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Short communication

Proteomic analysis of hemolymph from poly(I:C)-stimulated *Crassostrea gigas*

Timothy J. Green [a, b, *], Timothy Chataway [c], Aroon R. Melwani [a, b], David A. Raftos [a, b]

[a] Department of Biological Sciences, Macquarie University, Sydney, NSW, Australia
[b] Sydney Institute of Marine Science, Chowder Bay, Mosman, Sydney, NSW, Australia
[c] Department of Human Physiology and Centre for Neuroscience, Flinders University, Adelaide, SA, Australia

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ABSTRACT

Synthetic double stranded RNA (Poly(I:C)) injection of *Crassostrea gigas* results in a systemic antiviral response involving many evolutionary conserved antiviral effectors (ISGs). Compared to mammals, the timing of *C. gigas* ISG expression to viral or poly(I:C) injection is delayed (>12 h p.i.). It could be interpreted that a cytokine is responsible for the systemic, but delayed expression of *C. gigas* ISGs. We therefore analysed the acellular fraction of *C. gigas* hemolymph by two-dimensional electrophoresis (2-DE) to identify hemolymph proteins induced by poly(I:C). Poly(I:C) injection increased the relative intensity of four protein spots. These protein spots were identified by tandem mass spectrometry (LC-MS/MS) as a small heat shock protein (sHSP), poly(I:C)-inducible protein 1 (PIP1) and two isoforms of C1q-domain containing protein (C1qDC). RT-qPCR analysis confirmed that the genes encoding these proteins are induced in hemocytes of *C. gigas* injected with poly(I:C) ($p < 0.05$). Proteomic data from this experiment corroborates previous microarray and whole transcriptome studies that have reported up-regulation of C1qDC and sHSP during mass mortality events among farmed oysters.

1. Text

The genome of the Pacific oyster (*Crassostrea gigas*) encodes many interferon-stimulated genes (ISGs), which are up-regulated in response to infection with Ostreid herpesvirus type 1 (OsHV-1) [1,2]. The expression of these ISGs can also be induced by injecting *C. gigas* with virus mimics, such as poly(I:C) [3,4] and this response is protective against OsHV-1 infection [5]. Mammalian ISGs are induced rapidly after viral infection via a pathway dependent on type I interferons [6]. Type I interferons comprise of a large group of glycoproteins that are secreted from virus-infected cells and elicit a distinct antiviral response [7]. It is hypothesised that molluscs have a functionally equivalent of type I interferon and that mollusc cells infected with viruses or poly(I:C) secrete this cytokine into the hemolymph to induce the systemic transcription of ISGs [1,5,8]. The purpose of this study was to analyse the acellular fraction of the hemolymph proteome from *C. gigas* to identify proteins that are up-accumulated in response to poly(I:C)-stimulation.

Adult *C. gigas* had a notch filed in their shell adjacent to their adductor muscle to allow delivery of 100 μl of poly(I:C) (5 mg ml^{-1}) or sterile seawater (control) according to previous published procedures [4,9]. Hemolymph was collected from six oysters per treatment at 24 h post-injection using a sterile 21-gauge needle and syringe. Hemolymph samples was pooled (N = 6 per pool), centrifuged (2000 × g for 5 min) and filtered (0.2 μm) to obtain the acellular fraction. The experiment was repeated using a separate batch of adult *C. gigas*. Hemolymph proteins were purified using Tri-Reagent LS (Sigma–Aldrich) and analysed by two-dimensional electrophoresis (2-DE). A total of eight 2-DE gels were analysed: two batches of oysters x two treatments x two technical replicates. One hundred micrograms of precipitated hemolymph protein was separated in the first dimension by isoelectric focussing (IEF) using 7 cm immobilized pH gradient strips (Ready-Strip™ IPG strips; pH 4–7; Bio-Rad). IEF was performed using a Protein IEF Cell (Bio-Rad). After IEF, SDS-PAGE was performed using Mini-Protean® TGX™ Precast Gels (12%; Bio-Rad #456–1041). Gels were stained overnight with Sypro Ruby (Bio-Rad) and imaged using a Pharos FX™ Plus Molecular Imager (Bio-Rad). PDQuest™ Advanced 2-D Analysis Software version 8.01 (Bio-Rad) was used to automatically match corresponding proteins spots across separate 2-DE gels. Matched

* Corresponding author. Department of Biological Sciences, Macquarie University, Sydney, NSW, Australia.
E-mail address: tim.green@mq.edu.au (T.J. Green).

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Table 1
Hemolymph protein spots with different intensities between *Crassostrea gigas* injected with poly(I:C) or sterile seawater (controls). Two independent batches of *C. gigas* were analysed, representing experiment 1 and 2. Combined column represents protein spots observed in both experiments 1 and 2.

Protein spots	Experiment 1	Experiment 2	Combined
All spots	110	77	49
Poly(I:C)-specific	1	1	1
Up-regulated (≥2x fold)	4	14	3
Down-regulated (≥2x fold)	22	29	4

spots were checked manually to remove streaks, speckles and artifacts. Protein spots with ≥2.0-fold difference in intensity as identified by PDQuest's statistical package between poly(I:C) and control groups were considered to be hemolymph proteins that are regulated by poly(I:C)-injection.

A total of 110 and 77 protein spots were identified in the two separate batches of *C. gigas* hemolymph, respectively (Table 1). The number of distinct protein spots in the hemolymph of *C. gigas* is comparable to previous studies investigating the hemolymph proteome of Sydney rock oysters, *Saccostrea glomerata* [10,11]. The discrepancy in the number of protein spots between the two batches of *C. gigas* can be explained by the fact that oysters were collected at separate intervals and were in different stages of their reproductive cycle. We therefore chose to use the combined 2-DE dataset to identify proteins that were more or less abundant in poly(I:C) stimulated oysters relative to sterile seawater controls. The combined dataset consisted of 49 protein spots (Table 1), among which the relative intensities of four spots were significantly greater in the poly(I:C) treatment (≥2x fold increase). One of these protein spots was found only in poly(I:C)-injected oysters (Fig. 1). The four differential protein spots were excised from gels, digested with trypsin and subjected to mass spectroscopy on a Thermo Orbi-Trap XL mass spectrometer fitted with a nanospray source (Thermo Electron, San Jose, CA). The resulting spectra were searched against a fasta file of the translated oyster genome using Thermo Proteome Discoverer version 1.2 (Thermo Electron) and the SEQUEST algorithm [12]. Table 2 lists the proteins identified in the four differential protein spots. Spots p1 and p2 contained more than one distinct protein, whereas p3 and p4 matched single proteins. The poly(I:C)-induced proteins corresponded to a hypothetical protein (p1), small heat shock protein (p2) and a C1q domain containing protein (C1qDC) (p3 & p4). It is important to emphasize that protein identification was possible for spots that contained more than one protein (p1 and p2). It is likely that hypothetical and sHSP proteins in p1 and p2 (respectively) were the main proteins in these spots based on their high MS sequence coverage, molecular weight (MW) and isoelectric point (pI). Therefore, we considered the hypothetical and sHSP proteins were likely to account for the variation observed in protein spots p1 and p2, a conclusion that was supported by their differential expression identified by RT-qPCR (see below). Our results confirm the existence of the hypothetical protein (p1), which we have named poly(I:C)-inducible protein 1 (PIP1). BlastP analysis of the PIP1 against GenBank non-redundant database revealed a significant match (94% amino acid identity) to the C-terminus of *C. gigas* tripartite motif (TRIM)-containing protein 2 (GenBank EKC41504). The mRNA sequences for these two proteins also share exceptionally high homology (94% identity). TRIM proteins are implicated in multiple cellular functions, including cellular innate immunity towards viral infection [13]. The C1qDC protein was predicted to have a signal peptide and a tumor necrosis factor (TNF) superfamily domain (CDD: NCBI Conserved Domain Database, [14]). The crystalline structure of C1q and TNF domains are highly similar suggesting these innate immune proteins are evolutionary connected [15].

Proteomic analysis of up-regulated protein spots was confirmed by RT-qPCR analysis of *C. gigas* hemocytes treated with poly(I:C) or seawater (control). Hemocyte RNA was purified (TriSure, Bioline) and reverse transcribed (Tetro cDNA synthesis kit, Bioline). Real-time PCR was performed as previously described [3] using specific primers for C1qDC protein (5′-CATGATGACAGAGCTGTTG and 5′-GATTGCGCTGTGCCAACTC), small heat shock protein (5′-TGATCCTGATGGCTGTAAGAC and 5′-GTCCAGCCCATTCTTAGAC) and poly(I:C)-inducible protein 1 (5′-CACACACCCTCAAGATCAG and 5′-GAGATCCGCCTGATTGAC). Fig. 2 shows that mRNA levels of C1qDC, sHSP and PIP1 were all significantly elevated in *C. gigas* hemocytes

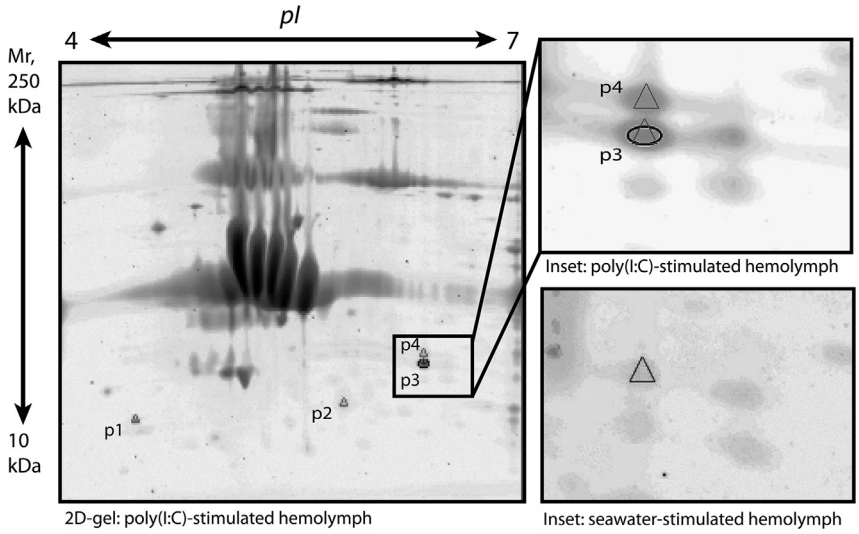

Fig. 1. Representative two-dimensional electrophoresis gel (2-DE) of hemolymph proteins from *Crassostrea gigas* injected with poly(I:C). Proteins were visualised by Sybro Ruby and gels imaged using Pharos FX Plus Molecular Imager (Bio-Rad). Protein spots that were more abundant in both batches of poly(I:C)-injected *C. gigas*, relative to seawater injected controls, were identified using PDQuest (Bio-Rad). Protein spots enclosed with an oblong indicate proteins identified only in poly(I:C)-injected oysters; triangles denote 3 protein spots that were more intense in poly(I:C)-injected oysters compared to controls. Protein spot numbers in the figure are labelled corresponding to Table 2. Inset provides a magnified view of the poly(I:C)-specific protein spot and its absence from the seawater-injected control.