Fish & Shellfish Immunology 28 (2010) 649–653

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

# Expression of immune-related genes in one phase of embryonic development of freshwater crayfish, *Pacifastacus leniusculus*

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ARTICLE INFO

*Article history:*
Received 13 October 2009
Received in revised form
14 December 2009
Accepted 21 December 2009
Available online 7 January 2010

*Keywords:*
Crayfish embryo
*Pacifastacus leniusculus*
Egg
Immune-related genes

ABSTRACT

Crayfish do not have larval stage as other crustacean such as penaeid shrimp they spawn their eggs until hatching and what hatches out from the eggs are miniature crayfish known as juveniles. In order to address the question whether immune genes are initially expressed during the embryo development in the egg stage, the expression of some immune-related genes: *prophenoloxidase* (proPO), *peroxinectin*, *hemocyanin*, *anti-lipopolysaccharide factor* (ALF), *plcrustin*, *astakine*-1, 2 and *transglutaminase* (TGase) were determined in the middle phase of crayfish embryo development. Furthermore, immune challenge was used to determine the immune response of eggs by immersing them in a solution of the highly pathogenic bacterium *Aeromonas hydrophila*. Semi-quantitative RT-PCR analysis showed that all tested genes are present except *proPO* in this phase of crayfish embryo development and none of the genes tested changed their expression following immersion in *A. hydrophila*. The proPO transcript has been reported from hemocytes in crustaceans and it plays crucial roles in crustacean immune response. This may indicate that the development of immune-competent hemocytes in this stage of crayfish embryo is not completed and the egg shell as such plays an important role as a shield in protecting the embryo from bacteria and maybe also other pathogens.



## 1. Introduction

*The freshwater crayfish Pacifastacus leniusculus*, is endemic to western North America between the Pacific Ocean and the Rocky Mountains, and introductions of *P. leniusculus* outside of North America to Europe began in the late 1960s, primarily to replace the plague-decimated *Astacus astacus* populations of Europe with a more plague tolerant species. It should be remembered that also *P. leniusculus* can die of crayfish plague when the immune system is suppressed [1].

Crayfish do not have larva, they spawn their eggs until hatching and what hatches out from the eggs are miniature crayfish known as juveniles that for the first two or three stages remain in a close connection to the mother [2]. This is totally different from the larval development of penaeid shrimp which have six nauplius, three protozoea, and three mysis larval stages, followed by the development of post-larvae to adult [3,4]. The duration of each phase of egg development in *P. leniusculus* was studied at the ultrastructural level by Celada et al. [5]. The immune defense system in adult crayfish has been studied intensively [6,7]. The proPO cascade is an efficient nonself-recognition system in invertebrates, which includes several proteins involved in the immune defense resulting in melanin production, phagocytosis, encapsulation, and cell adhesion. Several proteins that are involved in the proPO cascade have been described in detail [6,7]. Some are actors in the cascade, such as *Lipopolysaccharide and Glucan Binding Protein* (LGBP), *prophenoloxidase activating enzyme* (ppA), *serine proteinase homologue*1, 2 (SPH1, 2), whereas *pacifastin* and *melanization inhibitory protein* (MIP) function as regulators of the cascade to prevent unwanted activation of proPO and melanization [6,7]. It is well known that hemocytes in invertebrates play a crucial role in cellular defense mechanisms and that they are also responsible for releasing humoral defense molecules. Hematopoiesis is the progress where hemocytes form and mature and subsequently enter the blood circulation [8]. *Transglutaminases* (TGase) and *astakine* were recently reported to be involved in hematopoietic cell proliferation and/or differentiation [9,10]. TGase is involved in the coagulation system of the freshwater crayfish and shrimp [11,12], and can also prevent hematopoietic

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doi:10.1016/j.fsi.2009.12.021

stem cells from starting to migrate into the hemolymph [9]. *Astakine*, an endogenous cytokine like-factor of crayfish has been shown to play a critical role in the differentiation and growth of hematopoietic stem cells *in vitro* and *in vivo* [10]. *Hemocyanin*, is produced by the hepatopancreas and secreted into the plasma and its main role is as a respiratory protein, but this protein may also play critical roles in immune defense against microbial invasions in crayfish [13] and also in shrimp [14], since antimicrobial peptides can be produced from the large protein by a proteolytic cleavage. Crayfish as well as shrimp also produce a large array of different antimicrobial peptides (AMPs) in different organs [15].

The relationship between larva and their immune response has been reported in shrimp [3,16–19], however, no study has reported about the immune-related gene expression in embryo of any species of crayfish, crab or lobster. This may be because crayfish for example only produce eggs once per year, which makes studies on embryo development difficult and troublesome. It is interesting to compare these two kinds of larva/embryo between crayfish and shrimp, since larva of crayfish is similar to Nauplius stage 4 of shrimp. In this paper, we investigated the expression of some immune-related genes in the middle phase of the embryonic development of crayfish, and also studied the immune response of eggs to the crayfish pathogenic bacterium *Aeromonas hydrophila* [20].

## 2. Materials and methods

### 2.1. Animals

Egg-bearing female freshwater crayfish, *P. leniusculus*, were purchased from Torsäng, lake Vättern, Sweden, and were kept in aquaria in aerated tap water at 10 °C. The phase of embryonic development was determined according to Celada et al. [5].

### 2.2. Immersion

Female crayfish were held in 500 ml aerated saline (0.9% NaCl water) in each aquarium (10 cm × 10cm × 15 cm) for 24 h to acclimate to the experimental conditions, followed by immersion in saline with *A. hydrophila* (final concentration = $1 \times 10^8$ cfu/ml), 2 eggs were collected gently at 0, 6, 12, 24 h for RT-PCR detection. Each time point originated from at least two aquaria.

### 2.3. RNA isolation

Eggs from females were detached by sliding a blunt forceps smoothly from the base to the tip of the pleopods, and then they were homogenized in 1 ml of TRIzol Reagent (Invitrogen, USA) and stored at room temperature for 5 min. The mixture was then subjected to chloroform extraction, isopropanol precipitation and ethanol washing according to the manufacture's instructions. The precipitated RNA pellet was dissolved in RNase-free water, followed by RNase-free DNase I (Ambion, Austin, TX, USA) treatment.

### 2.4. RT-PCR

Equal amounts (2.0 μg) of total RNA was used for cDNA synthesis with ThermoScript™ (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions. PCR using 1 μl of cDNA was subsequently performed with specific primer sets as well as the thermal cycle number for the different genes (listed in Table 1). Crayfish 40 s

**Table 1**
Primers for RT-PCR used in this study.

| Gene | GenBank accession no. | Refs. | Primer sequence (5′–3′) | Size (bp) of PCR product |
|---|---|---|---|---|
| proPO | X83494 | [36] | F:CCAGAAGTTGCTGAGGAGAGACA<br>R:GCACTCTCCTTCACCATTCTTCG | 411 |
| ppA | AJ007668 | [37] | F: GGAAGCCAGCCGACCCACGCGA<br>R: GGTTTCCATTTGTCAGCCATT | 734 |
| Pacifastin | U81825 | [38] | F: GCACCAAGAGGCTTTGTCG<br>R:TTGGAGCCATCAGTACACACAGC | 512 |
| MIP | EU308499 | [39] | F: CCACTCACCTCAGCCGACAC<br>R: TCCTCCATTACGACTCTCAGCT | 502 |
| LGBP | AJ250128 | [40] | F: GCAGCAACTTCTGGGACTTTG<br>R: CGACACTCTCCATCTTCCACAC | 317 |
| Peroxinectin | X91409 | [41] | F:ATCTTCTCTGGTGCAACCGTCTCC<br>R:ACAGCAACCATACTCCTGACGAAGT | 799 |
| Hemocyanin | AF522504 | [13] | F: CCATTCTCCCTCAACATTC<br>R: TTAGTGATGGAAAATCTTGAC | 572 |
| Anti-LPS factor | EF523760 | [30] | F:TCCGGAATCTCCTGACAACC<br>R:TGCGAAGATCTCGGAACTAGGA | 450 |
| PlCrustin-1 | EF523612 | [15] | F:GGTAACCATGGCTCGATCAC<br>R:TGTAATGGTGAGACCGCTCC | 367 |
| PlCrustin-2 | EF523613 | [15] | F:CTGCAACTACTACTGCAAGAAGCCTGAAGGTC<br>R: GCATAACAAGCAAGTCAGCCA | 369 |
| PlCrustin-3 | EF523614 | [15] | F: AGCGCCCAGAACACTAACAC<br>R: GGCAGGTTTGCAGACGTAGT | 406 |
| Astakine-1 | AY787656 | [13] | F:ATGAAGATGCGAGGAGTTAGTGTG<br>R:CTAGTAGTAGGAGTCGAGCGTGTTGTC | 314 |
| Astakine-2 | EF568370 | Unpublished data | F: ATGCTGGAGCGCAGTGGAGTGATGGTG<br>R: AGTGAATGATGCCCAGAGTGTTGTC | 366 |
| TGase | AF336805 | [11] | F: TGGGYCTTCGGGCAGTT<br>R: CGAAGGGCACGTCGTAC | 642 |
| SPH1 | EU552456 | Unpublished data | F: TGTGGAACGTGTTGTGGAAG<br>R:TTGGGTGCCAACTCAAATGGTT | 629 |
| SPH2 | EU552457 | [42] | F: ATCATTTGCCTGCGCTGGAG<br>R: GATGGACCTGGACCTTTGTGTC | 600 |
| MBL | AY861653 | [42] | F: AAGTTCTATCCGGCGTACATCAG<br>R: CTCTGGACTTTGCTATACTCGCA | 472 |
| VMO-I | AY862390 | [42] | F: CATGAGGCTGCGAGTTGAGGGGA<br>R: CATTGTATGTGGGTGCCGTCTTT | 478 |