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RESEARCH ARTICLE

Prophenoloxidase Response to White Spot Syndrome Virus Infection in the Red Swamp Crayfish (*Procambarus clarkii*)

Yanhe Li[1,2,*], Lei She[1], Minglin Wu[1], Yongjie Wang[1], Suofei Ji[1], Keli Yang[3] and Weimin Wang[2]

[1]Institute of Fisheries, Anhui Academy of Agricultural Sciences, Hefei, Anhui 230031, PR China; [2]College of Fisheries, Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China; [3]Hubei Key Laboratory of Animal Embryo and Molecular Breeding, Institute of Animal Husbandry and Veterinary, Hubei Academy of Agricultural Sciences, Wuhan, Hubei 430064, PR China
*Corresponding author: yanhel6@126.com

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ABSTRACT

Invertebrates rely on innate immunity to respond to the entry of foreign microorganisms. The prophenoloxidase (*proPO*) system plays crucial roles in crustacean innate immunity. White spot syndrome virus (WSSV) infection has recently been responsible for significant economic losses in many crayfish-producing farms in China. We therefore aimed to examine the response of the proPO system to WSSV infection. The virulence of the virus, expression of the *proPO* gene, and phenoloxidase (PO) activity in six tissues from the red swamp crayfish *Procambarus clarkii* were investigated using LD_{50} tests, real-time polymerase chain reaction and spectrophotometry, after infection with different numbers of WSSV virions. The LD_{50} of the WSSV strain was 2.08×10^7 virions/crayfish. *proPO* mRNA expression was increased in all studied tissues after infection with WSSV, except in the cuticle epidermis after infection with 2×10^7 virions. *proPO* mRNA transcription was significantly increased in hemocytes and hepatopancreas, suggesting that *P. clarkii* might depend mainly on innate immunity for defense against viral pathogens. PO activity differed among different tissues during WSSV infection, suggesting that the proPO system might be activated by different mechanisms in different tissues in response to different viral stresses.

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INTRODUCTION

White spot syndrome virus (WSSV) belongs to the genus *Whispovirus* in family *Nimavirida* (Mayo, 2002). WSSV causes a serious disease in the shrimp aquaculture industry worldwide and is responsible for estimated annual global economic losses close to three billion dollars. Although the virus mainly causes high mortality in shrimps (Oidtmann and Stentiford, 2011), it has been reported to infect a wide range of other crustaceans, including crabs, lobsters, and crayfish (Liu *et al.,* 2011). Despite considerable progress in WSSV characterization, there are currently no effective preventive or therapeutic measures against WSSV infection (Wang *et al.,* 2013) and the understanding of the shrimp defense system in response to viral infection remains poor (Wang and Zhang, 2008).

The red swamp crayfish, *Procambarus clarkii,* was introduced from Japan into Nanjing, China, in 1929 (Yue *et al.,* 2010), since when it has become one of the most important economic species in China. The red swamp crayfish has recently been artificially propagated (Li *et al.,* 2012a; Shui *et al.,* 2012; Guan *et al.,* 2013), but the sustainability and healthy development of the crayfish aquaculture industry in China are threatened by disease, particularly WSSV infection, which has the potential to cause significant economic losses in many crayfish-producing farms (Li *et al.,* 2012b). The rapid spread of WSSV makes it difficult to control, and drug treatments are currently unable to eliminate the infection. Selective breeding of WSSV-resistant crayfish may thus represent an effective preventive measure for white spot disease outbreaks.

The prophenoloxidase (proPO) system is an important humoral immune factor in the innate immune

system. Crustacean innate immunity comprises humoral immunity represented by melanization via the proPO-activating system, and cellular immunity represented by phagocytosis. Activation of the proPO cascade triggers proPO synthesis in hemocytes and its storage in granules (Jearaphunt *et al.,* 2014). PO can catalyze the oxidation of phenolic substances to quinones, which are then further polymerized non-enzymatically to melanin (Cerenius *et al.,* 2008). The activated innate immune system also employs phagocytosis to eliminate microbes or foreign particles. Investigation of the proPO-activating system in *P. clarkii* could improve our understanding of this system, and may thus be useful for developing strategies to prevent infectious diseases (Li *et al.,* 2012b). In the previous study, *proPO* expression was increased in some tissues after stimulation by WSSV (Li *et al.,* 2012b). Furthermore, not all WSSV-infected crayfish died, indicating the existence of WSSV-resistant crayfish. However, the effects of WSSV infection on *proPO* expression and PO activity in *P. clarkii* remain unclear. In the present study, the virulence of the virus, and the time- and organ-dependence of *proPO* mRNA transcription and PO activity in six tissues from *P. clarkii* infected with WSSV were investigated to understand *proPO* expression and PO activity in freshwater crayfish. The results might help in the management of crayfish farming and contribute to the selective breeding of WSSV-resistant crayfish in the future.

MATERIALS AND METHODS

Experimental *P. clarkii* preparation: *P. clarkii*, 35-40 g in body weight, were kept in aquaria with a circulating biofilter system, and fed twice daily with commercial feed. Each crayfish was confirmed to be WSSV-free as reported (Park *et al.,* 2013).

Preparation of WSSV solution: Hemocytes of WSSV-infected crayfish were drawn and centrifuged at 3,000 *g* for 20 min at 4ºC. The supernatant was re-centrifuged and filtered through a 0.22-μm filter. WSSV in hemocytes was confirmed as above. WSSV-free hemocytes were prepared as a negative control.

Determination of LD_{50}: Dilutions of the WSSV strain were prepared. The desired numbers of virions were contained in 100 μL of PBS (pH 7.2). Each crayfish was infected with the WSSV strain from 6.25×10^6 to 1.0×10^8 virions/crayfish by intramuscular injection. The control was injected with 100 μL PBS. The LD_{50} was calculated using the method of Reed and Muench (1938).

WSSV infection: The viral inoculum was prepared as described (Wang *et al.,* 2009). The control group was injected with 100 μL PBS. The three experimental groups were injected with 100 μL PBS containing the WSSV strain at 1×10^7, 2×10^7, and 3×10^7 virions per crayfish, respectively.

Sample collection: Hemocytes were collected using anticoagulant-modified Alservier's solution (pH 7.0, 1:1), and isolated by centrifugation at 800 *g* at 4 ºC for 15 min. Total RNA was extracted. The tissues were dissected out, and preserved in RNA protection liquid until RNA extraction. Five crayfish from each group were collected for real-time PCR at 0, 3, 6, 12, 24, 48, 72 and 96 hpi.

Total RNA extraction and first-strand cDNA synthesis: Total RNA was extracted and first-strand cDNA was synthesized according to the protocols used by Li *et al.* (2012b).

Tissue tropism of *proPO*: For each tissue, three pooled samples were collected, with each pooled sample derived from five crayfish. β-actin was used as a control, and RT-PCR was performed using primers designed by Li *et al.* (2011) (Table 1).

Quantification of *proPO* mRNA expression: Real-time PCR assays were carried out following the protocols used by Li et al. (2012b). The primers were showed in Table 1. The *proPO* expression level was calculated by $2^{-\Delta CT}$ (Livak and Schmittgen, 2001). All data were given in terms of relative mRNA expressed as mean±SD.

PO activity assay: PO activity was measured using L-DOPA (L-3, 4-dihydroxyphenylalanine, Sigma) as the substrate and trypsin (Sigma) as the activator. L-DOPA buffer only was used as a control. One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1} mg^{-1} protein (Ji *et al.,* 2011). Tissues were homogenized in PBS (pH 7.2, 4ºC) and diluted to 0.1 g/ml. PO activity in tissues was measured as above.

Statistical analyses: For statistical analyses, mean±SD were calculated using Microsoft Excel 2003 and one-way analyses of variance (ANOVA) and cross-table analyses were performed using SPSS 13.0 software (SPSS). The data were analyzed by *t*-tests and $P<0.05$ was considered statistically significant.

RESULTS

LD_{50} of the virus: Virus virulence was assessed in five groups of 10 crayfish each. The LD_{50} of the WSSV strain was 2.08×10^7 virions/crayfish (Table 2).

Tissue tropism of *proPO*: The expression levels of *proPO* mRNA in different tissues collected from normal crayfish were investigated by RT-PCR analysis. *proPO* transcripts were expressed in all the collected tissues (Fig. 1), with higher levels in hemocytes and hepatopancreas, and lower levels in the branchia and heart.

Expression of *proPO* in tissues after infection with WSSV at 1×10^7 virions/crayfish: The expression of *proPO* mRNA in *P. clarkii* tissues after infection with WSSV at 1×10^7 copies/crayfish is shown in Table 3. *proPO* expression increased in all six studied tissues. Expression of *proPO* in the branchia increased significantly from 6 to 48 h post-infection (hpi) in the experimental group ($P<0.05$), while *proPO* expression in the cuticle epidermis increased slowly from 3 to 24 hpi ($P<0.05$) and then slowly decreased from 24 to 96 hpi in the experimental group. Expression in hemocytes increased significantly ($P<0.05$) from 6 to 96 hpi in the

Table 1: The primer sequences for analysis of *proPO* expression in the six tissues of *Procambarus clarkii*

Primer	Primer sequence (5'-3')	Position
proPO RT PCR-F	GCCAGGATAATACCCTACTC	801 820
proPO RT PCR-R	TGTCATGGCAGAATGCCAGC	1,094 1,075
proPO QPCR-F	GCACAAGTTTGTGGACGACGTC	1,174 1,195
proPO QPCR-R	GTCCATCTCAGCCCAGAGGAG	1,549 1,529
β-actin RT PCR-F	GAYGAYATGGAGAAGATCTGG	
β-actin RT PCR-R	CCRGGGTACATGGTGGTRCC	
β-actin QPCR-F	AGTAGCCGCCCTGGTTGTAGAC	
β-actin QPCR-R	TTCTCCATGTCGTCCCAGT	

Table 2: The duration of the LD_{50} study of WSSV in *Procambarus clarkii*

Group	Challenge dose (virions)	Dead %	Value of LD_{50} (virions)
1	1.0×10^8	100	
2	5.0×10^7	70	
3	2.5×10^7	60	2.08×10^7
4	1.25×10^7	40	
5	6.25×10^6	0	
control	0	0	

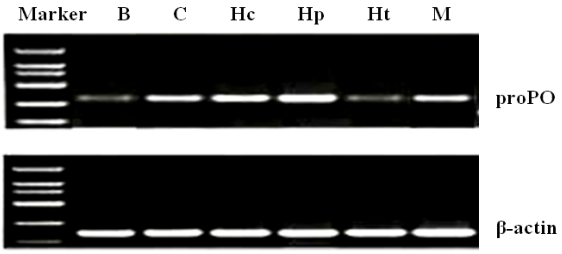

Fig. 1: Tissue tropism analysis of *Procambarus clarkii* prophenoloxidase (*proPO*) by a RT-PCR approach. Marker, DL2000; B, branchia; C, cuticle epidermis; Hc, hemocytes; Hp, hepatopancreas; Ht, heart; M, muscles. β-actin was used as an internal control to indicate and standardize the amount of the cDNA template in RT-PCR.

experimental group, while expression of *proPO* in the heart increased slowly from 3 hpi, peaked at 12 hpi ($P<0.05$), and then slowly decreased from 24 to 96 hpi in the experimental group. *proPO* expression in the hepatopancreas increased from 3 to 96 hpi in the infected group, and showed significant up-regulation at 48 hpi ($P<0.05$) compared with the control group. The expression of *proPO* in muscle tissue increased from 6 to 96 hpi in the experimental group, and then decreased from a maximum at 48 hpi to 96 hpi in the experimental group.

Expression of *proPO* in tissues after infection with WSSV at 2×10^7 virions/crayfish: The expression profiles of *proPO* after infection with 2×10^7 copies WSSV are shown in Table 3. The mRNA levels of *proPO* were increased in the branchia and heart at 3 hpi, and reached a maximum at 12 hpi, while levels in the hepatopancreas were decreased at 24 hpi ($P<0.05$) and up-regulated to a maximum at 48 hpi. The *proPO* levels in muscle and branchia tissues increased sharply to a peak at 12 hpi, and then declined slowly to normal levels at 96 hpi. In the cuticle epidermis, *proPO* expression slowly decreased to its lowest level at 12 hpi and was then up-regulated sharply to a maximum at 24 hpi. The expression levels in the hemocytes increased slowly up to 24 hpi and remained at a high level until 96 hpi.

Expression of *proPO* in tissues after infection with WSSV at 3×10^7 virions/crayfish: Expression levels of *proPO* increased in all six studied tissues after infection with 3×10^7 copies of WSSV. In the branchia, cuticle epidermis and hepatopancreas, *proPO* expression increased significantly ($P<0.05$) from 12 to 72 hpi in the experimental group, while levels increased significantly ($P<0.05$) from 6 to 48 hpi in the hemocytes and heart in the experimental group. The expression of *proPO* in the muscle increased slowly from 6 to 72 hpi in muscle, with a significant increase ($P<0.05$) from 12 to 48 hpi in the experimental group (Table 3).

Table 3: The mRNA level expression of *proPO* after WSSV infection in the six tissues of *Procambarus clarkii*

Tissue	Dose (virions)	Relative expression (hpi)							
		0	3	6	12	24	48	72	96
Branchia	1×10^7	0.20±0.014	0.32±0.016	0.35±0.011^a	0.46±0.020^b	0.48±0.015^b	0.36±0.012^a	0.28±0.014	0.23±0.012
	2×10^7	0.20±0.015	0.28±0.013	0.31±0.018	0.44±0.019^b	0.36±0.014^b	0.32±0.015^a	0.30±0.016^a	0.23±0.014
	3×10^7	0.22±0.014	0.32±0.012	0.39±0.016	0.67±0.021^b	0.56±0.014^b	0.49±0.014^b	0.42±0.014^a	0.32±0.015
	Control	0.20±0.016	0.24±0.014	0.26±0.021	0.25±0.016	0.20±0.011	0.21±0.010	0.21±0.013	0.20±0.015
Cuticle epidermis	1×10^7	0.34±0.018	0.42±0.022	0.46±0.013	0.5±0.018	0.60±0.016^b	0.53±0.014^a	0.42±0.017	0.36±0.018
	2×10^7	0.34±0.020	0.29±0.021	0.3±0.017	0.32±0.032	0.42±0.022^a	0.31±0.018	0.28±0.022	0.23±0.025
	3×10^7	0.33±0.017	0.39±0.024	0.45±0.016	0.52±0.019^a	0.59±0.015^b	0.50±0.012^a	0.48±0.024^a	0.36±0.022
	Control	0.36±0.015	0.38±0.022	0.40±0.014	0.34±0.024	0.33±0.014	0.34±0.016	0.35±0.018	0.33±0.024
Haemocytes	1×10^7	1.01±0.015	1.21±0.016	1.42±0.022	1.82±0.028^b	2.32±0.021^b	1.81±0.015^b	1.61±0.032^a	1.41±0.025^a
	2×10^7	1.12±0.022	1.22±0.018	1.33±0.019	1.41±0.025^a	1.61±0.016^a	1.22±0.024	1.42±0.024	1.21±0.018
	3×10^7	1.11±0.018	1.21±0.015	1.71±0.014^a	2.41±0.026^b	3.11±0.022^b	2.13±0.022^b	1.41±0.031	1.22±0.022
	Control	0.92±0.016	1.02±0.016	1.12±0.016	1.01±0.023	1.21±0.024	1.11±0.023	1.06±0.019	1.02±0.031
Heart	1×10^7	0.31±0.022	0.33±0.031	0.35±0.018	0.46±0.023^a	0.40±0.018	0.36±0.022	0.33±0.019	0.31±0.018
	2×10^7	0.32±0.025	0.34±0.022	0.35±0.022	0.46±0.025^a	0.40±0.015	0.36±0.031	0.33±0.024	0.32±0.014
	3×10^7	0.32±0.015	0.38±0.018	0.56±0.024^b	0.50±0.019^a	0.46±0.019^a	0.40±0.019	0.36±0.022	0.33±0.015
	Control	0.30±0.017	0.31±0.016	0.30±0.026	0.32±0.018	0.31±0.022	0.30±0.016	0.31±0.021	0.30±0.024
Hepatopancreas	1×10^7	0.81±0.022	0.84±0.031	0.89±0.026	0.94±0.019^a	0.99±0.028	1.20±0.017^a	0.90±0.017	0.84±0.016
	2×10^7	0.82±0.025	0.86±0.022	0.90±0.025	0.96±0.021	1.21±0.032^a	1.40±0.013^a	0.95±0.025	0.86±0.022
	3×10^7	0.79±0.019	0.96±0.018	0.99±0.022	1.32±0.023^a	1.58±0.026^b	1.36±0.015^b	1.25±0.027^a	0.98±0.024
	Control	0.80±0.023	0.81±0.026	0.82±0.024	0.80±0.020	0.81±0.022	0.82±0.021	0.81±0.023	0.81±0.018
Muscle	1×10^7	0.61±0.032	0.65±0.041	0.68±0.032	0.72±0.032	0.96±0.018^a	1.10±0.031^b	0.89±0.015	0.64±0.026
	2×10^7	0.63±0.019	0.66±0.036	0.74±0.012	1.05±0.026^b	0.97±0.015^a	0.88±0.028	0.76±0.023	0.68±0.022
	3×10^7	0.60±0.025	0.68±0.028	0.74±0.012	0.93±0.040^a	0.85±0.022	0.86±0.019	0.78±0.018	0.66±0.018
	Control	0.60±0.035	0.61±0.025	0.6±0.012	0.61±0.034	0.62±0.019	0.60±0.022	0.62±0.026	0.61±0.016

The superscript a means $P<0.05$, b means $P<0.01$ (n=5).

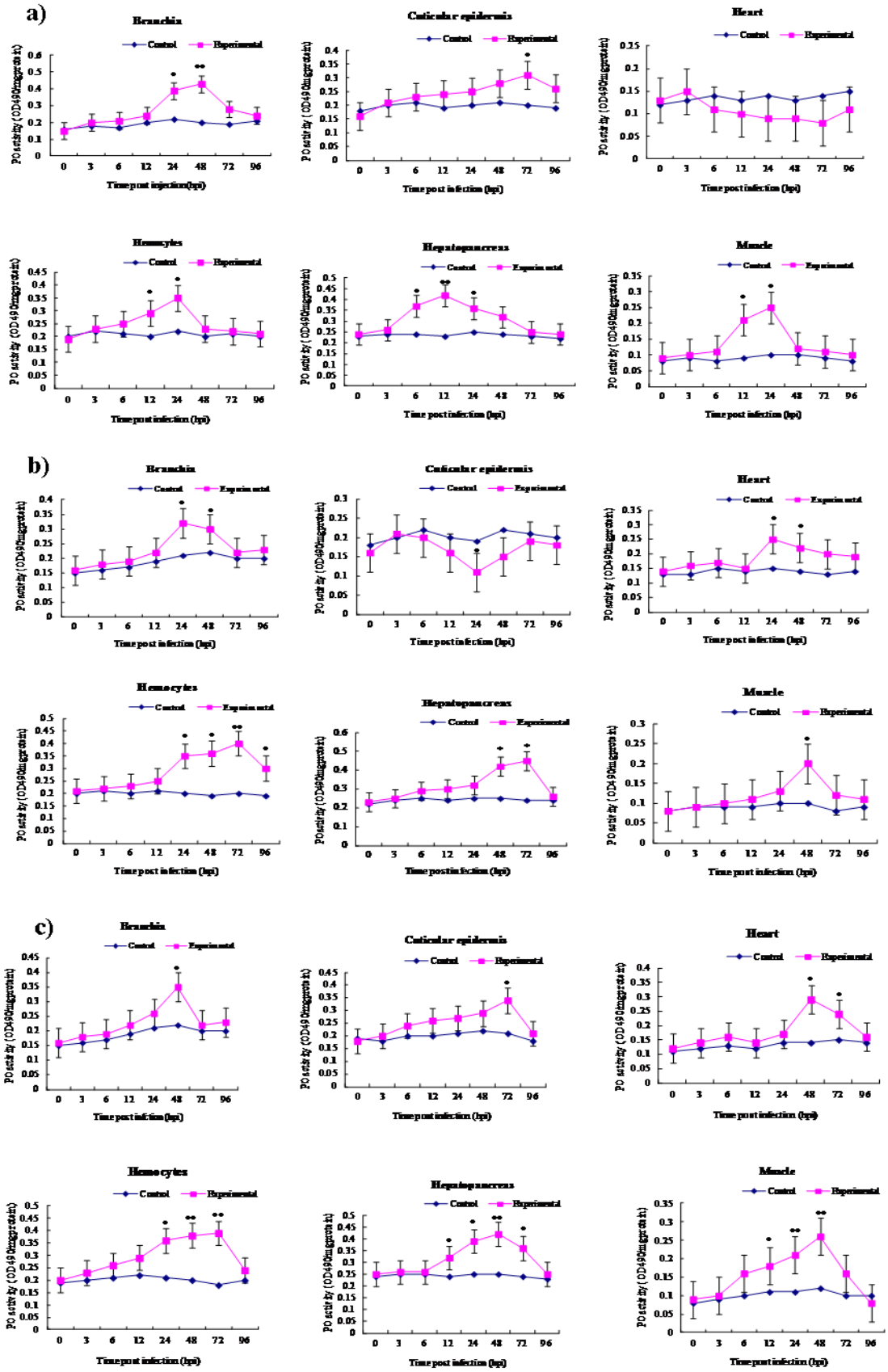

Fig. 2: PO activity after 1× 10^7, 2× 10^7 and 3 × 10^7 virions/crayfish WSSV infection in the six studied tissues of *P. clarkii* at 0, 3, 6, 12, 24, 48, 72, and 96 hpi. a) 1 × 10^7 virions/crayfish WSSV infected group; b) 2 × 10^7 virions/crayfish WSSV infected group; c) 3 × 10^7 virions/crayfish WSSV infected group. Error bars represent the ±SD of five crayfish across three independent measure (n=5, *P<0.05, **P<0.01).

PO activity in tissues after infection with WSSV at 1 × 10^7 virions/crayfish: The PO activities in tissues after treatment with WSSV at 1 × 10^7 virions/crayfish are shown in Fig. 2a. PO activities were higher in the branchia, cuticle epidermis, hepatopancreas, hemocytes, and muscle from 6 hpi, compared with the PBS-injected group. PO activity was significantly increased ($P<0.05$) in the branchia at 12 hpi and further increased at 24 hpi. PO was also significantly increased in cuticle epidermis and muscle in the experimental group from 12-24 hpi ($P<0.05$). PO activity was significantly increased in the hepatopancreas ($P<0.05$) at 6 and 24 hpi, and further increased at 12 hpi in the experimental group. However, PO activity in the heart was lower in the experimental group than in the PBS-injected group, reaching a minimum at 72 hpi.

PO activity in tissues after infection with WSSV at 2 × 10^7 virions/crayfish: PO activities in the branchia, hemocytes, heart, hepatopancreas, and muscle were increased from 6-96 hpi with WSSV at 2 × 10^7 virions/crayfish, but decreased in cuticle epidermis. PO activities were significantly increased in hemocytes ($P<0.05$) at 24, 48, and 96 hpi, and further increased ($P<0.01$) at 72 hpi in the experimental group. PO activities were significantly increased ($P<0.05$) in the branchia, heart, hepatopancreas, and muscle in the experimental group at 48 hpi. However, PO activity was significantly decreased in the cuticle epidermis ($P<0.05$) at 24 hpi (Fig. 2b).

PO activity in tissues after infection with WSSV at 3 × 10^7 virions/crayfish: PO activity increased in all six studied tissues of *P. clarkii* after infection with WSSV at 3 × 10^7 virions/crayfish. Activities in the branchia and cuticle epidermis were significantly increased ($P<0.05$) at 48 and 72 hpi, respectively. Activities in the hemocytes, hepatopancreas and muscle were highly significantly increased ($P<0.01$) at 48 hpi, and significantly increased in heart ($P<0.05$) from 48-72 hpi in the experimental group (Fig. 2c).

DISCUSSION

WSSV has caused significant economic losses to the shrimp-farming industry worldwide since 1993 (Musthaq *et al.,* 2011). The red swamp crayfish is a host of WSSV, which has recently become one of the most serious causes of disease in cultivated crayfish in China (Li *et al.,* 2012b). There are currently no effective measures for controlling virulence of WSSV which is dependent on its propagation ability and tissue tropism, and host resistance which plays significant roles in determining infection outcomes (Sun *et al.,* 2013). It is therefore essential to elucidate the interactions between WSSV and its host(s), in terms of both the host immune response and WSSV pathogenesis. In the present study, the virulence of WSSV and the molecular mechanisms in crayfish infected were investigated with different numbers of WSSV virions. The LD_{50} of WSSV was 2.08 × 10^7 virions/crayfish. The results suggested that the strain of WSSV used in the present study was not 100% lethal to crayfish under laboratory conditions, suggesting the existence of WSSV-resistant crayfish that may be suitable for selective breeding for WSSV resistance.

Analysis of the tissue distribution of *proPO* mRNA showed that it was more highly-expressed in hemocytes and hepatopancreas, which was consistent with previous reports (Liu *et al.,* 2006; Li *et al.,* 2012b; Liu *et al.,* 2013). In this study, *proPO* expression levels and PO activity post-WSSV infection varied among crayfish tissues in time- and concentration-dependent manners. These results indicated that the crayfish defense against the invading virus involves the induction and secretion of *proPO* into the circulating hemolymph, confirming the importance of *proPO* mRNA expression in hemocytes and the hepatopancreas as an important factor in the defense against WSSV infection in red swamp crayfish *P. clarkii* and other crustaceans.

The proPO system plays a vital role in antigen recognition and denaturation in innate immunity (Cerenius *et al.,* 2008), and is normally activated by invading microorganisms or parasites. Like other crustaceans, *P. clarkii* depends on its innate immune system to defend itself against pathogens. The apparent up-regulation of PO activity in hemocytes and the hepatopancreas in WSSV-infected *P. clarkii* in the present study indicated the involvement of these tissues in the crayfish antiviral immune response, in accordance with previous reports in *Scylla serrata* (Liu *et al.,* 2011).

The expression profiles of *proPO* mRNA after infection with different numbers of WSSV virions will help to clarify the implications of its function in the defense system (Jearaphunt *et al.,* 2014). In this study, *proPO* mRNA transcription was significantly increased in hemocytes and the hepatopancreas at most time points after infection with WSSV, confirming the idea that *P. clarkii* might depend mainly on the innate immune system to defend against viral pathogens. Furthermore, the results also suggest that *P. clarkii* attempted to control pathogenic microbes such as WSSV at various time points post-infection. Variations in *proPO* mRNA expression levels among tissues indicates that *proPO* was actively involved in the immune system of crayfish, thus helping it fight against the entry of pathogens.

Calreticulin responds to WSSV infection by increasing mRNA and protein expression and by delaying apoptosis (Watthanasurorot *et al.,* 2014). It is possible that proPO might interfere with WSSV replication through binding nucleocapsid proteins. This possibility should be investigated in future studies.

In the present study, PO activity was high in *P. clarkii* infected with different numbers of virions. These observations indicated that *P. clarkii* responded to WSSV injection, as shown by the detection of high proPO expression during the study period, and further suggested that high levels of proPO and PO may enhance the resistance of *P. clarkii* to WSSV (Clark *et al.,* 2013).

Conclusion: In this study, the results of tissue tropism, real-time PCR analysis and PO activity assays showed that the *proPO* played an important role in the crayfish immune system, and that its expression might be activated by different mechanisms in different tissues in response to viral stress. However, further studies are needed to determine if there are other gene(s) trigger an immune

response or antiviral activity in response to WSSV infection in *P. clarkii*. More work is also needed to identify the mechanism of resistance against WSSV in *P. clarkii*. Such information would help to develop WSSV resistance in freshwater crayfish.

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Author's contribution: YL and WW conceived and designed the experiments, analyzed the results, and wrote the paper. LS, MW, YW, SJ and KY performed the experiments. All authors have read and approved the final manuscript.

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