Available online at www.sciencedirect.com
SCIENCE DIRECT®
Developmental and Comparative Immunology 28 (2004) 565–569
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Short communication

Phenoloxidase and QX disease resistance in Sydney rock oysters (*Saccostrea glomerata*)

Kathryn Newton, Rodney Peters, David Raftos*

Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia

Received 30 January 2003; revised 19 September 2003; accepted 21 October 2003

Abstract

QX is a fatal disease in Sydney rock oysters (*Saccostrea glomerata*) that results from infection by the protistan parasite, *Marteilia sydneyi.* Since 1997, the New South Wales Fisheries Service has bred *S. glomerata* for resistance to QX disease. The current study shows that the QX resistance breeding program has selected oysters with enhanced phenoloxidase (PO) activities. The third generation of QX-selected oysters was compared to *S. glomerata* that had never been selected for disease resistance. PO enzyme assays showed that oysters bred for resistance had significantly higher PO activities than the non-selected population. There was no difference between populations in the activities of a variety of other enzymes. Native polyacrylamide gel electrophoresis identified a novel form of PO in QX-selected oysters that contributes to their enhanced PO activities. This novel form of PO may represent a specific QX disease resistance factor.

Keywords: *Saccostrea glomerata*; Phenoloxidase; Disease resistance; Oyster

1. Introduction

The Sydney rock oyster, *Saccostrea glomerata* (previously *Saccostrea commercialis*), is endemic to Australia [1]. It has been farmed on Australia's east coast since the 1870s, and on the west coast since the 1980s [1]. Even though it remains the cornerstone of the Australian oyster industry, rock oyster production has declined dramatically in recent years. Approximately 14.5 million dozen *S. glomerata* were produced annually during the 1970s, but only 7.9 million dozen were harvested in the 2000/2001 growing season [2].

Declining rock oyster production has resulted, at least in part, from mortality associated with infection by two microbial parasites—*Mikrocytos roughleyi*, which causes Winter Mortality disease, and *Marteilia sydneyi*, the etiological agent of QX disease [1,3]. *M. sydneyi* is a haplosporidean protozoan that invades the digestive gland of susceptible oysters leading to complete disorganization of the infected tissue [3,4]. Mortality associated with QX disease results from starvation within 60 days of infection. QX disease causes up to 98% mortality among rock oysters during

Abbreviations: DHPPA, 3-(2,4-dihydroxyphenyl)propionic acid; FSW, filtered seawater; MAC, marine anticoagulant; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PO, phenoloxidase; PAGE, polyacrylamide gel electrophoresis; QXR_3, third generation oysters bred for QX disease resistance.

* Corresponding author. Tel.: +61-2-9850-8402; fax: +61-2-9850-8245.
E-mail address: draftos@rna.bio.mq.edu.au (D. Raftos).

0145-305X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2003.10.004

late summer (January to April) [5]. The only current control method is the removal of oysters from rivers prone to QX disease during the infective period.

Recently, we have found that the phenoloxidase (PO) cascade of susceptible oysters is suppressed prior to *M. sydneyi* infection [5]. In many invertebrates, the PO cascade represents a critical host defense response. Recognition components of the PO cascade can identify a variety of potentially pathogenic microorganisms and activate PO via proteolysis of its zymogen, prophenoloxidase [6–8]. PO, also known as tyrosinase, is a bifunctional enzyme with both monophenol monooxygenase (monophenolase) and *o*-diphenoloxidase (*o*-diphenolase) activities [9,10]. Monophenolase activity leads to the hydroxylation of substrates such as tyrosine to *o*-diphenols like L-3,4-dihydroxyphenylalanine (L-DOPA). PO's *o*-diphenolase activity then converts L-DOPA to quinones such as dopaquinone. This cascade of enzymatic reactions ultimately results in the formation of the pigment, melanin [11]. Melanin and other products of the PO pathway are involved in host defense reactions such as wound healing, cytotoxicity, phagocytosis and the encapsulation of pathogens [8,12–14].

In 1997, the New South Wales (NSW) Fisheries Service began a rock oyster breeding program to develop QX disease resistant oysters. This program has been based on interbreeding the survivors of annual QX disease outbreaks [15]. After just two generations of selection, mortality from QX disease was reduced from more than 90% to 64% [16]. Given the apparent association between suppressed PO activity and the establishment of QX disease, the study reported here tests whether oysters bred for QX disease resistance have enhanced PO activities relative to non-selected oysters.

2. Materials and methods

2.1. Oysters

Two types of *S. glomerata*, designated QXR_3 and wild type, were used in this study. Wild type oysters were collected from commercial oyster leases in Porto Bay on the Hawkesbury River, NSW. These oysters had not been selected for resistance to QX disease and the Hawkesbury River has never suffered a QX disease outbreak. QXR_3 oysters were the third generation of *S. glomerata* bred for QX resistance in the Georges River, NSW. They were kindly supplied by Dr John Nell, NSW Fisheries. The parental generation of the QXR strain came from the same brood stock as the wild type oysters from the Hawkesbury River.

All oysters were collected during the period July–October, 2002, when there was no active *M. sydneyi* infection in the Georges River. Oysters were approximately 2-years-old when they were collected.

After collection, oysters were held for up to 3 weeks in 50 l aquaria filled with sea water collected from The Spit, Sydney Harbor. They were maintained at a constant temperature of 15 °C and were fed Aquasonic invertebrate food supplement (1.5 ml/50 l) at regular intervals.

2.2. Oyster hemolymph and hemocytes

Oysters were removed from aquaria 30 min prior to hemolymph extraction to drain seawater from their mantle cavities. They were then shucked with an oyster knife and the exuding hemolymph was removed with a pipette.

For native polyacrylamide gel electrophoresis (native PAGE), 1 ml of hemolymph was removed from each oyster and centrifuged for 10 sec at 12,000*g* so that the serum (supernatant) could be collected. To prepare hemolymph for microplate enzyme assays, 2 ml of hemolymph from each oyster was mixed with 2 ml of marine anticoagulant (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl, pH 7.0) before being centrifuged for 5 min at 3000*g*. The serum was then removed and placed in separate tubes. Hemocytes were resuspended in 4 ml of a 1:1 mixture of MAC and filtered seawater (FSW; 0.45 μm filter) and allowed to agglutinate. The suspensions were then centrifuged for another 5 min at 3000*g* to obtain hemocyte supernatants. Serum and hemocyte supernatants were kept on ice before use. The total protein contents of sera were measured using a BioRad Protein Determination kit with BSA as a standard (BioRad, Regent's Park, NSW). Cell numbers in hemocyte suspensions were calculated with a Neubauer hemocytometer.

2.3. Enzyme assays

Two substrates identified by Espin et al. [17] were used to test independently for the monophenolase and *o*-diphenolase activities of PO. Hydroquinone monomethyl ether (Fluka, Switzerland) was used to measure monophenolase activity and 3-(2,4-dihydroxyphenyl)propionic acid (DHPPA; Fluka) was employed to quantify *o*-diphenolase activity. Both substrates were prepared in FSW. The chromogenic nucleophile, 3-methyl-2-benzothiazolinone hydrazone (MBTH; Sigma Aldrich, NSW) was added to substrate solutions at a final concentration of 1 mM [10]. In some cases, the PO-specific inhibitor, tropolone (Sigma Aldrich) was added (1 mM final concentration) to sera and hemocyte supernatants 15 min prior to the addition of PO substrates [18].

The activities of six other enzymes were tested using the following substrates (all purchased from Sigma Aldrich): 4-nitrophenyl acetate (general esterase), 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (*N*-acetyl-glucosaminidase), 4-nitrophenyl β-D-galactopyranoside (β-galactosidase), 4-nitrophenyl α-D-maltopentanoside (α-amylase), 4-nitrophenyl phosphate (alkaline phosphatase) and 4-nitrophenyl β-D-triacetylchitotriose (lysozyme). These substrates were prepared in FSW and used at a final concentration of 2.5 mg/ml.

Enzyme activities were determined spectrophotometrically in 96 well microtest plates (Sarstedt, Technology Park, SA). In each microplate well, 100 μl of serum or hemocyte supernatants were mixed with 100 μl of substrate solution. Absorbance at 490 nm (substrates containing MBTH) or 415 nm (4-nitrophenol based substrates) was measured using a microplate spectrophotometer (BioRad). Control wells containing 100 μl of substrate solution mixed with 100 μl of MAC:FSW were included in all plates. Data were corrected for spontaneous hydrolysis of substrates in these control wells.

2.4. Native PAGE

Differences between the PO proteins of QXR_3 and wild type oysters were identified by native PAGE according to the method of Nellaiappan and Vinayakam [19]. Native PAGE gels had a lower resolving layer of 5% acrylamide and an upper stacking layer of 4% acrylamide. Samples consisted of 16 μl of oyster serum mixed with 4 μl of 0.35 M Tris-HCl (pH 6.8) containing 10% glycerol. Gels were stained with 20 mM hydroquinone in FSW containing 5 mM MBTH overnight before being rinsed with FSW.

2.5. Statistical analyses

Enzyme assays of sera or hemocyte supernatants from individual oysters were performed in triplicate. Data from triplicates were averaged to provide enzyme activities for individual oysters. Mean values were calculated from the enzyme activities of at least 10 separate oysters ($n \geq 10$) per treatment.

Differences between mean enzyme activities were tested for significance using Student's *t*-tests. The frequencies of PO protein banding patterns in different populations were compared by contingency Chi-squared analysis. Differences were deemed to be significant if $p < 0.05$.

3. Results

3.1. QXR_3 oysters have higher phenoloxidase activities than wild type oysters

Substantial PO (monophenolase and *o*-diphenolase) activity was detected by microplate assays of serum and hemocyte supernatants from QXR_3 and wild type oysters. Three to four times more PO activity was evident in hemocytes when compared to serum ($p < 0.001$). The PO-specific inhibitor, tropolone, eliminated activity against the monophenolase susbtrate, hydroquinone, and the *o*-diphenolase substrate (DHPPA) ($p > 0.05$ vs substrate only controls). This confirms that the conversion of both substrates measured true PO activity.

Microplate assays also revealed a significant difference in PO activity between the two oyster populations. Sera and hemocyte supernatants from QXR_3 oysters had approximately two times more monophenolase and *o*-diphenolase activity than the wild type population ($p < 0.001$).

Unlike PO, the activities of six other enzymes (esterase, *N*-acetyl-glucosaminidase, β-galactosidase, α-amylase, alkaline phosphatase and lysozyme) in

hemocyte supernatants did not differ significantly between QXR_3 and wild type oysters ($p > 0.05$). There was also no significant difference between the two oyster populations in the total protein content of serum or in the number of cells in hemocyte suspensions ($p > 0.05$). Serum from QXR_3 oysters contained 38 ± 3 μg protein/ml, and their hemocyte suspensions averaged 5.4 ± 0.9 × 10^5 cells/ml. Wild type oysters yielded serum samples with total protein contents of 36 ± 4 μg protein/ml, and their hemocyte suspensions contained 5.3 ± 0.7 × 10^5 cells/ml.

3.2. QXR_3 oysters express a novel form of PO

Native PAGE identified two distinct PO protein banding patterns among QXR_3 oysters (Fig. 1A). Twenty six percent (12/46) of QXR_3 oysters exhibited two distinct PO bands after native PAGE. Only the lower of these two bands could be detected among the remaining 74% of QXR_3 oysters (Fig. 1B). Three

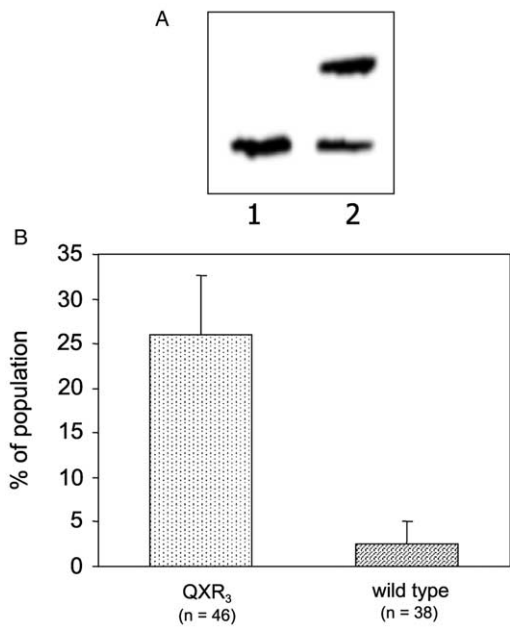

Fig. 1. A. Phenoloxidase staining patterns of serum from QXR_3 that had been subjected to native PAGE and stained with hydroxyquinone and MBTH. Two phenotypes, one PO band (lane 1) or two PO bands (lane 2) were evident among QXR_3 oysters. B. Percentage of QXR_3 and wild type oysters in which two PO bands could be detected by native PAGE (n = sample size, bars = binomial standard error).

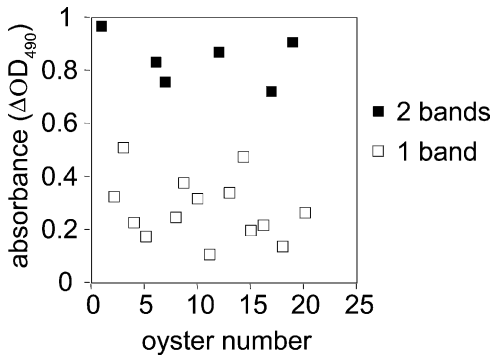

Fig. 2. Monophenoloxidase activities in hemocyte supernatants from individual QXR_3 oysters (identified by arbitrarily assigned numbers) that had been shown by native PAGE to express either one or two forms of PO. Activities are shown as change in absorbance at 490 nm after 1 h (ΔOD_{490}).

percent (1/38) of wild type oysters exhibited two PO bands, whilst the remainder (97%) had only the lower PO band. Chi-squared analysis confirmed that there was a significant difference in the frequency of the two PO banding patterns between QXR_3 and wild type oysters ($p < 0.001$).

Native PAGE of a further 20 QXR_3 oysters identified six oysters that exhibited two PO protein bands after native PAGE (Fig. 2). The mean PO activity in hemocyte supernatants from these six oysters ($\Delta OD_{490} = 0.84 \pm 0.04$) was significantly higher ($p < 0.001$) than that for the remaining 14 oysters ($\Delta OD_{490} = 0.31 \pm 0.04$), which exhibited just one PO protein (lower band) after native PAGE.

4. Discussion

This study has shown that oysters selected for resistance to QX disease have significantly higher PO (monophenolase and *o*-diphenolase) activities than wild type oysters. The difference between populations cannot be explained by variation in total protein expression or circulating hemocyte frequencies. Wild type and QXR_3 oysters had very similar total protein contents in their serum and comparable numbers of cells in their hemocyte suspensions.

We have also demonstrated that increased PO activity in QXR_3 oysters is associated with the expression a novel form of PO protein. Native PAGE showed that 26% of QXR_3 oysters have a second form

of PO, in addition to the enzyme that is common among wild type oysters. Only 3% of wild type oysters exhibited this additional form of PO. Expression of the second PO protein is clearly associated with the increased enzyme activities of QXR_3 oysters. The mean PO activity of QXR_3 oysters bearing the addition PO protein was almost three times greater than that of oysters exhibiting a single (wild type) form of the enzyme.

All of this evidence suggests that the QX disease resistance breeding program has selected oysters bearing an additional PO protein that enhances their PO enzymatic activities. That selection does not appear to be a chance effect of inbreeding that might have occurred during the intensive development of the QXR strain. The activities of other enzymes (esterases, β-galactosidase, α-amylase, *N*-acetyl-glucosamidase, alkaline phosphatase and lysozyme) do not differ significantly between the QXR and wild type populations, indicating that the breeding of QXR oysters has not resulted in chance genetic drift. It is also unlikely that the appearance of the novel PO protein in QXR_3 oysters simply reflects the genetic background of that population. The QXR_3 and wild type populations are derived from the same original brood stock and so have the same genetic origin. The lack of inbreeding effects and the common genetic origin of the QXR and wild type populations implies that the increased PO activity of QXR_3 oysters represents a specific, directionally selected trait that protects Sydney rock oysters from QX disease.

Acknowledgements

We thank Dr John Nell, from NSW Fisheries, Robert and Lynn Drake of Drake Oyster Farm, and Kevin and Sue Buie of K and S Buie Oyster Farm for their generosity in the provision of oysters used in this study.

References

[1] Nell J. Farming the Sydney rock oyster (*Saccostrea commercialis*) in Australia. Rev Fish Sci 1993;1:97–120.

[2] Nell J. Oyster industry report. In: Status of fisheries resources 2001/2002, Sydney: NSW Fisheries; 2002. p. 103.

[3] Perkins FO, Wolf PH. Fine structure of *Marteilia sydneyi*—haplosporidan pathogen of Australian oysters. J Parasitol 1976;62:528–38.

[4] Kleeman SN, Adlard RD, Lester RJG. Detection of initial infective stages of the protozoan parasite *Marteilia sydneyi* in *Saccostrea glomerata* and their development through to sporogenesis. Int J Parasitol 2002;32:767–84.

[5] Peters R, Raftos D. The role of phenoloxidase suppression in QX-disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). Aquaculture 2003;223:29–39.

[6] Asokan R, Arumugam M, Mullainadhan P. Activation of prophenoloxidase in the plasma and haemocytes of the marine mussel *Perna viridis* Linnaeus. Dev Comp Immunol 1997;21:1–12.

[7] Söderhall K. The prophenoloxidase activating system. In: Söderhall KS, Iwanga S, Vasta G, editors. New directions in invertebrate immunology. Fair Haven: SOS Publications; 1996. p. 229–53.

[8] Söderhall K, Cerenius L. Role of the prophenoloxidase activating system in invertebrate immunity. Curr Opin Immunol 1998;10:23–8.

[9] Chase MR, Raina K, Bruno J, Sugumaran M. Purification, characterisation and molecular cloning of prophenoloxidase from *Sarcophaga bullata*. Insect Biochem Mol Biol 2000;30:953–67.

[10] Dicko M. Zymography of monophenolase and *o*-diphenolase activities of polyphenol oxidase. Anal Biochem 2002;306:336–9.

[11] Prota G. Melanins and melanogenesis. San Diego: Academic Press; 1992.

[12] Ashida M, Brey P. Role of the integument in insect defence: prophenoloxidase cascade in the cuticular matrix. Proc Natl Acad Sci USA 1995;92:10698–702.

[13] Gillespie JP, Kanost MR, Trenczek T. Biological mediators of insect immunity. Ann Rev Entomol 1997;42:611–43.

[14] Lai-Fook J. The repair of wounds in the integument of insects. J Insect Physiol 1966;12:195–206.

[15] Nell JA, Smith IR, McPhee CC. The Sydney rock oyster *Saccostrea glomerata* breeding programme: progress and goals. Aquaculture Res 2000;31:45–9.

[16] Nell J. Selective breeding for disease resistance and fast growth in Sydney rock oysters. NSW Fisheries Advisory # Aqu2000/007, Sydney: NSW Fisheries; 2000.

[17] Espin JC, Garcia-Ruiz PA, Tudela J, Varon R, Garcia-Canovas F. Monophenolase and diphenolase reaction mechanisms of apple and pear polyphenol oxidases. J Agric Food Chem 1998;46:2968–75.

[18] Kahn V, Andrawis A. Inhibition of mushroom tyrosinase EC-1.10.3.1 by tropolone. Phytochemistry 1985;24:905–8.

[19] Nellaiappan K, Vinayakam A. A method for demonstrating prophenoloxidase after electrophoresis. Biotech Histochem 1993;68:193–5.