Host oyster tissue extracts modulate *in vitro* protease expression and cellular differentiation in the protozoan parasite, *Perkinsus marinus*

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SUMMARY

Perkinsus marinus is responsible for a chronic disease (Dermo) of the Eastern oyster, *Crassostrea virginica*. In order to simulate the *in vivo* environment more closely, a chemically defined medium (JL-ODRP-3) was supplemented with tissue homogenate extracts or plasma from oysters possessing varying degrees of susceptibility to *P. marinus* infection. In media supplemented with extracts from highly susceptible oysters (*C. virginica*), *P. marinus* cells secreted elevated amounts of a set of low molecular weight serine proteases (LMP: 30–45 kDa) as assessed by enhanced digestion within gelatin-substrate SDS–PAGE gels. Oyster species of low susceptibility (*C. gigas* and *C. ariakensis*) did not exhibit this ability to upregulate *P. marinus* LMP expression. Oyster extract supplementation also led to pronounced changes in *P. marinus* cellular morphology, such that the cells were comparable to those observed within naturally infected oysters.

Key words: *Perkinsus marinus*, protease, *Crassostrea* sp., differentiation, *in vitro* culture.

INTRODUCTION

Perkinsus marinus has been a significant factor in recent widespread mortalities of the Eastern oyster within the Chesapeake Bay (Andrews, 1988; Burreson & Ragone Calvo, 1996). Of considerable concern is its wide geographical range (Burreson *et al*. 1994; Soniat, 1996), particularly its present northerly expansion along the Atlantic coast (Ford, 1996). *P. marinus* is a unique protistan parasite that has recently been grouped within a new Phylum, Perkinsoa (Noren, Moestrup & Rehnstam-Holm, 1999). It causes a chronic wasting disease in oysters, with severe impairment of growth and eventual mortality (Dwyer & Burnett, 1996; Paynter, 1996). Within the last 10 years, efforts to understand the pathogenic mechanisms have been greatly facilitated by development of *in vitro* culture systems for *P. marinus* (La Peyre, Faisal & Burreson, 1993; Kleinschuster & Swink, 1993; Gauthier & Vasta, 1993). Through *in vitro* culture techniques, generation of the large quantities of cells required for genetic analysis, artificial infection and analysis of extracellular products (ECP) has been possible (La Peyre, Yarnall & Faisal, 1996; Oliver *et al*. 1999; Reece *et al*. 2001; Ottinger *et al*. 2001). *In vitro* generated ECP have been shown to enhance *P. marinus in vivo* infectivity (La Peyre *et al*. 1996), suppress host cellular defences (Tall *et al*. 1999; Garreis, La Peyre & Faisal, 1996) and neutralize humoral defence factors *in vitro* (Garreis *et al*. 1996). Of particular relevance to the studies reported here are the roles of serine proteases within *P. marinus* ECP, which have been proposed as possible virulence factors responsible for tissue degradation in infected Eastern oysters (La Peyre *et al*. 1996). Indirect support of this hypothesis has come from studies wherein addition of the protease inhibitor, bacitracin, was found to significantly reduce both *P. marinus* infectivity and *in vitro* growth (Faisal *et al*. 1999).

Unfortunately *P. marinus* grown *in vitro* has been reported to undergo attenuation (Ford, Chintala & Bushek, 2002). It was reasoned that if *in vitro* culture can result in attenuation, the cause may be a reduced expression of factors important for infectivity (i.e. proteases). Thus supplementation of defined medium with oyster tissue extracts was examined to determine if protease expression profiles could be modulated.

MATERIALS AND METHODS

Oysters

Specific pathogen-free (SPF) Eastern oysters (*Crassostrea virginica*) were kindly provided by Dr John Scarpa (Harbor Branch Oceanographic Institute, Fort Pierce, FL). Twelve of these oysters were immediately processed for body burden analysis (below) to confirm their *Perkinsus*-free status and the remaining oysters were gradually acclimated over a period of 3 weeks to the temperature and salinity of 1 μm filtered York River (Gloucester County, VA) water. Tissues from these oysters were used in all

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experiments except for the dose–response experiment depicted in Figs 3 and 4. The latter oysters (*C. virginica*) were obtained from Pemaquid Oyster Company, Waldoboro, ME.

Pacific (*Crassostrea gigas*) and Suminoe oysters (*Crassostrea ariakensis*), which exhibit low susceptibility to *P. marinus* (Barber & Mann, 1994; Calvo *et al*. 1999, 2001), were kindly provided by Dr Standish Allen Jr (Aquaculture Genetics and Breeding Technology Center; Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA) and were determined to be free of *P. marinus* by body burden analysis (see below). All 3 oyster species had a shell height of approximately 5–7 cm, were maintained in 10 l aquaria under quarantine conditions, and fed daily with *Isochrysis* sp. strain number CCMP 1324 (Reed Mariculture Inc., San Jose, CA) at a rate of 0·1 g/oyster/day. Water was exchanged every 3 days.

Oyster extracts and plasma

Oyster plasma was withdrawn from the adductor sinus using a 25 G needle after notching the shell adjacent to the adductor muscle with a lapidary saw. Haemocytes were removed from haemolymph by centrifugation at 400 ***g*** at 4 °C for 10 min. Pooled samples were then sterilized by passage through a 0.22 μm syringe filter (Gelman Sciences, Ann Arbor, MI), and stored at 4 °C until used.

Following plasma collection, all soft tissues were removed, weighed, then finely minced with a razor blade. One half of each tissue sample was homogenized with a Ten-Broeck homogenizer in 10 ml of 4 °C artificial seawater (22 g/l: Forty Fathoms Marine Mix, Marine Enterprises International, Baltimore, MD). This suspension was initially centrifuged at 1500 ***g*** at 4 °C for 10 min to remove cells and debris. The supernatant fraction was then centrifuged at 10 000 ***g*** for an additional 10 min to pellet particulate matter further. Each resulting supernatant fraction (from individual oysters) was passed through a fibreglass filter (Gelman Sciences), sterilized using a 0·22 μm filter (Gelman Sciences), and stored for up to 48 h at 4 °C until used. Protein concentrations of all samples were determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Screening of oysters for disease status

The *Perkinsus*-free status of all oysters used in these studies was ascertained by performing body burden analysis as described by Fisher & Oliver (1996), with the modification of using one half of the minced oyster tissue rather than the entire oyster. Briefly, oyster tissues were placed in 20 ml of sterile Ray's Fluid Thioglycollate Medium (RFTM) supplemented with 0·5 mg/ml penicillin and 500 U/ml streptomycin (Sigma, St Louis, MO), and incubated in the dark at room temperature for 1 week. Samples were then centrifuged at 1500 ***g*** for 10 min, and the resultant pellet digested in 2 M NaOH for 4 h at 60 °C. The pellet was then washed 3 times with distilled deionized water, and resuspended in 1 ml of a 1/20 dilution of Lugol's solution (Sigma). The entire sample was filtered over a 47 mm diameter, 0·45 μm pore size filter disk (MSI, Westboro, MA) which was subsequently examined by light microscopy (400×) for stained parasites. Prior to homogenate or haemolymph preparation, a subsample of 4 oysters/species were also histologically examined for the presence of *Haplosporidium nelsoni* (MSX) by the laboratory of Dr Eugene Burreson (VIMS). All oysters used were found to be negative for *H. nelsoni*.

Culture of Perkinsus marinus *cells*

Oyster tissue extract supplementation: *P. marinus* (P-1 isolate; La Peyre *et al*. 1993) was seeded in triplicate wells of a 24-well tissue culture plate (Costar, Corning Inc., Corning, NY) in a volume of 2 ml and at an initial density of 2×10^5/ml. Cells were cultured in chemically defined medium, JL-ODRP-3 (La Peyre & Faisal, 1997) or JL-ODRP-3 supplemented with cell-free tissue extracts at final protein concentrations of 0·3 or 0·03 mg/ml. Osmolality, pH and salinity of all media were determined to be approximately the same as that of the seed culture (540 mOsm, 7·35, and 22 ppt respectively) using a vapor pressure osmometer (Wescor, Logan, UT), pH meter (Corning, Corning, NY) and a temperature compensated Reichert refractometer (Cambridge Instruments, Cambridge, MA). Duplicate control wells containing unseeded medium were also prepared. Cultures were incubated at 27 °C in a 5% CO_2/95% air, humidified incubator for 6 weeks. Cell density and viability were determined using neutral red stain on a Neubauer haemacytometer. Culture supernatants were collected from the wells and centrifuged at 800 ***g*** for 10 min at room temperature. Resulting supernatants were filtered (0·22 μm) to remove *P. marinus* cells, aliquoted and stored at −20 °C until used.

Dose–response analysis. P-1 cells were seeded at 10^6/ml in duplicate wells containing 2-fold dilutions of homogenate or plasma (final concentrations; 4·8 mg/ml to 0·01 mg/ml) in JL-ODRP-3 medium. Duplicate control wells containing unseeded medium were also prepared. Incubation time and conditions as well as cell counts and harvesting of supernatants were as described above.

Co-incubation studies. Culture supernatant containing ECP from a 6-week-old culture of *P. marinus* in JL-ODRP-3 medium was seeded and harvested as

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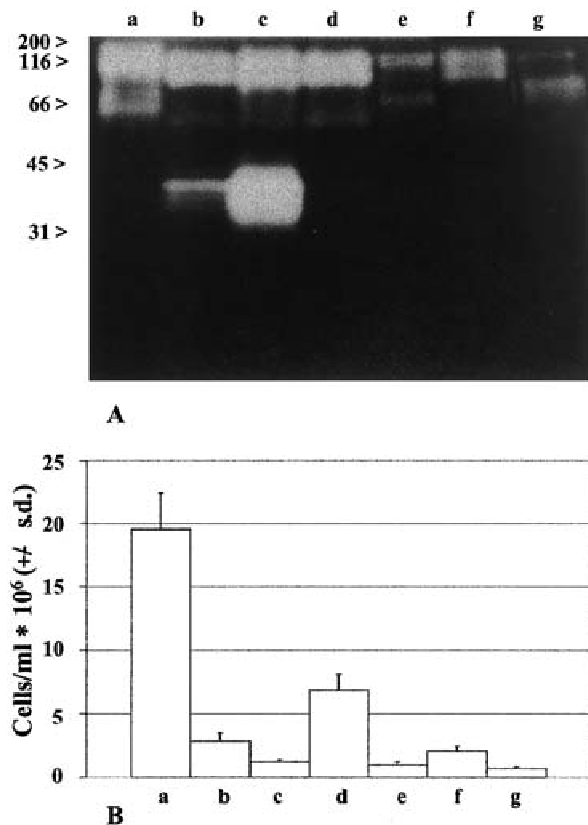

Fig. 1. Low molecular weight protease activity and cell growth in supplemented *Perkinsus marinus* cultures. (A) *P. marinus* were cultured in JL-ODRP-3 (defined medium) that was either unsupplemented (a), or supplemented with 0·03 mg/ml or 0·3 mg/ml *Crassostrea virginica* homogenate (b, c respectively), *C. gigas* homogenate (d, e), or *C. ariakensis* homogenate (f, g). All cultures were incubated for 6 weeks at 27 °C under 5% CO_2 in air. Proteolytic activity was assessed by use of gelatin gel electrophoresis. (B) Four days prior to supernatant harvest, cell counts of individual cultures were taken. The cell counts appear directly under their respective supernatant protease profiles. ANOVA revealed that there was a statistical difference between all treated versus untreated cultures ($P<0·0001$) with pairwise analysis by Fisher's PLSD demonstrating no statistical difference between species at the 0·3 mg/ml supplement concentration. At the 0·03 mg/ml supplement concentration there were statistical differences between species as follows; *C. ariakensis*, *C. gigas* $P<0·0001$; *C. ariakensis*, *C. virginica* $P=0·0074$; *C. gigas*, *C. virginica* $P<0·0001$.

previously described. *C. virginica* homogenate extract (above) was added to culture supernatant to a concentration of 0·3 mg/ml. Control wells were also prepared containing culture supernatant, JL-ODRP-3 medium, or JL-ODRP-3 medium supplemented with homogenate (0·3 mg/ml). All samples were incubated at 27 °C, 5% CO_2 in air for 3 weeks.

Assessment of LMP (low molecular weight protease) inhibitors in C. ariakensis *and* C. gigas *homogenates*. Six-week-old culture supernatant of *P. marinus* (ECP) grown in JL-ODRP-3 medium supplemented

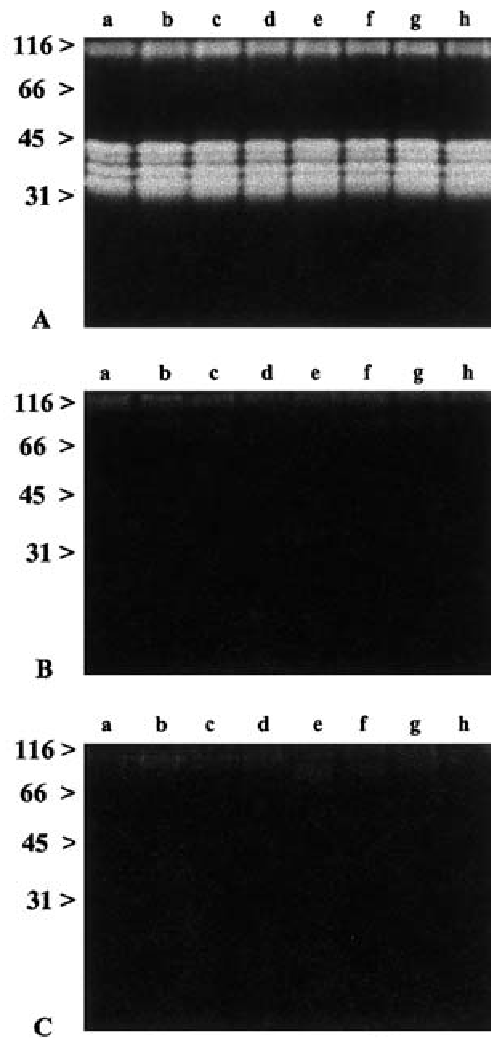

Fig. 2. Individual oyster assessments for inductive activity. Homogenates from 8 (lanes a–h) individual *Crassostrea virginica* (A), *C. gigas* (B) and *C. ariakensis* (C) were used to supplement JL-ODRP-3 cultures to a final concentration of 0·3 mg/ml. Cultures were assessed for proteolytic activity by gelatin gel analysis of the culture supernatants.

with 0·3 mg/ml *C. virginica* homogenate, was combined with an equal supplement of homogenate from either *C. ariakensis* or *C. gigas*. Control wells of JL-ODRP-3 medium supplemented with 0·3 mg/ml of *C. ariakensis* or *C. gigas* homogenate, and *P. marinus* culture supernatant from *C. virginica* supplemented medium were also prepared. All samples were incubated at 27 °C, 5% CO_2 in air for 24 h.

Varied protein supplementation. *P. marinus* was seeded into 24-well tissue culture plates at 2×10^5 cells/ml, 2 ml/well in triplicate sets of JL-ODRP-3 medium or JL-ODRP-3 medium supplemented with the following proteins at 0·3 mg/ml; foetal calf serum (Irvine Sci., Santa Ana, CA), yeastolate (GIBCO-BRL, Grand Island, NY), fetuin, bovine serum albumin, or ovalbumin (Sigma). Duplicate wells of the various unseeded media were included as controls.

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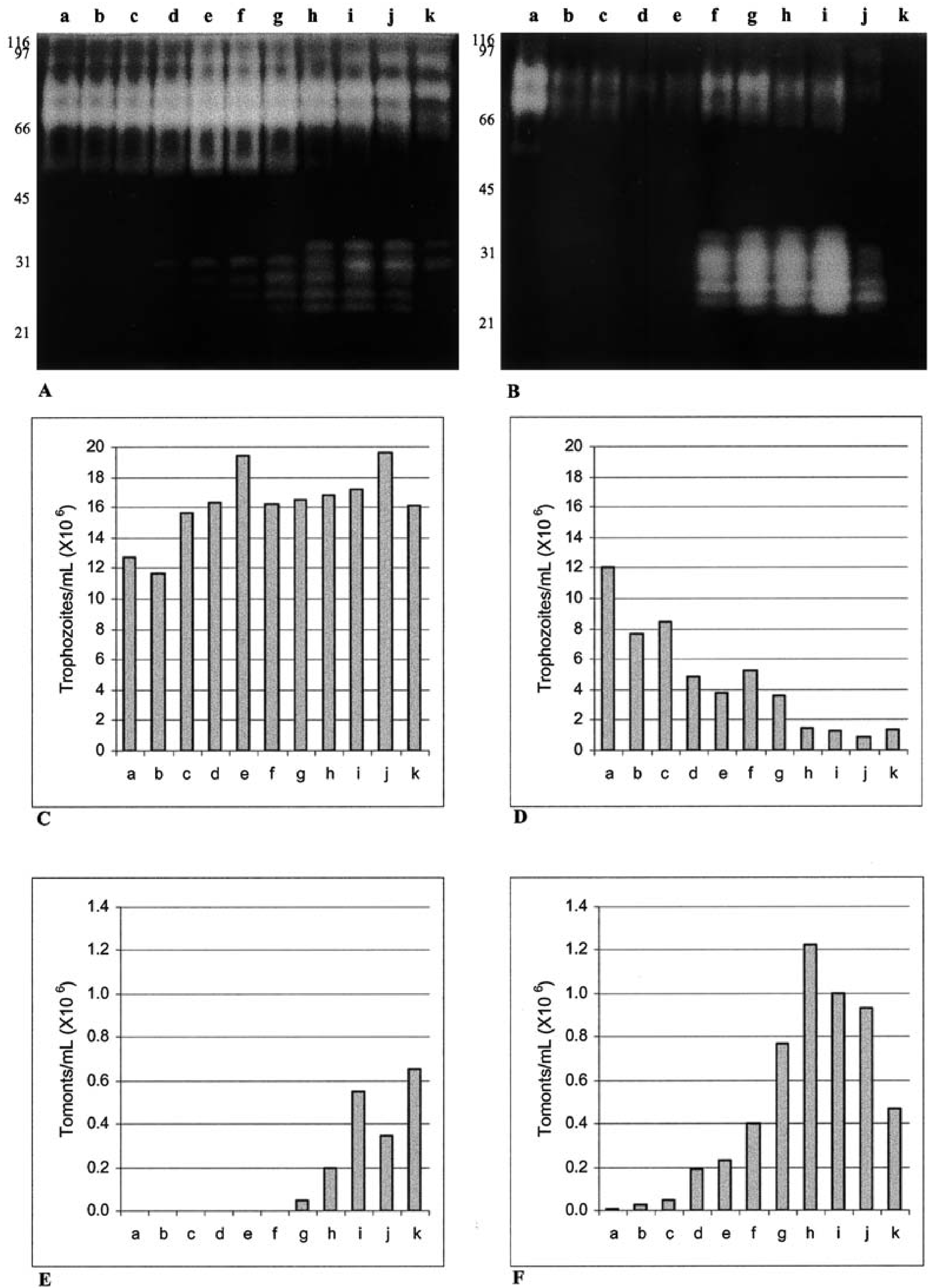

Fig. 3. For legend see opposite page.

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Cultures were incubated at 27 °C, 5% CO_2 in air for 6 weeks. Culture supernatants were collected and stored as described above.

Substrate gel analysis

Proteolytic profiles were analysed using gelatin substrate sodium dodecylsulphate polyacrylamide gel electrophoresis (Rockey, Fryer & Rohovec, 1988) incorporating 8% or 10% acrylamide, 0·2% gelatin, and SDS with a 4% stacking gel. Samples were mixed separately with non-reducing (without 2-mercaptoethanol) Laemmli sample buffer (Laemmli, 1970). One lane containing Bio-Rad broad range molecular weight standards (Bio-Rad, Richmond, CA) were run with each gel. Electrophoresis was performed on a Bio-Rad mini-Protean II unit (Bio-Rad) or Hoefer SE-250 (Amersham Pharmacia, Piscataway, NJ), at 4 °C, 100 V constant voltage for approximately 2 h, using a Tris–glycine buffer system (Laemmli, 1970). Gels were then washed 3 times for 10 min in 2·5% Triton–X-100 at 4 °C to remove the SDS. A final 10 min wash at room temperature in 100 mM Tris, pH 8·0, was followed by incubation for 3 h at 37 °C in fresh Tris buffer. Gels were stained overnight in Coomassie Brilliant Blue-G250, and destained in 40% methanol, 10% acetic acid.

Protease inhibitor analysis

The effect of protease inhibitors on LMP (30–45 kDa) activity was assessed by incubating gelatin gel strips of electrophoresed *C. virginica*-supplemented culture supernatants in inhibitor solutions. Gels were introduced to inhibitor or control solutions immediately after Tris wash and incubated for 3 h at 37 °C.

Phenylmethylsulphonyl fluoride (PMSF) stock solution (100 mM) was prepared in absolute ethanol, 3,4-dichloroisocoumarin (3,4-DCI) stock solution (10 mM) was prepared in 99·5% dimethyl sulphoxide, benzamidine (1 M) and soybean trypsin inhibitor (40 mg/ml) in 50 mM phosphate buffer, pH 7·5. All inhibitors were diluted to their final concentrations in phosphate-buffered saline. Comparably diluted vehicle stock solutions were also employed as controls. All inhibitors were purchased from Sigma.

RESULTS

Culture of *P. marinus* cells in the presence of fresh *C. virginica* extracts (Fig. 1A, lanes b, c) elicited a striking elevation of LMP (30–45 kDa) which was not observed when *P. marinus* was grown in JL-ODRP-3 medium alone (lane a), nor with fresh extracts derived from oysters with low susceptibility to *P. marinus*; *C. gigas* (lanes d, e) and *C. ariakensis* (lanes f, g). This elevation was observed with as little as 0·03 mg/ml of *C. virginica* protein, while extracts of the latter oyster species were incapable of detectable elicitation at either 0·03 or 0·3 mg/ml. This expression of LMP in supplemented cultures appears to be independent of the amount of cell growth (Fig. 1B), as there were no statistical differences in cell numbers between homogenate cultures derived from oysters of different species at a concentration of 0·3 mg/ml. However, total cell counts were reduced from that observed with defined medium alone. These LMP profiles were not present within any of the oyster extracts alone (data not shown). These protease profiles were also obtained from 8 independent cultures using 8 individual *C. virginica* (Fig. 2A), *C. gigas* (Fig. 2B), and *C. ariakensis* (Fig. 2C) extracts, demonstrating the unique consistency of induction by *C. virginica* extracts. Comparable results were seen for individual oysters with medium supplemented at 0·03 mg/ml (data not shown). Long-term storage or freezing of homogenates has not consistently provided material capable of supporting induction of protease activity of *P. marinus* growth to the same degree as freshly processed extracts (data not shown).

Incorporation of *C. virginica* plasma was also observed to elicit detectable LMP, and therefore both plasma and tissue extracts were examined to ascertain the relative differences in their inductive abilities (Fig. 3A, B). Although plasma of the same protein concentration as homogenate can elicit comparable bands (Fig. 3A), they are generally of lesser intensity than those elicited by homogenates (Fig. 3B). Simultaneous analysis of the *P. marinus* cells present at the point of harvest revealed a striking difference in cellular forms between supplemented and unsupplemented media (Fig. 3C–F). Extract or plasma supplementation resulted in heightened numbers of multicellular tomonts (Fig. 3E, F). These tomonts (Fig. 4C) are not typically observed when *P. marinus* is cultured in JL-ODRP-3 medium, (Fig. 4A), or are present to a lesser degree with plasma supplementation (Fig. 4B). However, they commonly are observed in homogenate supplemented media (Fig. 4C) and in infected oyster tissue (Fig. 4D). Although the LMP profiles occurred with *C. virginica* extracts

Fig. 3. Effect of oyster plasma and homogenate dose on protease expression, cell proliferation and differentiation. *Crassostrea virginica* plasma (A, C, E) or homogenate (B, D, F) at: 0 mg/ml (a); 0·01 mg/ml (b); 0·02 mg/ml (c); 0·04 mg/ml (d); 0·08 mg/ml (e); 0·15 mg/ml (f); 0·3 mg/ml (g); 0·6 mg/ml (h); 1·2 mg/ml (i); 2·4 mg/ml (j); 4·8 mg/ml (k) were added to JL-ODRP-3 cultures of *Perkinsus marinus* seeded at 10^6 cells/ml. ECP from each were assessed for proteolytic activity via gelatin gel analysis, and microscopically (Improved Neubauer haemacytometer, 400 ×, with neutral red stain) for cell type (trophozoite or tomont) and number at week 4 of culture.

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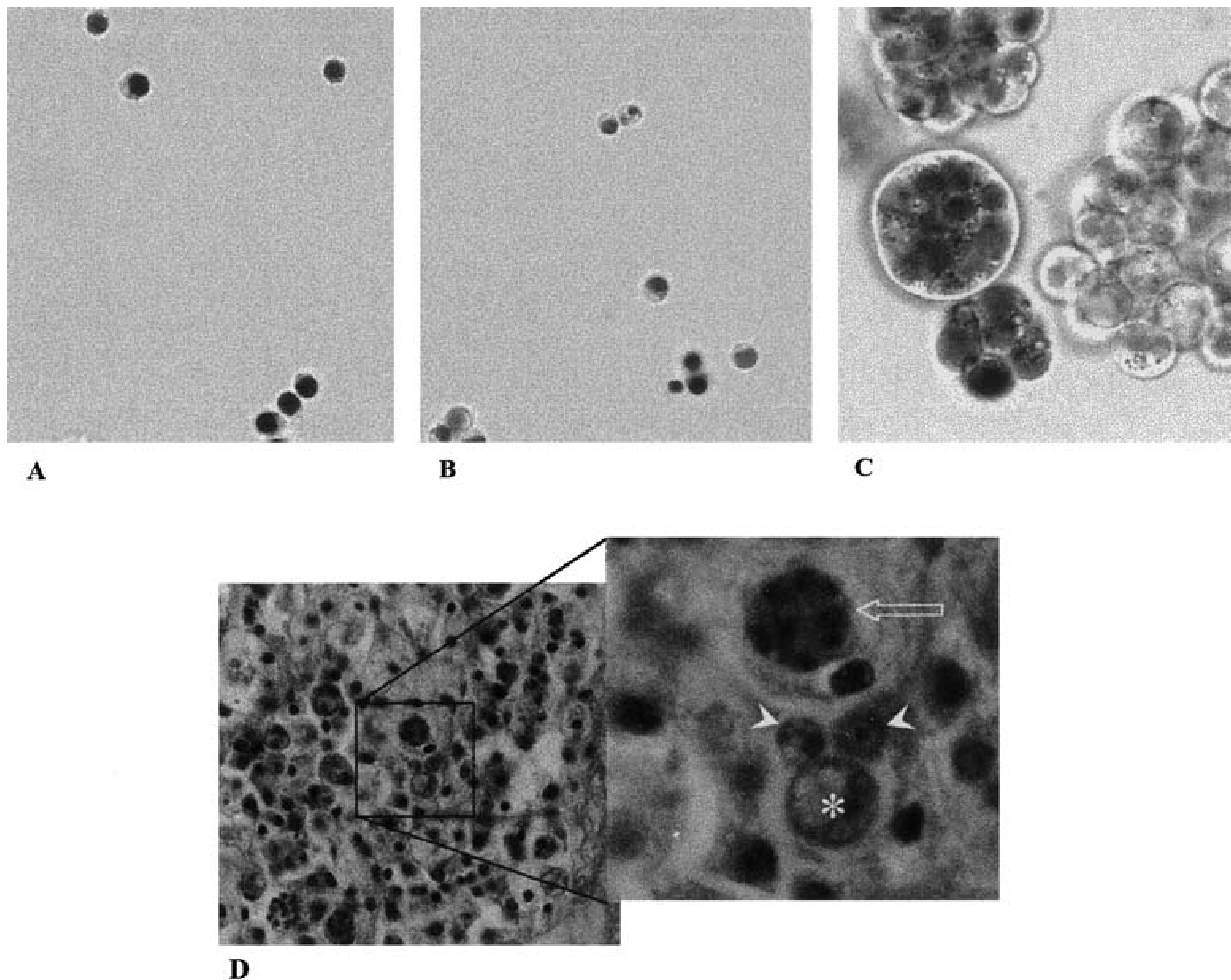

Fig. 4. Morphological comparison of *in situ* and *in vitro Perkinsus marinus*. *P. marinus* grown in JL-ODRP-3 medium (A) or medium supplemented with *Crassostrea virginica* plasma (B) or homogenate (C) (0·3 mg/ml). (D) A photomicrograph of *P. marinus* found within a naturally infected *C. virginica* digestive gland (from the archive of Dr E. M. Burreson). Developmental stages within this paraffin-embedded section (400 ×) are noted within the enlargement (1300 ×). Immature trophozoites (arrowheads), mature trophozoite (asterisk), and multicellular tomont (arrow) are noted.

only, increases in the numbers of multicellular tomonts also occurred in the presence of *C. gigas* and *C. ariakensis* tissue extracts (data not shown).

Possible causes for the appearance of LMP in *P. marinus* ECP from *C. virginica* supplemented media, other than the *de novo* induction of proteases, were investigated. LMP may have been the result of activation of endogenous pro-enzymes in the homogenate extract by proteases from *P. marinus*. Alternatively, pro-enzymes produced by *P. marinus* could have been activated by components of the *C. virginica* extract. To test these possibilities, *P. marinus* ECP supernatants from cultures of unsupplemented JL-ODRP-3 medium were co-incubated with 0·3 mg/ml of *C. virginica* homogenate extract (Fig. 5A). The inability to generate LMP under these conditions indicated that the oyster extracts were not simply modifying higher molecular weight or inactive *P. marinus* proteases generated within JL-ODRP-3 medium, nor were *P. marinus* ECP activating oyster pro-enzymes.

The inability of *C. gigas* or *C. ariakensis* supplements to elicit LMP bands did not preclude the possibility that these extracts may induce LMP but contain protease inhibitors which would block their activity within the gel. To test this possibility, *C. virginica*-induced supernatants were co-incubated with either *C. gigas* or *C. ariakensis* extracts prior to gel analysis to determine if the proteolytic activity seen in *C. virginica* cultures could be inhibited (Fig. 5B). However, addition of either of the latter two extracts did not impact LMP activity of *C. virginica*-induced cultures (lanes b, c).

Alternative protein supplements were tested to determine possible specificity in the elicitation of these proteases. It was found that some xenogeneic protein sources were capable of eliciting LMP activity (Fig. 6). *C. virginica* homogenate at a concentration of 0·3 mg/ml could strongly induce the series of 5 resolvable LMP. Yeastolate, a yeast extract supplement used in JL-ODRP-1 (La Peyre *et al.* 1993) could elicit 2 resolvable LMPs (lane h), as could

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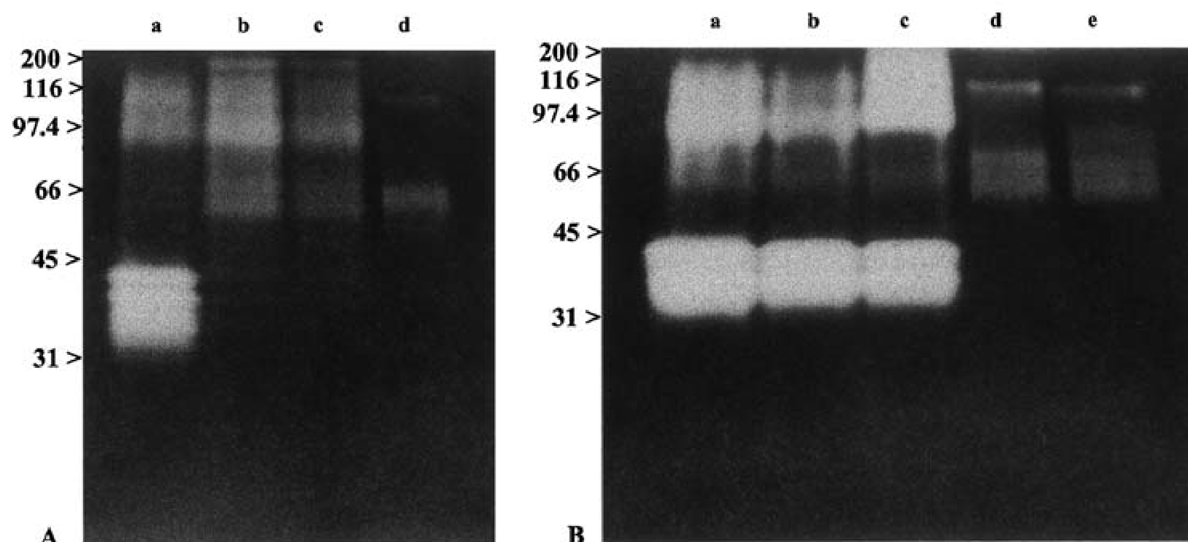

Fig. 5. Origins of *Crassostrea virginica*-induced LMP activity. (A) Effect of oyster homogenate on *Perkinsus marinus* protease produced in unsupplemented medium. Supernatant from *P. marinus* cultured in JL-ODRP-3 was co-incubated with *C. virginica* homogenate (0·3 mg/ml) and assessed for the production of LMP via gelatin substrate gel electrophoresis. Supernatant from *P. marinus* grown in homogenate-supplemented JL-ODRP-3 (lane a); supernatant from *P. marinus* grown in JL-ODRP-3 (lane b); co-incubation of unsupplemented supernatant from lane b with homogenate (lane c); homogenate supplemented medium alone (lane d). All homogenate supplements were at a final concentration of 0·3 mg/ml. (B) Effect of resistant oyster homogenate on *C. virginica*-induced protease activity. Gelatin substrate gel electrophoresis of supernatant from *C. virginica*-supplemented cultures of *P. marinus* (lane a); supernatant from *C. virginica* supplemented cultures co-incubated with *C. gigas* homogenate (lane b); supernatant from *C. virginica*-supplemented cultures co-incubated with *C. ariakensis* (lane c); *C. gigas*-supplemented medium only (lane d); *C. ariakensis*-supplemented medium only (lane e). All homogenate supplements were at a final concentration of 0·3 mg/ml.

fetuin, albeit to a lesser extent (lane e). Foetal calf serum (lane d), bovine serum albumin (lane f), and ovalbumin (lane g) were all relatively weak in elicitation as was unsupplemented medium (lane a). Thus although the inducing activity appeared to be species-specific with respect to oyster whole tissue extracts, specific xenogeneic protein sources could selectively elicit enhanced expression of these same protease bands elicited by *C. virginica* extracts. Of additional interest is the differential impact of supplementation in the modulation of high molecular weight proteases (HMP; >60 kDa) expression. In some cases a uniformly higher degree of expression appeared to be manifested than was observed with homogenate supplementation (lanes e, h), and in other cases even less activity than that expressed in unsupplemented medium (lanes d, f).

Various serine protease inhibitors were employed to characterize the induced LMP (Fig. 7). These studies revealed that complete inhibition occurred with PMSF (10 mM), while only marginal inhibition occurred with 3,4 dichlororisocumarin (DCI; 10 μM) and soybean trypsin inhibitor (SBTI; 0·8 mg/ml). No sensitivity was observed to benzamidine (100 mM).

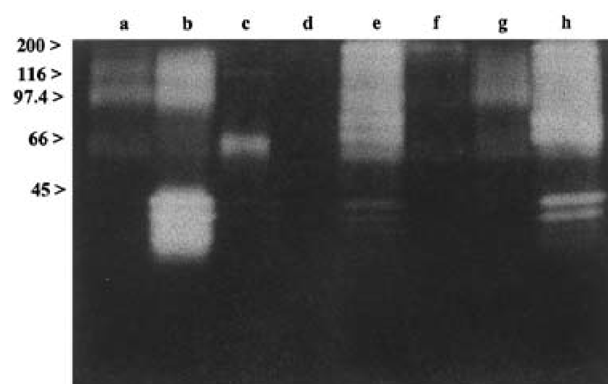

Fig. 6. Effect of alternate protein sources on the elicitation of LMP. Gelatin substrate SDS–PAGE of supernatants of *Perkinsus marinus* cultured in JL-ODRP-3 (lane a) or JL-ODRP-3 supplemented with the following proteins; *Crassostrea virginica* supplemented medium (lane b), foetal calf serum (lane d), fetuin (lane e), bovine serum albumin (lane f), ovalbumin (lane g), yeastolate (a JL-ODRP-1 supplement) (lane h). A sample of unseeded *C. virginica* supplemented JL-ODRP-3 medium was also run as a control (lane c). All protein or homogenate supplements were added to a final concentration of 0·3 mg/ml.

DISCUSSION

Although the use of protein-free, chemically defined culture medium permits purification and study of virulence factors, antigens, and other pathogen-derived molecules, there is an inherent risk of significantly altering parasite function *in vitro* by the exclusion of requisite host-derived material. Pathogens are often exquisitely programmed to respond to

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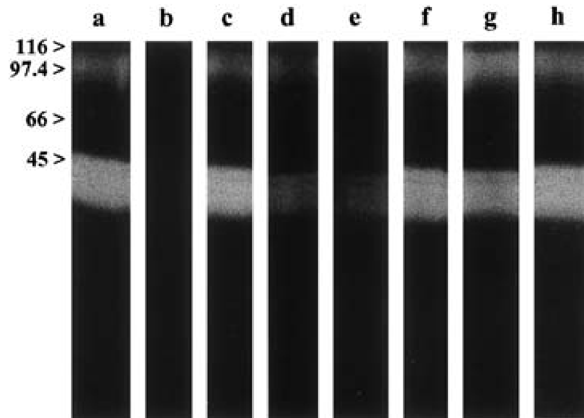

Fig. 7. Effect of protease inhibitors on LMP activity. After electrophoresis of *Crassostrea virginica*-supplemented JL-ODRP-3 culture supernatants into gelatin substrate gels, each lane was incubated in a solution containing the following: 0·1 M Tris buffer, pH 8·0 (lane a), 10 mM PMSF (lane b), 100 mM benzamidine (lane c), 100 μM 3,4-DCI (lane d), 0·8 mg/ml soybean trypsin inhibitor (lane e), phosphate-buffered saline control (lane f) ethanol control (lane g), and dimethyl sulphoxide control (lane h). All incubations were for 3 h at 37 °C.

molecular signals within the host in order to effect colonization, pathogenicity, or invasiveness. For example, selective protease induction has been observed with parasites such as *Plasmodium falciparum*. This protozoan differentially expresses proteases as it matures from trophozoite into schizont and later into merozoite life-stages within the host (Rosenthal *et al*. 1987; Braun-Breton & Pereira da Silva, 1998). Comparable differentiative schema are also observed with *Schistosoma mansoni*, which expresses a specific serine protease during the development of cercariae in the host (Fishelson *et al*. 1992). Such regulated protease expression has also been associated with the breakdown of host tissues (Kapur *et al*. 1993; McKerrow *et al*. 1993) and has been postulated for *P. marinus* (La Peyre *et al*. 1995). In the current studies the induction of LMP appears to be associated with developmental processes yielding morphologically distinct forms (i.e. tomonts); however, this association may not be causally related. Although *C. virginica* homogenate induces heavy expression of LMP and tomonts, *C. gigas* and *C. ariakensis* homogenates only lead to expression of tomonts, not LMP. Thus, it is likely that induction of morphological changes and protease expression are not linked.

The anecdotal, as well as published, observations (Ford *et al*. 2002) on the attenuation of *P. marinus* due to extensive *in vitro* culture become highly relevant to the *in vitro* analysis of *P. marinus* pathogenesis. As completely defined media may reduce parasite infectivity or critical maturational events, we examined the possibility that this pathogen may only express important functional and structural characteristics when cultured in the presence of host-derived nutrients/factors. Not surprisingly, co-culture with homogenate extract from the host, *C. virginica*, elicited a significant shift in *P. marinus* function, most notably a substantially greater expression of LMP. This is in direct contrast to the exclusive production of high molecular weight proteases (HMP; >60 kDa) observed when cultured in JL-ODRP-3 medium alone. Plasma was also found to elicit LMP, although to a much lesser degree. It is striking that equivalent amounts of homogenate protein from oysters, *C. gigas* and *C. ariakensis*, did not elicit detectable levels of this activity. These species have been found to possess low susceptibility to *P. marinus* in that, although they may have comparable prevalences, the intensity of infection remains at the lowest detectable levels (Mann, Burreson & Baker, 1991; Barber & Mann, 1994; Calvo *et al*. 1999). It has been suggested that rather than being resistant, these oysters may be tolerant of the disease (Calvo *et al*. 2001).

The questions of whether this absence of LMP activity was due to the presence of inhibitors within these oyster extracts, which would prevent their detection, or if these extracts were incapable of eliciting LMP, were addressed by inclusion of *C. gigas* and *C. ariakensis* extracts in *C. virginica*-induced culture supernatants. It was demonstrated that the latter extracts were incapable of blocking the expressed proteolytic activity of the *C. virginica* supernatants. Thus, simple blocking of universally induced proteases could not explain the lack of activity observed with *C. gigas* and *C. ariakensis* extracts.

The inability of frozen extracts to support induction of protease activity or *P. marinus* growth suggests that these rather gross preparations of whole tissue homogenate are far too complex to permit prolonged storage and still yield the precise levels of activity. Studies are currently underway to ascertain the precise molecular signals the elicit this activity and the means by which long-term preservation of their activity may be accomplished.

It is tempting to consider this selective ability to induce LMP as a potential prognostic indicator of *P. marinus* susceptibility. The mechanism of induction has not been experimentally addressed, but specific models yield intriguing possibilities. One possibility may be that only *C. virginica* bears a specific signal molecule that triggers secretion of this set of proteases, while less susceptible species lack this inductive signal. Alternatively, all tissue extracts may possess a putative inductive signal, but those that are less susceptible may also possess suppressive signal(s) that counteract this induction.

Oyster tissue extracts also exhibited a potent effect on the differentiation of *P. marinus* cells. Whereas *P. marinus* growth in JL-ODRP-3 medium, or in plasma supplemented medium, yielded only small trophozoites, growth in extracts promoted development of tomonts and a reduction in the number of

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trophozoites. These tomonts are morphologically comparable to those observed within infected oyster tissues. This effect on the growth and differentiation was observed with extract from both susceptible and resistant oysters.

Studies of Gauthier & Vasta (2002) have demonstrated that co-incubation with plasma from varied sources can have an impact on the proliferation of *P. marinus* trophozoites with inhibition of proliferation being observed with the plasma of heavily infected oysters and lesser inhibition with plasma from uninfected or lightly infected oysters. Plasma from the resistant oyster *C. gigas* enhanced proliferation, but plasma from other non-host bivalves also demonstrated a high degree of inhibition. In our studies non-infected plasma (all sources of plasma and homogenates were procured from *P. marinus*-negative oysters) had little relative effect on trophozoite proliferation. However, we do not incorporate foetal bovine serum within our standard tissue culture medium, which may contribute to the differences observed between these systems. Examination of the effects of extract supplements on cell proliferation was confounded by the differentiation of trophozoites to multicellular tomont stages. Thus, the simple measurement of cellular proliferation was insufficient for a direct correlation to be made.

Our observation that other, non-oyster-derived proteins are capable of eliciting LMP suggests that inductive signals may be more universal, and selective suppression of protease expression could be a critical factor with respect to species specificity of protease elaboration. Use of proteins for which specific function(s) are known (albeit derived from non-oyster sources) may provide insight into the functional requisites for LMP expression within the oyster. Interestingly it has also been reported that inclusion of fetuin within a defined culture medium enhances cell growth and proliferation (Gauthier, Feig & Vasta, 1995). This was contrasted with the inability of bovine serum albumin and transferrin to accomplish the same effect. In our studies, although no differences in cell proliferation were noted at the fetuin dose studied, the induction of LMP was apparent. The form and function of the inducing agent(s), regardless of their origin, can provide important clues as to the mechanisms by which this activity is elicited. In summary, the ability to consistently induce cellular proliferation and/or differentiation as well as predictably modulate levels of protease expression may provide a tool for investigation of not only *P. marinus* gene expression, life-cycle, and virulence mechanisms, but also of tolerance in *Crassostrea* oyster species.

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