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Methods

The expression of prophenoloxidase mRNA in red swamp crayfish, *Procambarus clarkii,* when it was challenged

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ABSTRACT

The expression of the prophenoloxidase (*proPO*) gene was investigated in nine tissues of red swamp crayfish *Procambarus clarkii*, by real-time PCR after challenges by CpG oligodeoxynucleotide (ODN), *Aeromonas hydrophila* and white spot syndrome virus (WSSV). The results can be summarized as follows: (i) the expression level of the *proPO* gene in haemocytes was highest among nine studied tissues before the challenge; (ii) the expression of *proPO* increased in all studied tissues after stimulation by CpG ODN and WSSV, and also increased in all tissues, except the ovary, after the *A. hydrophila* challenge; (iii) the whole expression profiles were different, suggesting that different immune mechanisms may exist for crayfish that are resistant to WSSV and *A. hydrophila,* although the expression in haemocytes was similar before and after the WSSV and *A. hydrophila* challenges.

1. Introduction

Highly evolved organisms have developed unique immune systems and mechanisms to control and eliminate pathogenic invaders into their systems. Arthropods lack the highly sophisticated adaptive immune system of vertebrates. Instead, they have to rely solely on their innate immune system to fight against invading microorganisms [1]. Crustaceans have an innate immune system, including both cellular and humoral factors. Humoral factors comprise plasma proteins and peptides, including recognition proteins, clotting proteins, antimicrobial peptides, and the prophenoloxidase (proPO) system [2,3]. There are many genes within the crustacean innate immune system that are possibly involved in interactions between hosts and pathogens [4]. The various expression patterns of these genes indicate differential reactions of hosts toward different types of pathogens and immunostimulators [5].

The red swamp crayfish, *Procambarus clarkii,* is indigenous to south-central USA and Northeastern Mexico [6]. It was introduced to Nanjing, China from Japan in 1929 [7]. This crayfish is one of the important economic species in China and has been artificially propagated in recent years. Generally, it has great resistance to diseases in natural environments. Nevertheless, the present sustainability and healthy development of the crayfish aquaculture are at risk due to significant problems with pathogens. This has the potential to result in significant economic losses in many crayfish-producing farms in China. Under these circumstances, an investigation into the mechanisms of immune defense mechanisms against diseases of *P. clarkii* might be beneficial to the management of crayfish farming. The *proPO* system, as one of important humoral immune factors in the innate immune system, may therefore provide some insights into *proPO* gene expression interactions between the host and different pathogens. It should be noted that immunostimulators are regarded as the basis for understanding the response of crayfish towards various pathogens. Investigating the similarities and differences between the proPO activating system of *P. clarkii* and that of other crustaceans could serve to advance the understanding of factors that activate this system [8]. In our previous research, we successfully cloned the *proPO* gene from *P. clarkii* (GenBank ID: EF595973) [9] and also studied the expression profile of *proPO* in *P. clarkii*.

Results from a number of research projects have shown that CpG oligodeoxynucleotide (ODN) can be used as vaccine adjuvants, antiallergens and medicines for the treatment of infectious diseases and cancer [10–12]. Investigating the expression profile of *proPO* in crayfish challenged by CpG ODN will therefore also be useful for the general development of control strategies for preventing infectious diseases.

Abbreviations: proPO, prophenoloxidase; ODN, oligodeoxynucleotide; WSSV, white spot syndrome virus; hpi, hours post-infection.

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To enhance understanding of the expression profile of *proPO* in freshwater crayfish, we investigated transcripts-level changes in *proPO* in *P. clarkii* after challenges by *Aeromonas hydrophila*, white spot syndrome virus (WSSV) and ODN. We also investigated the expression of *proPO* prior to such challenges.

2. Results

2.1. Expression of proPO before challenge

The mRNA transcripts of *proPO* at various expression levels were widely detected in all of the tissues that we examined (Fig. 1). The highest level of mRNA was observed in haemocytes, followed by those in the hepatopancreas, ovary, muscles, spermary, cuticular epidermis, branchia, intestines, and stomach sequentially.

2.2. Expression of proPO in tissues after CpG ODN stimulation

The expression of *proPO* in *P. clarkii* tissues after CpG ODN stimulation is shown in Fig. 2. The increase of *proPO* gene expression was first observed at 3 h after injection and the highest expression was reached at about 12–24 hpi in the intestine, muscle, stomach, cuticular epidermis and haemocytes. The highest gene expression of *proPO* in branchia and spermary were, however, measured at about 24–48 hpi. In the hepatopancreas, the highest level of *proPO* gene expression was at 6 hpi whereas those in the ovary were at 6 hpi and 24 hpi.

Compared to the control, the expression of *proPO* increased in all of the nine studied tissues. In the intestine, muscle and stomach, the expression of *proPO* increased significantly ($P<0.05$) from 6 hpi to 24 hpi, and in the haemocytes, cuticular epidermis, branchia and spermary, the expression of *proPO* increased significantly ($P<0.05$) from 12 hpi to 48 hpi. In the hepatopancreas, the *proPO* expression level was up-regulated to its peak at 6 hpi and then decreased slowly to normal levels. In the ovary, there significant differences between the control groups and the experimental groups were noted at 6 hpi and 24 hpi ($P<0.01$) .

2.3. Expression of proPO in tissues after A. hydrophila injection

Expression profiles of *proPO* after *A. hydrophila* challenge in the nine tissues studied are shown in Fig. 3. After an injection of *A. hydrophila* at 3 h, the mRNA levels of *proPO* increased in the intestine and stomach, reaching the highest level at 12 hpi. In the hepatopancreas, the *proPO* level sharply decreased at 6 hpi ($P<0.05$) and up-regulated to its highest level at 24 hpi. In muscle, cuticular epidermis and haemocyte tissues, the *proPO* levels slowly increased to the highest level at 24 hpi and then slowly declined to normal levels at 96 hpi. In the ovary, the expression of *proPO* slowly decreased to the lowest level at 24 hpi and up-regulated slowly to normal levels at 96 hpi.

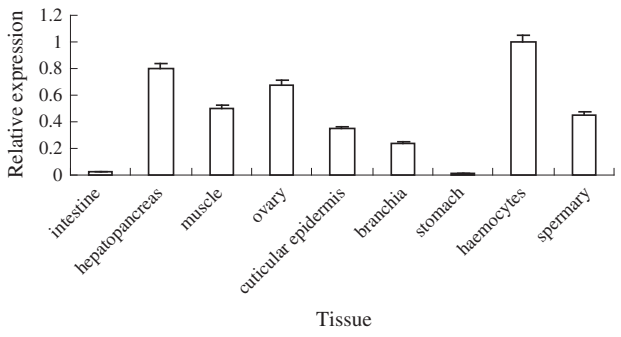

Fig. 1. The *proPO* mRNA expression level in the nine studied tissues of *P. clarkii*. The relative expression in haemocytes, hepatopancreas, ovary and spermary were higher, that in muscle, epidermis and branchia were lower, and that in intestine and stomach were almost none.

In the branchia, the expression of *proPO* sharply increased to the highest level at 12 hpi and remained at the higher level until 96 hpi. In the spermary, the expression of *proPO* slowly increased to the highest level at 48 hpi and remained at this level until the end of the experiment (96 hpi).

In the intestine, cuticular epidermis, branchia and spermary, the expression of *proPO* increased significantly ($P<0.05$) from 12 hpi to 96 hpi in the experimental group. The expression of *proPO* increased significantly ($P<0.05$) at different levels in experimental groups of different tissues: from 6 hpi to 96 hpi in the haematocytes; and from 6 hpi to 48 hpi in the stomach, and muscles. In the hepatopancreas, *proPO* expression in the infected group decreased sharply at 6 hpi ($P<0.05$) and showed a high level of up-regulation (from 12 hpi to 48 hpi) compared to that of the control group. In the ovary, the expression of *proPO* decreased significantly ($P<0.05$), from 24 hpi to 48 hpi, in the experimental group.

2.4. Expression of proPO in tissues after WSSV injection

After the challenge by WSSV, the expression of *proPO* increased in all of the nine studied tissues. In the branchia, the expression of *proPO* increased significantly ($P<0.05$) from 12 hpi to 96 hpi in the experimental group. In the hepatopancreas, cuticular epidermis and spermary, the expression of *proPO* increased significantly ($P<0.05$) from 12 hpi to 48 hpi in the experimental group. In the ovary, the expression of *proPO* increased slowly from 3 hpi to 96 hpi and sharply increased at 24 hpi ($P<0.01$) in the experimental group. In the intestine, stomach and muscle tissue, the expression of *proPO* increased from 3 hpi to 96 hpi in the experimental group, but this was not significant ($P>0.05$). Very little change was noted in the control group. In the haemocytes, the *proPO* expression in the infected group decreased at 3 hpi but showed much up-regulation, from 6 hpi to 96 hpi, compared to that of the control group (Fig. 4).

In the intestine, muscle and spermary, the *proPO* levels slowly increased to the highest level at 24 hpi and dropped slowly to normal levels at 96 hpi. In the hepatopancreas, ovary, cuticular epidermis and haemocytes, *proPO* levels increased rapidly at 6 h after the WSSV challenge and then followed an upward trend to the highest levels at 24 h. In the stomach, the *proPO* levels increased slightly until 24 h, followed by a downward trend, while in the branchia these levels up-regulated significantly at 12 hpi followed by a down-regulation trend.

3. Discussion

Results indicated that the expression of *proPO* in haemocytes was the highest among the nine studied tissues, which was consistent with previous reports [13–16]. The distributions of *proPO* have been studied in some other species [15–19]. In the black tiger shrimp, *P. monodon,* and the giant freshwater prawn, *M. rosenbergii*, *proPO* mRNA is synthesized in the hemocytes and not in the hepatopancreas [14,16]. The *proPO* of mud crab *S. serrata* is strongly expressed in haemocytes, but not in the tissues of the heart, eyestalk, gill, muscle, ovary, hepatopancreas, stomach and intestine [19]. In the Pacific white shrimp *L. vannamei*, *proPO* expression has been widely detected in haemocytes, gills, heart, the lymphoid organ, stomach, midgut, anterior midgut caecum and in the ganglia but lower expression levels have also been measured in the hepatopancreas, muscle and cuticular epidermis of this organism [17]. In the Chinese mitten crab *E. sinensis* the mRNA transcripts of *proPO*-specific activities were detected in all the examined tissues, with the highest level being in the hepatopancreas [18].

CpG ODNs are known to enhance the activity of B cells, which have the capacity to secrete cytokines and protect IgG [20]. Many studies have indicated that CpG ODN can be used as vaccine adjuvants, anti-allergens and medicines for the treatment of infectious diseases and cancer [10–12]. In our experiment, we found that the *proPO* gene