The Effects of β-Cyclodextrin on the Immune System of the Pacific Oyster (*Crassostrea gigas*) using Gene expression

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**Abstract:**

Aquaculture could benefit greatly from an FDA approved immunostimulant. Cyclodextrin has been shown to boost the immune response in aquatic vertebrates, specifically the proinflamitory response, but what is not known is what effect if any it has on vertebrates such as Crassostrea Gigas, the Pacific Oyster. Using quantitative PCR we can start to gain an understanding of whether immune related genes are being up regulated when exposed to cyclodextrin.

**Introduction:**

There are few chemicals commercially available that can be used in the aquaculture industry due to FDA regulations on food fish (HUANG et al., 2005; Sakai, 1999). There are no immunostimulants approved for the use as preventative treatments for mitigating mortality due to stress(Aquatic Animal Drug Approval Partnership Program, 2011). Having a FDA approved drug commercially available for the use in food fish would be a huge step forward in the aquaculture industry and could potentially save a lot of money. One chemical, Cyclodextin, is a sugar based ring molecule in which 3 forms are used in many food products and has been indicated as safe for human consumption by the FDA (Szejtli, 1998). Unpublished research done by Dr. Goetz has shown cyclodextrin stimulates proinflamitory gene expression in trout and has protected Zebra fish against gram-negative bacteria LPS in a challenge. We don’t however know if this treatment would be effective in stimulating the immune response in invertebrates, although it is likely considering the Gram negative bacteria recognition system is more closely related to fish than it is to that of mammals (MacKenzie et al., 2010). One first step in determining if there is any response is the analysis of gene expression using QPCR.

Two genes related to the immune system status are Interleukin 17 (IL17) and prostaglandin E2 receptor (PGE2) (Roberts et al., 2009, 2008). IL17 is a cytokine and used in the immune response to a pathogen and PGE2 is used in the inflammatory response of the organism. Based on what was seen in aquatic vertebrates, if there is an immune response to the cyclodextrin it is highly likely we would see increases of expression of theses two genes.

Since we don’t know if there will be a pathogen like response in the immune system. Analyzing the expression of the gene Cytochrome P450, a response to xenobiotics, could give some insight to the mechanism behind the response of exposure to cyclodextrin, if not related to the innate immune response

**Methods:**

Treatment and sampling:

30 Pacific oysters (Crassostreas Gigas) were received from Taylor shellfish farm in Washington. 15 were kept as a control and held at 14 ° C. The experimental individuals were kept in identical conditions as the controls but were also exposed to Beta cyclodextrin at 250 μg/ml for 24 hours prior to sampling.

The Oysters were removed from the water and kept on ice while processing. They were shucked and small tissue samples were taken from the gill and placed into 1.5 ml tubes and immediately placed on dry ice and transferred to the -80° C freezer where they were stored until RNA extraction.

QPCR analysis:

The tissue was then thawed and .02-.03 g of tissue was placed into 1.5 ml tubes and the RNA was extracted using trireagent per the manufacturer’s protocol. The RNA extracted was then used to create CDNA by means of rtPCR. The RNA was incubated for 60 minutes at 42° C and then heated for 3 minutes at 70° C on the thermocycler.

The CDNA was then analyzed by QPCR in a Biorad light cycler with a mastermix listed in table 1 and a run schedule of 95°C for 10 minutes 1 time for initial denaturation and then 39 repetitions of 95°C for 15 seconds to 55°C for 15 seconds and 72°C for 30 seconds and one repetition of 95°C for 10 seconds with a melt curve from 65°C to 95°C at0,5°C for 5 seconds. For IL17 the forward and reverse primers were IL17 ISO D-F (5’ GGTGGTGTGTAGCGTGATTG 3’) and IL17 ISO D-R (3’ AACTCTTGGGGTCAGTGGTG 5’) respectively (Genbank Accession #EF190193). For the PGE2 the forward and reverse primers were PGE2-F (5’ ACCGAGAGTGCTGAGTGGTT 3’) and PGE2-R (3’ GGCAAACTGTAAGCCAGGAG 5’) (Genbank Accession #EW777722). For the Cytochrome P450 the forward and reverse primers used were Cyp1A1-F (5’ AATTTCAAGTGGCCCGTGTGGT 3’) and Cyp1A1-R (3’ ATGCCATGCGCAGAGTCTCTTT 5’) (Genbank Accession #EW778340) (Roberts et al., 2009, 2008).

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|  | Volume | Final Conc. |
| 2X Sensimix Sybr (Bioline) | 12.5μl | 1x |
| SYBR 25X dye | 1 μl | 1X |
| upstream primer, 10 uM | 1.25 μl | 2.5 uM |
| downstream primer, 10 uM | 1.25 μl | 2.5 uM |
| Ultra Pure Water | 7 μl | NA |
| CDNA | 2 μl |  |

Table 1 QPCR Mastermix

The QPCR data was then analyzed using PCR Miner and the data was normalized using the gene encoding elongation factor 1A (GenBank Accession #AB122066). The forward and reverse primers were EF1A-F (5’ AACAAGGAGCAACGATGGGT 3’) and EF1A-R (5’ GGTGATACCACGTTCACGCT 3’).

Processing Data:

The data was processed using Real-time PCR Miner (Zhao and Fernald, 2005). Using the equation R0= 1/(1+E) ^Ct, where E represents the gene efficiency calculated by the average of of all samples efficiencies for a plate and Ct is the cycle number where the threshold is surpassed (Zhao and Fernald, 2005). The R0 was then normalized by dividing the R0 value of each sample by the R0 of the normalizing gene for each corresponding sample. All values in this paper are the expression value means ± one standard deviation error.

**Results:**

QPCR analysis indicated a mean expression level for all individuals at .002 (± .002) and .006 (±.009) of IL17 gene in the control and cyclodextrin treated oysters respectively. The PGE2 indicated a mean expression level at .0007 (± .0009) and .003 (± .004) for control and treatment. There was not enough expression of the P450 to warrant any statistical analysis. A T-test was performed for determination of statistical significance. IL17 yielded a p value of .085 while PGE2 was .077 illustrating the statistical insignificance of the data collected.

Fig. 1 Expression values of all samples for control and Cyclodextrin treated groups for Il17 and PGE2

When looking at the results (Fig 1.) there is an obvious pattern based on sample individual which could be attributed to who extracted that sample. To reduce this error only samples I extracted were analyzed. For control samples 1-5 were used and for the cyclodextrin treatment samples 23-27 were used.

For the samples only I extracted, the QPCR analysis indicated a mean expression level for the individuals at .002 (± .002) and .012 (±.01) of IL17 gene in the control and cyclodextrin treated oysters respectively. The PGE2 indicated a mean expression level at .0005 (± .0007) and .005 (± .004) for control and treatment (Fig. 2). There was not enough expression of the P450 to warrant any statistical analysis with this reduced sample number either. A T-test was performed for determination of statistical significance. IL17 yielded a p value of .024 while PGE2 was .028 indicating statistical significance of the subsampled data.

Fig. 2 Means and standard deviations of the subsample data for IL17 and PGE2

**Discussion:**

In Dr. Goetz’s study gene expression was analyzed as well as a bacterial challenge was performed. The challenge against the bacterial pathogen LPS showed decreased mortality from 80-100% down to almost zero at any dose above 250 μg/ml. Using QPCR Goetz was able to determine that β-Cyclodextrin stimulated a number of genes associated with immune response. Immune related genes that were stimulated were Interleukin 1, Interleukin 6 and the toll-like receptor. Based on this information it is possible that there is a similar response in invertebrates like C. Gigas.

There is a trend in the data that suggests an increase in gene expression of IL17 and PGE2 though the data is not statistically significant when looked at as a whole. When subsampled to reflect similar extraction techniques across the set, the data shows a significant increase in expression of IL17 and PGE2.

Some underlying sources of variance include a low number of samples, not quantifying and standardizing the amount of RNA used to create CDNA, not running the normalizing gene at the same time as the samples on the same plate, not running samples in duplicate, and not using DNAse on the samples to remove DNA carryover. If a study is done with an increased number of samples and with the aforementioned issues taken into account it is likely that there will be an increase in expression of the innate immune markers of C. Gigas.

There was no expression in either the controls or the treatment group of the gene Cytochrome p450. This coupled with the increase in expression of the innate immune genes suggests that the exposure of C. Gigas to cyclodextrin does not initiate a xenobiotic response.

Over all this data suggests further research into the effects of cyclodexrin on invertebrates, specifically the pacific oyster, could show strong immune boosting properties and could be a potential viable option for use as an immunostimulant in the aquaculture industry.

IN GENERAL

There needs to be more expounding on increased expression of two genes and why they are regulated with comparison to similar studies ie oysters – from there should be able to infer meaning

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