Protease inhibitors and haemagglutinins associated with resistance to the protozoan parasite, *Perkinsus marinus*, in the Pacific oyster, *Crassostrea gigas*

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SUMMARY

Perkinsus marinus is a protozoan responsible for dramatic mortality in the Eastern oyster, *Crassostrea virginica*, but not in the Pacific oyster, *C. gigas*. To understand the host–parasite relationship, we inoculated *P. marinus* trophozoïtes into the shell cavity of *C. gigas* and measured, over 2 months, (i) intensity of infection, (ii) protease inhibitory activities against *P. marinus* proteases and against bovine α-chymotrypsin, (iii) plasma haemagglutinin titre, (iv) plasma protein concentration, (v) plasma lysozyme activity and (vi) total haemocyte count. We observed that the highest protease inhibitory activities and haemagglutinin titres (3–10 days post-challenge) preceded parasite elimination (initiated 7 days post-challenge). In contrast, plasma protein concentration, lysozyme activity and total haemocyte count showed no significant modification following the challenge. It is hypothesized that the capacity of *C. gigas* to increase its protease inhibitors represents the key event in resistance to parasite infection by neutralizing the proteases secreted by *P. marinus*, thus preserving the oyster haemagglutinins from degradation. Such haemagglutinins will be ready to act as opsonins stimulating phagocytosis of parasites.

Key words: protease inhibitors, *Crassostrea gigas*, *Perkinsus marinus*, haemagglutinin, oyster, immunity.

INTRODUCTION

The protozoan *Perkinsus marinus*, causing perkinsiosis, is considered the most important pathogen affecting the Eastern oyster, *Crassostrea virginica* (Burreson & Ragone-Calvo, 1996). With the development of methods for the culture of *P. marinus* (Gauthier & Vasta, 1993; La Peyre, Faisal & Burreson, 1993), it was found that proteases released from the protozoan might be virulence factors (La Peyre *et al*. 1995*a*; Faisal *et al*. 1999). These proteases are responsible for damaging the immune defence capacities of *C. virginica*, that is lysozyme activity, haemagglutinin titre and haemocyte motility (Garreis, La Peyre & Faisal, 1996). In addition, these proteases facilitate the propagation of parasites in oysters (La Peyre, Yarnall & Faisal, 1996) and might be implicated in the modification of mechanisms controlling cell membrane permeability (Paynter, Pierce & Burreson, 1995), decreasing the level of circulating free amino acids (Soniat & Koenig, 1982).

The non-indigenous species, *C. gigas*, is significantly more resistant to *P. marinus* than to *C. virginica* (Meyers *et al*. 1991; Barber & Mann, 1994). The mechanisms mediating the resistance are unknown but preliminary studies revealed the presence of protease inhibitors in the plasma of both *C. gigas* and *C. virginica* (Faisal *et al*. 1998). The protease inhibitory activity against the proteases of *P. marinus* is greater for *C. gigas* than for *C. virginica* and the activity is still detectable 3 weeks post-infection only in the plasma of *C. gigas*. Since protease inhibitors are well known for their protective role against several protozoan parasites (Rosenthal, 1999), they may provide a key to understanding resistance of *C. gigas* to *P. marinus*.

Previous studies involving *C. gigas* challenged by *P. marinus* were mainly focused on the kinetics of infection and only one study considered parameters such as concentration of circulating haemocytes, plasma lysozyme activity, protein concentration and haemagglutinin titre, but measured 3 months post-challenge (La Peyre, Chu & Meyers, 1995*b*).

Our aims were to determine the role of protease inhibitors and how they combine with other immune factors to eliminate parasites. In *C. gigas*, experimentally challenged by inoculation of *in vitro*-cultivated *P. marinus* trophozoïtes into the shell cavity, we measured over 2 months: (i) intensity of infection, (ii) protease inhibitory activity against *P. marinus* proteases and against bovine α-chymotrypsin, (iii) plasma haemagglutinin titre, (iv) plasma protein concentration, (v) plasma lysozyme activity and total haemocyte count.

MATERIALS AND METHODS

Crassostrea gigas

Adult oysters of about 10 cm length (3 years old) were obtained from Thau lagoon, France (Mediter-

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ranean sea) in April 1999 and quarantined for 2 weeks in 200 l tanks containing seawater at 18 °C and a salinity of 37 ‰. One week before inoculation, they were separated into 2 groups and transferred to different 200 l tanks with seawater at 18 °C and salinity at 37 ‰. Throughout the experiment, oysters were fed twice a week with living algae, *Tetraselmis suecica*.

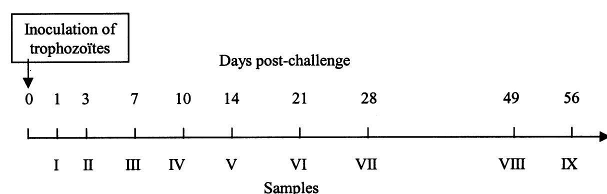
Perkinsus marinus

The cloned isolate *Perkinsus*-1 was cultivated for 6 weeks according to the method of La Peyre, Faisal & Burreson (1993). Parasites at the trophozoïte stage were collected and washed 3 times in phosphate-buffered saline, pH 7·2 (PBS). Cell viability was measured using trypan blue dye exclusion as described by Tirard *et al.* (1995) and density was determined using a Malassez haemocytometer. Parasites were suspended in PBS and adjusted to a density of 10^7 cells/ml.

Experimental protocol

Experimental oysters (90) were challenged by carefully introducing 200 μl of PBS containing 10^6 trophozoïtes into the shell cavity without penetrating soft tissues as recommended by Bushek *et al.* (1997). Parasites were deposited near gills to facilitate contact and penetration. Control oysters (90) received 200 μl of PBS alone. After inoculation, both challenged and control oysters were left overnight, out of seawater, to promote retention of the parasites, and then returned to seawater.

The duration of the experiment was 2 months, with 9 samplings of 5 challenged and 5 control oysters on each occasion. After sampling, the superior valve was removed and haemolymph withdrawn from adductor muscle sinus. Protein concentration was measured according to the Bradford method (Bio-Rad) and total haemocyte count (THC) was established using an automatic haemocytometer (COBAS, Roche Diagnostic). For each oyster, gills, rectum and a sample of the mantle were collected, weighed, and incubated in Ray's fluid thioglycollate medium (RFTM) (Ray, 1966). Infection intensity was then scored according to the method of Ray (1966) on a scale from 0 (negative) to 5 (heavy) based on the number of parasites per mg of tissues that inflate in thioglycollate medium.



Preparation of P. marinus *proteases*

Total *P. marinus* proteases were obtained by bacitracin affinity chromatography as described by La Peyre *et al.* (1995*b*). Culture supernatants from 6-week-old cultures of *P. marinus* cultivated on JLODRP3 medium were concentrated 30 times on a Centriprep concentrator (Amicon) at a molecular weight cut-off of 10 kDa, followed by overnight dialysis with 3 changes against equilibration buffer (50 mM $C_2H_3O_2NH_4$, 10 mM $CaCl_2$, 10 % (w/v) sucrose, pH 6·5). After equilibration of two 5 ml bacitracin-sulpho-NHS Sepharose columns (Pharmacia) with 60 ml of equilibration buffer, 25 ml of concentrated dialysed supernatant were loaded. Unbound materials were washed with 60 ml of equilibration buffer and total proteases were then eluted with 60 ml of equilibration buffer containing 1 M NaCl in 12 % (v/v) isopropanol (Sigma). Eluted fractions were dialysed against 20 mM NH_4Cl, pH 7·0, and freeze-dried. Solutions at 0·2 mg/ml of proteins were prepared in PBS.

Protease inhibitory activity assay

Protease inhibitory activity (PIA) was measured on a spectrophotometer using 0·4 % azocasein labelled with Resorufin (Boehringer Mannheim). Since proteases that may be related to α-chymotrypsin have been described among the proteases of *P. marinus* (Faisal *et al.* 1999), we measured PIA against a solution of bovine α-chymotrypsin (Sigma) at 1 mg/ml in PBS and against a solution of *P. marinus* proteases at 0·2 mg/ml in PBS. PIA was determined as described by La Peyre *et al.* (1995) with modifications. Protease solution (100 μl) was pre-incubated for 30 min at 37 °C with 100 μl of plasma from individual oysters. Then, 100 μl of the mixture were added to 50 μl of substrate solution with 50 μl of 0·2 M Tris–HCl, 20 mM $CaCl_2$, pH 7·8, and incubated for 3 h at 37 °C. The reaction was stopped by addition of 480 μl of 5 % trichloroacetic acid (10 min at 37 °C) followed by 5 min centrifugation at 2000 ***g***. Finally, 400 μl of the supernatant were added to 600 μl of 0·5 M Tris–HCl, pH 8·8, and absorbency was measured at 545 nm (SAFAS Double Energy spectrophotometer).

One unit of protease activity was defined as the amount of enzyme required to produce an absorbency of 1·0 in a 1 cm cell under assay conditions (Sarath, De La Motte & Wagner, 1989). Percentages of PIA were determined and adjusted to the plasma protein concentration and expressed as percentage of inhibition/mg of proteins. Each assay was done in triplicate and the arithmetic mean was attributed to the corresponding oyster.

Haemagglutinin titre

Haemagglutinin titre was measured as described by

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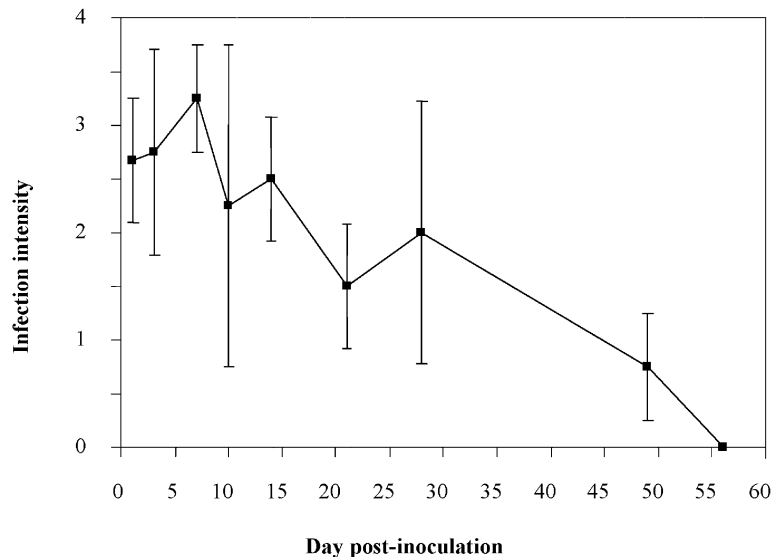

Fig. 1. Kinetics of infection intensity in *Crassostrea gigas* based on the number of parasites per mg of tissues. Disease code numbers were scored as: 0 = none; 1 = light; 2 = light to moderate; 3 = moderate; 4 = moderate to heavy; 5 = heavy. Each value represents the arithmetic mean ($n = 5$) ± S.E.M. as vertical bars.

Fisher & Di Nuzzo (1991) with slight modifications. Sheep red blood cells (SRBC) (Sanofi Diagnostics Pasteur) were centrifuged at 200 ***g*** for 15 min, washed once in Alsever's solution, then twice in PBS and finally adjusted to 0·5 % suspension.

Serial doubling dilutions of plasma collected from 1 oyster were made in PBS from 1/100 to 1/200000 and 50 μl of each dilution were mixed with 50 μl of 0·5 % SRBC suspension in microtitre plates. Plates were incubated overnight at 4 °C and haemagglutinin titres were recorded as the reciprocal of the highest dilution showing a distinct increase in sediment granularity and a decrease in pellet size. Each assay was done in triplicate and the mean value attributed to the corresponding oyster after adjustment to plasma protein concentration. Non-agglutinated controls consisted of 50 μl of 0·5 % SRBC suspension and 50 μl of PBS alone.

Plasma lysozyme activity

Plasma lysozyme activity (PLA) was determined according to the method of Chu & La Peyre (1989). Briefly, a standard suspension of 0·2 mg/ml of *Micrococcus lysodeikticus* powder was prepared in PBS. Plasma (100 μl) was added to 1·4 ml of bacterial suspension, and decrease in absorbency was recorded at 450 nm (SAFAS Double Energy spectrophotometer) over 2 min at 25 °C. Each assay was done in triplicate and the mean value attributed to the corresponding oyster. Lysozyme activity was expressed in μg/ml of hen-egg-white lysozyme equivalent and adjusted to plasma protein concentration.

Statistics

Each parameter was measured in 5 different oysters. Except for infection intensity, measurements were done in triplicate and the mean value attributed to the corresponding oyster. For each sampling day, arithmetic mean value was calculated on the 5 data corresponding to 5 oysters and statistical significance of differences between challenged and control oysters was determined by Student's *t*-test. Differences were considered statistically significant if P values were $< 0{\cdot}05$.

RESULTS

Infection intensity

Direct inoculation of *P. marinus* trophozoïtes into the shell cavity of *C. gigas* established an infection. Important differences in the number of parasites were observed between the 5 oysters of each sampling day. Infection intensity, expressed as mean of disease code number, is presented on Fig. 1. The highest value (3·25 ± 0·5) was obtained 7 days post-inoculation after a regular increase. This value corresponded to a moderate level of infection. From day 10 (2·25 ± 1·5) until day 49 (0·75 ± 0·5), infection intensity slowly decreased, being undetectable with the RFTM method on day 56, suggesting elimination of parasites. Four oysters out of 90 died among the challenged group but no mortality was observed among the control group. The dead oysters were found to have light to moderate infection.

Protease inhibitory activity (PIA)

PIA was generally superior in *P. marinus*-challenged oysters than in controls. Statistically significant differences ($P < 0{\cdot}05$) were observed only on day 10 (80·7 ± 4·89 %) when tested against *P. marinus* proteases, and on days 3 (34·0 ± 3·26 %), 7 (37·18 ± 5·07) and 10 (37·93 ± 4·52) when tested against bovine α-

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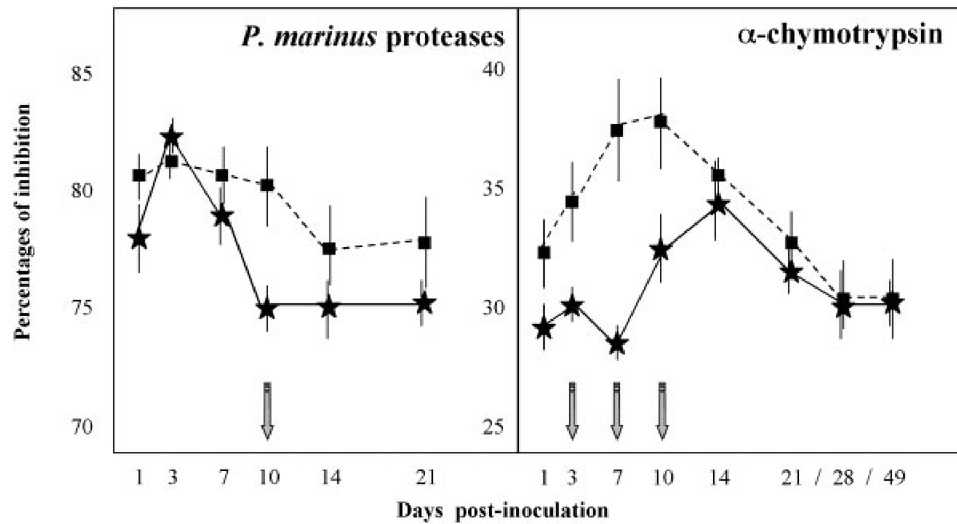

Fig. 2. Kinetics of protease inhibitory activity (PIA) of control (★) and *Perkinsus marinus* challenged oysters (■), expressed as the percentages of inhibition towards *P. marinus* proteases and α-chymotrypsin. Each value represents the arithmetic mean ($n = 5$) ± S.E.M. as vertical bars. Arrows indicate statistically significant mean differences according to Student's *t*-test ($P < 0{\cdot}05$).

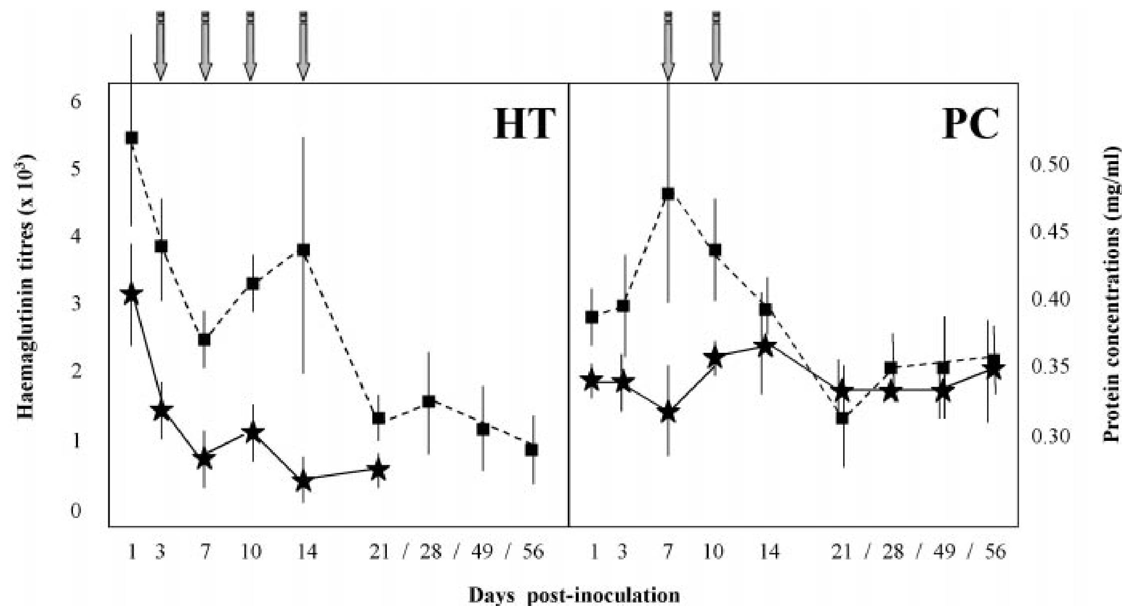

Fig. 3. Kinetics of haemagglutinin titres (HT) and protein concentrations (PC) of control (★) and *Perkinsus marinus*-challenged oysters (■). Each value represents the arithmetic mean ($n = 5$) ± S.E.M. as vertical bars. Arrows indicate statistically significant mean differences according to Student's *t*-test ($P < 0{\cdot}05$).

chymotrypsin (Fig. 2). In challenged oysters, PIA was maintained (against *P. marinus* proteases) or increased (against bovine α-chymotrypsin) from day 1 until days 10–14. Control oysters reacted to the deposit of PBS and overnight emersion by a rapid but evanescent increase in PIA, peaking on day 3.

Haemagglutinin titre (HT)

Challenged oysters presented higher HT than naive ones, at least until 21 days post-inoculation and the difference between the 2 groups remained statistically significant ($P < 0{\cdot}05$) from day 3 until day 14 (Fig. 3). In both groups, an important but temporary increase in HT was observed on day 1, as the result of the treatments. Such increase rapidly returned to baseline without other significant evolution in controls. In contrast, a second peak of HT was observed on day 14 in challenged oysters, rapidly returning to nearly the baseline by day 21.

Protein concentration

In challenged oysters, protein concentration increased from $0{\cdot}38 \pm 0{\cdot}02$ (day 1) to $0{\cdot}47 \pm 0{\cdot}05$ (day

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7), then returned to background (0·31 to 0·35) on day 21 (Fig. 3). Statistically significant differences ($P < 0·05$) were observed only for days 7 and 10. No significant variation was observed in control oysters.

Plasma lysozyme activity (*PLA*) *and total haemocyte count* (*THC*)

Kinetics of both PLA and THC did not show remarkable trends and differences between challenged and control oysters for each sampling day were not statistically significant. Moreover, the values for these two parameters were characterized by an extended individual variability, both in challenged and in control oysters, which may hinder differences.

DISCUSSION

In the present report, we demonstrated that direct inoculation of *P. marinus* into the shell cavity of *C. gigas* induced the classical pattern of infection intensity. In the few studies conducted so far, *C. gigas* were challenged by immersion in seawater containing *P. marinus* (Meyers *et al*. 1991; Barber & Mann, 1994). Using this delivery technique, authors observed similar trends of infection intensity consisting in a short period of increase with a light infection intensity followed by a gradual decrease leading to parasites no longer being detectable. The higher infection intensity reported here may be related to the route of inoculation. Indeed, inoculation of parasites into the shell cavity is efficient for *C. virginica* unlike the method of feeding oysters with trophozoïtes mixed with a daily alga diet (Bushek *et al*. 1997). As reported for *C. virginica* (Ford, Schotthoefer & Spruck, 1999), we also observed a non-random distribution of parasites within *C. gigas* populations, characterized by few oysters with heavy infection intensity whereas other oysters were only slightly infected, explaining the extended S.E.M.

After establishment of the laboratory infection method, our aims were to determine whether protease inhibitors are associated with resistance of *C. gigas* to *P. marinus* infection, and how they interact with other immune factors to eliminate parasites. We found that PIA increased in response to challenge suggesting that protease inhibitors constitute a major defence system against *P. marinus*. We demonstrated also that oyster protease inhibitors can disable the proteases elaborated by parasites, which constitute a major pathogen factor (La Peyre *et al*. 1995; Faisal *et al*. 1999). Because PIA increase just preceded gradual decline of infection intensity, such decline might result from oyster protease inhibitors counteracting the parasite proteases. This is in agreement with Faisal *et al*. (1998) showing higher PIA in *C. gigas* than in *C. virginica* against *P. marinus* proteases and suggesting a possible role of protease inhibitors in parasite elimination.

The delays between the highest values of PIA tested against *P. marinus* proteases (day 3) and against α-chymotrypsin (day 10), demonstrated that *P. marinus* protease activity cannot be limited to a bovine α-chymotrypsin type. In fact, *P. marinus* proteases are multiple, some of them being reported as α-chymotrypsin-like (La Peyre *et al*. 1995; Faisal *et al*. 1999). Also, *C. gigas* protease inhibitors were reported as multiple (Faisal *et al*. 1998).

HT remained elevated as long as parasites could be detected. This correlation suggests a direct link between oyster haemagglutinins, which are lectins (Fisher & Di Nuzzo, 1991), and parasite elimination. Because the highest values of PIA just preceded those of HT, the synthesis of protease inhibitors may be the triggering factor leading to parasite elimination. Positive correlation between HT and infection intensity has been previously reported for *C. gigas* (La Peyre *et al*. 1995*b*) but not for *C. virginica* (Chintala *et al*. 1994; Anderson, 1996). Our results emphasize the role of this type of lectin in the response of *C. gigas* against *P. marinus*. Haemocytes from infected *C. gigas* and *C. virginica* are more efficient at phagocytosis than haemocytes from non-infected oysters (Anderson, Paynter & Burreson, 1992; La Peyre, Chu & Vogelbein, 1995*c*) and the opsonizing role of lectins is well known. Because lectins are disabled by *P. marinus* proteases, survival and proliferation of the parasite within *C. virginica* might be the consequence of a decrease in lectin activity (Garreis, La Peyre & Faisal, 1996; Oliver *et al*. 1999). In *C. gigas*, due to synthesis of protease inhibitors, lectins are protected and may participate in *P. marinus* elimination. The type of lectin involved, their role and the way they react against *P. marinus*, remain unknown.

In contrast to La Peyre *et al*. (1995*b*), we detected PLA both in challenged and in control *C. gigas*, characterized by considerable individual variability, as reported in *C. virginica* (Chu *et al*. 1993). Such PLA was not statistically different between both groups, as previously reported for *C. virginica* parasitized by *P. marinus* (Chu *et al*. 1993) suggesting that lysozyme plays little role in the resistance of *C. gigas* against *P. marinus*.

A surprising result was the absence of significant difference in the THC between challenged and control oysters, suggesting a limited role of haemocytes in parasite elimination. Previous work reported a limited but significant increase in circulating haemocytes, particularly granulocytes, only in *C. gigas* infected with *P. marinus*, suggesting that a slight change in circulating haemocytes is sufficient to elaborate an anti-parasite response (La Peyre *et al*. 1995*b*). In contrast, the significant increase in protein concentration on days 7–10, at the time both

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PIA and HT were significantly enhanced, suggested the release of active proteins in the plasma.

In conclusion, we provided observations towards understanding the mechanisms involved in immune defence reactions of *C. gigas* challenged by *P. marinus*. Our observations are in agreement with the hypothesis that the Pacific oyster, *C. gigas*, responds to *P. marinus* infection by increasing its production of protease inhibitors. Such inhibitors may protect the oyster haemagglutinins by inactivating the proteases secreted by the parasite. Thus, haemagglutinins will participate to parasite opsonization, leading to more efficient elimination by phagocytosis.

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