Chemosphere 93 (2013) 931–938

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Screening for antioxidant and detoxification responses in *Perna canaliculus* Gmelin exposed to an antifouling bioactive intended for use in aquaculture

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HIGHLIGHTS

- Used biochemical biomarkers to screen for effects of polygodial on *Perna canaliculus*.
- Examined markers of oxidative stress and a detoxification pathway.
- Exposure to the IC_{99} against fouling ascidians had no effect in *P. canaliculus*.
- Antioxidant enzyme activity increased in *P. canaliculus* exposed to 10× the IC_{99}.

ARTICLE INFO

Article history:
Received 21 September 2012
Received in revised form 13 May 2013
Accepted 25 May 2013
Available online 2 July 2013

Keywords:
Antioxidant
Biofouling
Biomarker
Detoxification
Perna canaliculus
Polygodial

ABSTRACT

Polygodial is a drimane sesquiterpene dialdehyde derived from certain terrestrial plant species that potently inhibits ascidian metamorphosis, and thus has potential for controlling fouling ascidians in bivalve aquaculture. The current study examined the effects of polygodial on a range of biochemical biomarkers of oxidative stress and detoxification effort in the gills of adult *Perna canaliculus* Gmelin. Despite high statistical power and the success of positive controls, the antioxidant enzymes glutathione reductase (GR), glutathione peroxidase (GPOX), catalase (CAT), and superoxide dismutase (SOD); thiol status, as measured by total glutathione (GSH-t), glutathione disulphide (GSSG), and GSH-t/GSSG ratio; end products of oxidative damage, lipid hydroperoxides (LHPO) and protein carbonyls; and detoxification pathways, represented by GSH-t and glutathione S-transferase (GST), were unaffected in the gills of adult *P. canaliculus* exposed to polygodial at 0.1 or 1 × the 99% effective dose in fouling ascidians (IC_{99}). Similarly, GR levels, thiol status, and detoxification activities were unaffected in mussels exposed to polygodial at 10 × the IC_{99}, although GPOX, CAT, and SOD activities increased. However, the increases were small relative to positive controls, no corresponding oxidative damage was detected, and this concentration greatly exceeds effective doses required to inhibit fouling ascidians in aquaculture. These findings compliment a previous study that established the insensitivity to polygodial of *P. canaliculus* growth, condition, and mitochondrial functioning, providing additional support for the suitability of polygodial for use as an antifouling agent in bivalve aquaculture.

1. Introduction

Polygodial is a drimane sesquiterpene dialdehyde produced by several terrestrial plant species that has potential as a specific antifouling remedy for the costly problem of fouling ascidians in bivalve aquaculture. Polygodial potently inhibits metamorphosis in ascidian larvae (Cahill et al., 2012), and initial screens of organism-level PB (growth, survival, and mitochondrial functioning) detected no negative effects in cultured bivalves exposed to polygodial at the 99% effective dose against ascidian metamorphosis (i.e., IC_{99}; Cahill et al., 2013). However, while PB respond to a broad range of xenobiotic stressors (Depledge et al., 1995; Lam and Gray, 2003) and are relevant to whole-organism consequences (De Coen et al., 2000), they can have relatively limited sensitivity for detecting low-level negative effects within a practicable timeframe (Van der Oost et al., 2003; Venturino et al., 2003).

Abbreviations: BB, biochemical biomarker; CAT, catalase; EtOH, ethanol; FSW, filtered seawater; GPOX, glutathione peroxidase; GSH, glutathione; GSH-t, total glutathione; GSSG, glutathione disulphide; GST, glutathione S-transferase; IC_{99}, 99% inhibition of metamorphosis; LHPO, lipid hydroperoxides; PB, physiological biomarker; SOD, superoxide dismutase.

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http://dx.doi.org/10.1016/j.chemosphere.2013.05.058

Conversely, BB – biochemical components, processes, and functions – are inherently sensitive proxies that often react to stressors long before organism-level impacts become apparent (Walker, 1995; Madden and Gallagher, 1999; Van der Oost et al., 2003). Although the relevance of BB to whole-organism consequences must be interpreted carefully (Forbes et al., 2006), they can respond to a broad range of environmental stressors and are routinely utilized for ecotoxicological studies on marine bivalves (e.g., Regoli and Principato, 1995; Walker, 1995; De Lafontaine et al., 2000). Thus, BB provide an ideal secondary screen to compliment the previously established lack of detectable effects on PB and further evaluate possible negative effects of polygodial on adult bivalves. While the exact mechanism by which polygodial inhibits ascidian metamorphosis is unknown, a range of biochemical effects have been reported in other species, including non-ionic surfactant properties (Kubo et al., 2001); ATP synthase antagonism (Lunde and Kubo, 2000); tachykinin NK_2 receptor agonism (El Sayah et al., 1998); and interaction with the opioid system (Mendes et al., 2000), nitric oxide, endogenous prostaglandins, sulfhydryl compounds, and vanilloid receptors (Matsuda et al., 2002). In light of the plethora of conceivable biochemical modes of action for polygodial in bivalves, the current study calls for widely responsive and generally applicable BB that reflect general environmental stress.

Markers of oxidative stress are among the most widely responsive and generally applicable class of BB (Almeida et al., 2007) because of the close relationship between general environmental stress and the generation rate of cellular reactive oxygen and nitrogen species (i.e., oxidative stress) in an organism (Storey, 1996). The three main classes of BB of oxidative stress are antioxidant enzymes, thiol status, and end products of oxidative damage. Antioxidant enzymes of interest in mussels include GR, GPOX, CAT, and SOD (e.g., Solé and Albaigés, 1995; Lionetto et al., 2003; Bocchetti and Regoli, 2006; Box et al., 2007; Vlahogianni et al., 2007). These enzymes are integral components of the cellular antioxidant defense system: GR catalyzes the conversion of GSSG to GSH (Mannervik, 1987); GPOX, the reduction of LHPO and hydrogen peroxide (Battin and Brumaghim, 2009); CAT, the decomposition of hydrogen peroxide (Chelikani et al., 2004); and SOD, the dismutation of superoxide (McCord and Fridovich, 1988). Thiol status also plays a central role in antioxidant defense (Finkel and Holbrook, 2000; Dafre et al., 2004). Integral measures of thiol status that have been identified as useful BB of oxidative stress in bivalves are GSH-t, GSSG, and the GSH-t/GSSG ratio (e.g., Regoli and Principato, 1995; Cheung et al., 2001; Dafre et al., 2004; Franco et al., 2006). Likewise, the end products of oxidative damage LHPO and protein carbonyls reflect the ultimate consequences of oxidative stress for cells (Doyotte et al., 1997; Almeida et al., 2007; Verlecar et al., 2007).

Comparable to oxidative stress, detoxification effort is a useful indicator of environmental stress that provides direct evidence of exposure to toxins (Lagadic et al., 1994). In addition to its antioxidant roles, GSH is a central component of cellular detoxification, owing to its ability to conjugate some toxins (Viarengo and Nott, 1993). Consequently, levels of GSH-t, and the associated conjugation enzyme GST (Douglas, 1987), are effective BB of detoxification effort in bivalves (e.g., Hai et al., 1997; Kaaya et al., 1999; Akcha et al., 2000; Manduzio et al., 2004).

The aim of this study was to quantify the effects of polygodial on the BB outlined above in the gills of an economically important aquaculture species in New Zealand, the green-lipped mussel (*Perna canaliculus* Gmelin). This mussel is the main target aquaculture species for the application of polygodial and the optimal culture parameters for this species are well established (Vakily, 1989). Furthermore, gills are the ideal target tissue for biomonitoring and toxicological studies in mussels because they are the first barrier to potential contaminants, owing to their role as the filter feeding apparatus and respiratory organ (Bolognesi et al., 2004).

Due to the previously established lack of detectable effects on the physiological health of bivalves (Cahill et al., 2013), it was hypothesized that polygodial would not affect antioxidant enzymes, thiol status, end products of oxidative damage, or detoxification pathways in adult *P. canaliculus*. The results from this study provide an important next step towards developing polygodial as an antifouling agent for use in bivalve aquaculture.

2. Methods

2.1. Experimental organisms

Adult *P. canaliculus* (24.2 ± 8.3 mm shell height) were obtained from a commercial mussel farm in Pelorus Sound, Marlborough, New Zealand and held in a 5000-L recirculating seawater system according to Cahill et al. (2013).

2.2. Experimental design

Randomly selected mussels were housed in pairs in high density polyethylene containers (130 × 130 × 150 mm), prepared according to Cahill et al. (2013) and each containing 2.0 L of 0.4-μm FSW (salinity: 34 ± 1 ppt). The culture containers were held at 18 ± 1 °C and mussels were fed daily with 50 mL of a 1:1 mix of *Isochrysis galbana* Parke ($8–9 \times 10^6$ cells mL^{-1}) to *Pavlova lutheri* Green ($10–12 \times 10^6$ cells mL^{-1}).

Treatments consisted of polygodial (ENZO Lifescience, Farmingdale, NY; yellow solid comprising 97% polygodial; extracted from *Polygonum hydropiper* L.) dosed at 0.1, 1, or 10× the IC_{99} in ascidian larvae, corresponding to 0.3 (Pg_{low}), 3.0 (Pg_{mid}), and 30.0 ng mL^{-1} (Pg_{high}), respectively (Cahill et al., 2012). Stock solutions of polygodial were prepared in EtOH and dosed into the culture vessels at a final concentration of 0.1 μL EtOH mL^{-1} of FSW. Positive controls utilized zinc dosed at and above concentrations previously shown to negatively affect antioxidant status in a closely related mussel species, *Perna perna* L. (Franco et al., 2006). Accordingly, zinc was dosed at 0.7 (Zn_{low}), 7.0 (Zn_{mid}), and 70.0 μg mL^{-1} (Zn_{high}). Negative controls consisted of 0.1 μL EtOH mL^{-1} of FSW and FSW only. Ten replicates ($n = 10$) were performed simultaneously for all treatments and controls. The experiment was run for 14 d following the first introduction of the chemical treatments. Chemical treatments in all containers were refreshed daily with FSW changes.

2.3. Gill tissue preparation

At 7 and 14 d after the chemical treatments began, one mussel was removed from each culture vessel and immediately dissected on ice. Gills were excised, divided into 100–150 mg subsamples, and placed in Nunc™ CryoTube vials (Thermo Fisher Scientific, Waltham, MA), which were flushed with nitrogen gas and then sealed. Subsamples were frozen in liquid nitrogen, and stored at −80 °C until further analysis.

All extraction steps were performed at 4 °C. Total protein was extracted for determination of enzyme activities and protein carbonyls according to Franco et al. (2006) with minor modifications. Briefly, gill tissue subsamples were homogenized in 900 μL of HEPES buffer using a bead beater (3 × 2.4 mm BioSpec™ zirconia beads; BioSpec Products, Bartlesvile, OK; 3 × 15 s cycles). The homogenate was centrifuged at 20,000 *g* for 30 min and the supernatant was stored in 150-μL aliquots at – 80 °C. For cellular thiol analysis, the same procedure as outlined for total protein extraction was used, except 5% salicylic acid was substituted for HEPES