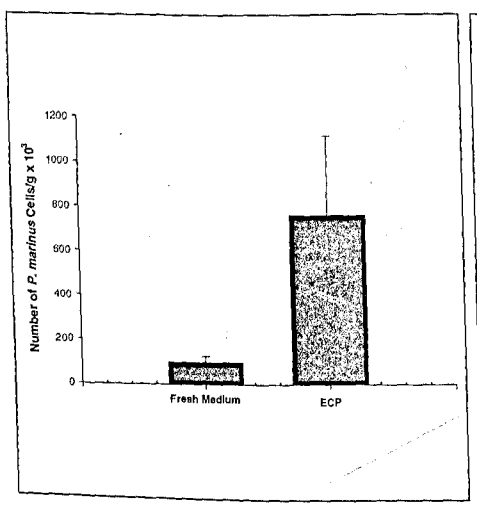
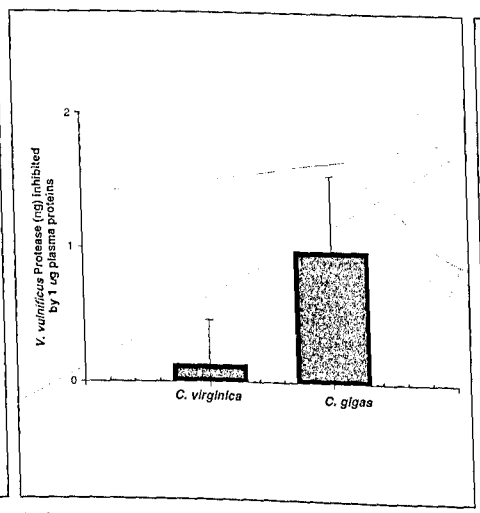


Fig 6



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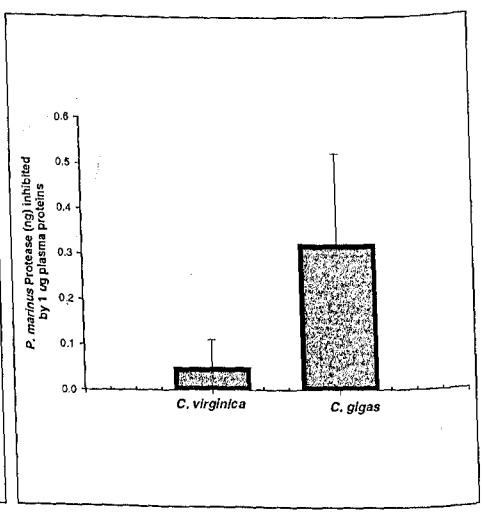

Fig 8

Figure 6: *P. marinus* burden (number of parasite/g wet oyster tissue) in oysters infected with 10^7 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).

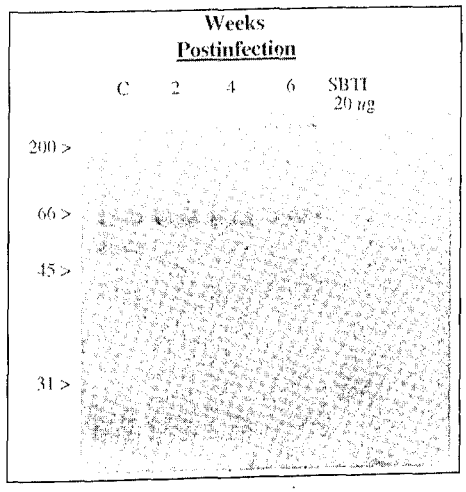

Fig 9

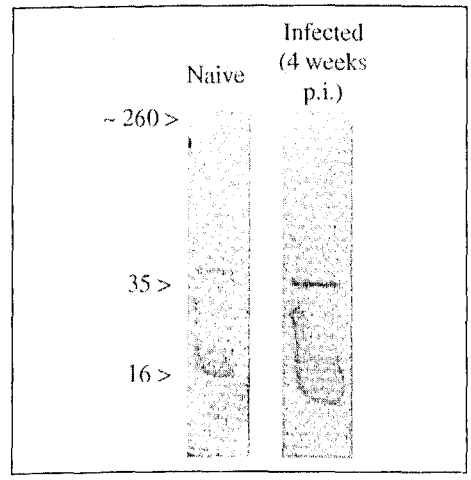

Fig 10

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. Bands/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.

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.

Visualisation of Putative *P. marinus* Protease Inhibitors (PMPI) in Oyster Plasma:

In order to analyse protease inhibitor induction in oyster plasma, *P. marinus*-free eastern and Pacific oysters were divided into three groups each. The first group was sampled immediately; the second group was inoculated with 10^5 *P. marinus* merozoites/oyster into the adductor muscle

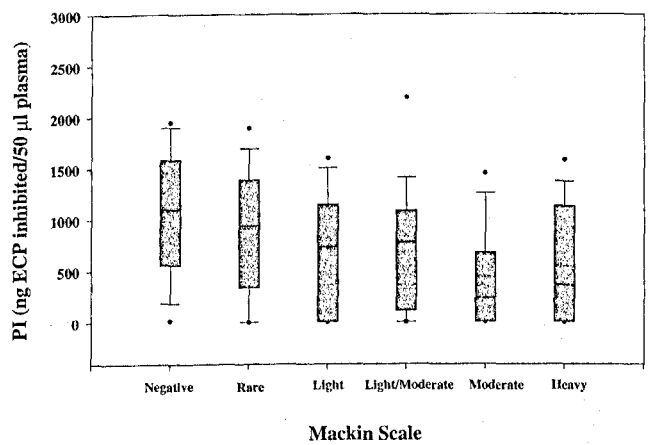

Figure 11. Correlation between low molecular weight *P. marinus* protease inhibitory activity (LMW-PMPI) of *C. virginica* and Dermo intensity as rated on the Mackin scale (Mackin, 1962). Individual oysters from selectively bred families were plotted according to their LMW-PMPI and rating on the Mackin 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.

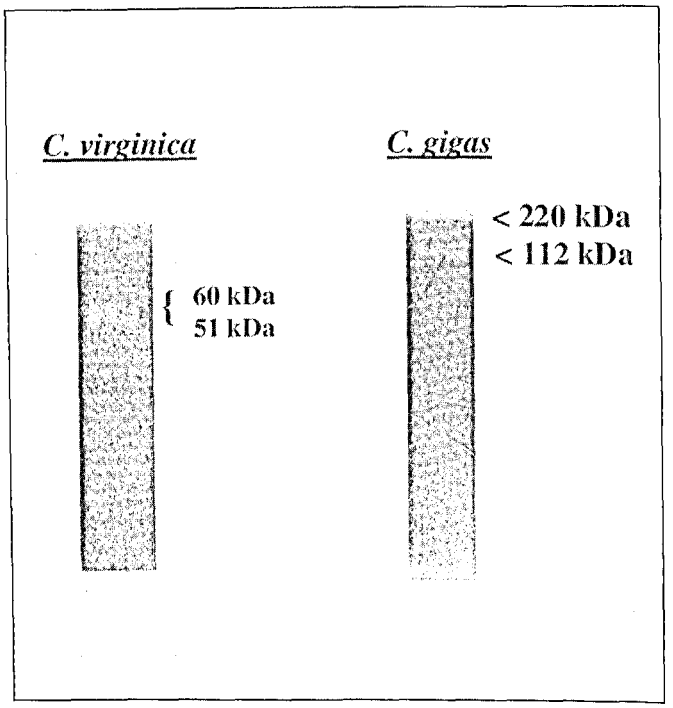

Fig 12

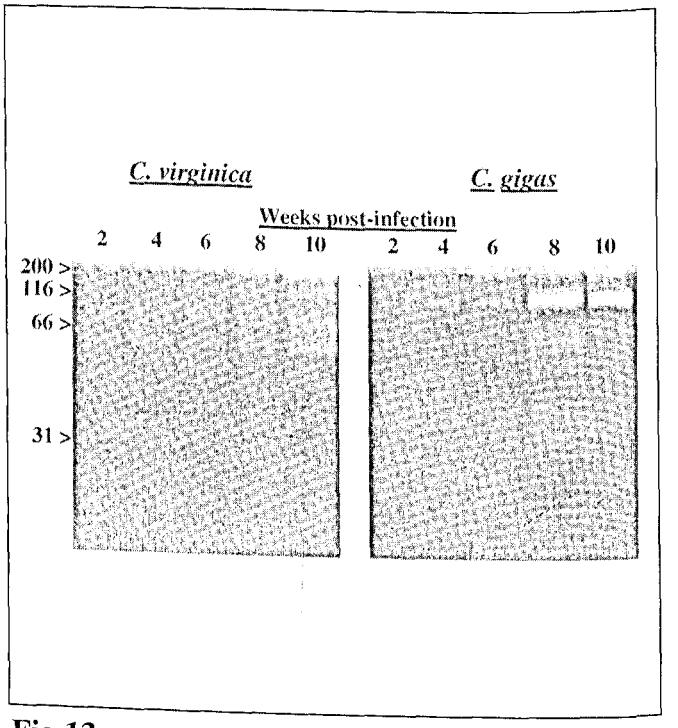

Fig 13

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. **Figure 13.** Detection of metalloproteases in the plasma of *P. marinus* infected oysters. Plasma samples were loaded at 5 µg/lane in the case of *C. virginica* and at 1 µg/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 appearance of two additional proteases in *C. virginica*.

sinus (challenged), and; the third group was inoculated with artificial seawater (ASW, control group). Infected and control oysters were then divided into two groups. Staggered, biweekly sampling of hemolymph from the two alternate groups allowed for weekly average assessments, while minimising trauma to the oyster. After 10 weeks, the total number of protozoal cells/g tissue was determined in surviving oysters.

Visualisation of PMPI activity was achieved by the use of gelatine impregnated SDS-PAGE according to the methods modified after Uriel and Bergen (1968) and Eguchi *et al.* (1982). After electrophoresis, the gels were incubated for 60-120 min with *P. marinus* protease(s), which entered the gel by diffusion and interacted with the electrophoresed PMPIs in the gel. The position of putative PMPIs appeared as darkly stained bands on a colourless background (hydrolysed gelatine).

C. virginica exhibited three serine protease inhibitors (p16, p31, p35) constitutively. *C. gigas* exhibited six serpins (p 253, p 120, p65, p56, p43, p25). Exposure to *P. marinus* was associated with the appearance of an additional band (p260) in *C. virginica*, and the loss of p56 in *C. gigas* (Fig 9 and10).

Correlation Between LMW-PMPI Levels and Resistance to Natural *Perkinsus marinus* infection:

In a collaborative study, 10 *C. virginica* families with varying degrees of resistance to *P. marinus* were analysed for LMW-PMPI levels. Oysters were challenged by placing them in *P. marinus* endemic areas. Their survival and disease intensity were then recorded. Three of these families had the highest survival, lowest average disease intensity, and in two independent studies, the highest PMPI levels (Oliver *et al.*, 1999b). Among all families, there was an inverse correlation between disease intensity and PMPI levels (Figure 11).

2. Metalloproteases in Oyster Plasma.

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 1g/lane, displayed an obvious band of gelatine degradation with an 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 1g protein/lane. However, when the sample concentrations were increased to 5:g 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 (Fig. 13). These new bands were not observed in control *C. virginica*. Like other oyster gelatinases, the new bands are also zinc proteases. No new protease bands were detected in the plasma of infected *C. gigas* (Fig. 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 demonstrated (Adema *et al.*, 1991; Anderson 1994; 1996; Bachere *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 oyster 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 peptide. Identification of factors controlling expression of these molecules may provide explanation to disease resistance in the absence of adaptive immunity.

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