Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(7):432-436

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Silencing of profilin in red swamp crayfish *Procambarus clarkii*

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ABSTRACT

Profilin is an actin-binding protein and is involved in controlling T-lymphocyte activation and effector functions in invertebrate immune response. In this study, RNA interference (RNAi) technique was used to elucidate the role of profilin in the ovarian development in Procambarus clarkii. RNAi-mediated silencing of profilin gene, performed by injection of double-stranded RNA (dsRNA) corresponding to the gene, significantly increased the mortality and additionally decreased vitellogenin (Vg) transcripts level (80.2%) in this crayfish, implied that the profilin knockdown seems inhibited the synthesization or transportation of Vg in the process of ovarian development and maturation. Our results suggest an important role for profilin in ovarian development and maturity in crustaceans.

Key words: Procambarus clarkii; profilin; RNA interference; ovarian development

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INTRODUCTION

Profilin is a small ubiquitous protein originally described as actin-binding protein and is critical for cytoskeletal dynamics [1]. Profilins are potent regulators of actin filament dynamics, by promoting the exchange of ADP to ATP on actin and by the affinity of profilin-actin complexes for actin filament ends [2].

Apart from actin binding, profilins also have a role in cellular processes such as membrane trafficking, small-GTPase signaling and nuclear activities, in addition to neurological diseases and tumor formation [3]. Genetic studies have shown the importance of profilins for cell proliferation and differentiation. Profilin gene disruption leads to grossly impaired growth, motility and cytokinesis, and embryonic lethality in multicellular organisms, such as insects and mice [4,2]. Profilin-mutant hemocytes in *Drosophila* exhibited an increased phagocytic activity, showing that profilin is a critical regulator of phagocytosis in *Drosophila* [5]. Human breast cancer cell lines contain less profilin than normal breast epithelial cells, and raising the intracellular profilin level by transfection abolishes tumor growth in nude mice, suggesting that profilin plays a role as a tumor suppressor [6, 7].

A series of studies indicated that profilin, like other actin-binding proteins, might be involved in controlling T-lymphocyte activation and effector functions, through participating in the reorganization of cytoskeleton dynamics at the immune synapse [8]. There is also some indirect evidence of a role of profilin in invertebrate immune response. Transcriptomic analysis of gills from the Pacific white shrimp *Litopenaeus vannamei* infected with white spot syndrome virus (WSSV) revealed an increase in the profilin mRNAs level at 6 h after infection [9]. In plant cells, the actin and actin-binding partners (including profilin) performed reorganization of the cytoskeleton in response to external and internal cues, and thus regulates several vital cellular processes, including reactions to the pathogen attack and wounding [10]. Whereas, very little is known about the profilin in ovarian development and maturity in crustaceans.

In a previous report, we have observed that profilin expression level was been up-regulated in stage IV ovaries

compared to stage III by using 2D-gel electrophoresis analysis in *P. clarkii* [11]. Furthermore, we have isolated the cDNA sequence for profilin and have characterized the gene and its predicted protein sequence in *P. clarkii* (to be published). In this study, RNA interference technique was used to elucidate the role of profilin in the ovarian development and maturity in *P. clarkii*.

EXPERIMENTAL SECTION

2.1. Sample preparation

Domesticated female crayfish *P. clarkii* broodstock (length 10.5-12.5 cm, weight 28.2-35.5 g) were collected from the Jiangsu Baolong Breed Aquatics Company in Dafeng city, Jiangsu province, China. Brooders were maintained in 40-L aquaria in filtered aerated water at room temperature (25°C). A commercially available diet (Minghui Feed Co., Ltd., ZJ) for crayfish broodstock was fed to the animals twice daily.

2.2. Synthesis of dsRNA

Double-stranded RNA (dsRNA) corresponding to the *Procambarus clarkii* profilin (pc-profilin) sequence was generated by in vitro transcription. The T7 promoter sequence was incorporated into the DNA templates using primers Ppcpro-F: 5'-TAATACGACTCACTATAGGGATGTCTTGGAACACATAC-3' and Ppcpro-R: 5'-TAATACGACTCACTATAGGGTCAGTAATTCTGGCCCT-3', which had a T7 promoter in their 5'-ends (the sequence of T7 promoter is underlined). The sequence of primers was designed according to the *P. clarkii* profilin gene (GenBank accession no. KJ150295). For an exogenous gene, a 740 bp fragment of the green fluorescent protein (GFP) was amplified with the pEGFP-1 vector as template using primers GFP-F: 5'-TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA-3' and GFP-R: 5'-TAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA-3' (the sequence of T7 promoter is underlined). One microgram of each template was used in an in vitro transcription using the T7 RiboMAX^{TM} Express RNAi Systems (Promega, USA), according to the manufacturer's instructions. Equal amounts of sense and anti-sense single stranded RNA were annealed to produce dsRNA and the remaining DNA template in the solution was digested with RNase-free DNase I. The dsRNA specific for the GFP gene was generated via the same procedure and used as control to exclude the non-specific effect of dsRNA. The quality and amount of dsRNAs was verified by TBE-1.5% agarose gel electrophoresis with UV visualization following ethidium bromide staining, and UV spectrophotometry, respectively.

2.3. In vivo gene silencing

Red swamp crayfish (10.5-12.0 g, fresh weight) was intramuscularly injected with 5 ug of either pc-profilin (0.5 ug dsRNA per 1 g crayfish) or GFP dsRNA dissolved in 25 ul of phosphate buffer saline (PBS), using a 0.5-ml syringe with a 29-gauge needle, to assess the sequence-specific knockdown effect of dsRNA. Injection of PBS alone was done as an additional control for handling and injection induced mortality. The injection of dsRNA (5 ug) or PBS was repeated together with 20 mg of the lipopolysaccharide (LPS) (Sigma) and laminarin (b-1,3-glucan chain with some b- 1,6-linked glucose units) (Sigma) at 24 h after the first injection.

Extraction of RNA samples from frozen ovaries using TRIzol reagent was done according to manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The contaminating genomic DNA was removed by DNase I at 0.15 U mg^{-1} total RNA treatments at 37 °C for 30 min. Total RNA samples was collected and extracted from the ovary of crayfish from all of three groups at 48 h after the second dsRNA/PBS injection. From this total RNA preparation, the pc-*profilin* transcript level was determined by RT-PCR analysis. A 150 bp fragment of 18S rRNA was amplified using the primer pair Pr-F: 5'-TGGTGCATGGCCGTTCTTA-3' and Pr-R: 5'-AATTGCTGGAGATCCGTCGAC-3' according to the *P. clarkii* 18S rRNA gene (GenBank accession no. AF436001) as an internal control.

2.4. Cumulative mortality assay

Crayfish, each of ~10 g in size, were each intramuscularly injected with 5 ug of pc-*profilin* dsRNA (0.5 ug dsRNA per 1 g crayfish) or PBS in 25 ul volume. Injection of GFP dsRNA was included as sequence-independent dsRNA control. The cumulative mortality was recorded twice a day for 4 days post second injection. The experiment was performed in triplicate groups, with each group consisting of 8-12 healthy crayfish. Statistical analysis of the mortality test was performed using a one-way ANOVA test.

2.5. Real-time RT-qPCR assay

The level of Vitellogenin (Vg) gene transcript in each RNA sample was recorded by real-time RT-qPCR analysis respectively on the second, fourth and sixth day post second injection. The Vg specific primers, PVg-F: 5'-AGTCATCAGTGGTGACAGATGTA-3' and PVg-R: 5'-GGTATCATATTGACAAGCTTGAGAT-3', were designed according to the partial cDNA sequence of *P. clarkii* obtained from previous study by Shui in 2012 (data

not shown). 18S rRNA was amplified as a partial gene fragment of 150 bp using the primer pair Pcon-F: 5'-TGGTGCATGGCCGTTCTTA-3' and Pcon-R: 5'-AATTGCTGGAGATCCGTCGAC-3' according to *P. clarkii* 18S rRNA gene (GenBank accession no. AF436001) as an internal control. DEPC-water for the replacement of RNA template was used as negative control. The SYBR Green RT-qPCR assay was conducted in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data analysis of RT-qPCR was performed using the SDS software V2.0 (Applied Biosystems) according to the $2^{-\Delta\Delta Ct}$ algorithm [12]. The finally obtained data were subjected to one-way ANOVA followed by Duncan's test ($P < 0.05$).

RESULTS AND DISCUSSION

3.1. Pc-*profilin* silencing

Double-stranded RNA-mediated RNAi was used to determine the role of profilin in ovarian development in *P. clarkii*. To determine the efficiency of dsRNA mediated knockdown of pc-*profilin* transcript levels, crayfish (~10 g) were injected with 5 ug of dsRNA specific for the pc-*profilin* gene twice and the level of its expression was determined 2 days after the second dsRNA injection. It was further verified by RT-PCR using gene-specific primers for 18S RNA of *P. clarkii* (Fig. 1). Its data shows that the transcript level of pc-*profilin* gene was decreased in pc-*profilin* knockdown crayfish, whereas injection of GFP dsRNA had no discernable effect on the pc-*profilin* transcript levels. Furthermore, it demonstrated that injection of pc-*profilin*-dsRNA did not detectably suppress transcription of 18S rRNA, which supports the likely specificity of pc-*profilin* RNAi knockdown.

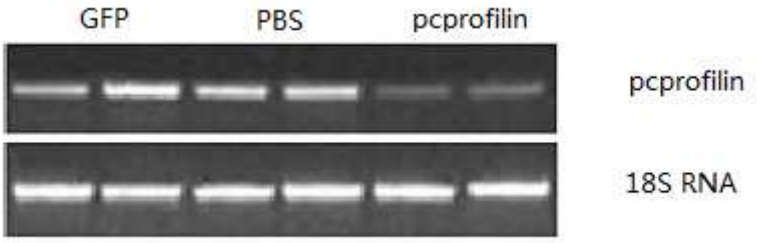

Fig. 1 Gene-specific silencing of pc-*profilin* transcript levels in *P. clarkii*. Crayfish (~10 g) were injected twice with 5 ug of pc-profilin or GFP dsRNAs. Total RNA was extracted from ovary 48 h after the second injection and was subjected to analysis using RT-PCR. Each lane represents cDNA from an individual crayfish. The effect of pc-*profilin*-dsRNA injection on the expression level of pc-*profilin* and 18S rRNA of *P. clarkii* was further examined by RT-PCR using gene-specific primers. 18S rRNA was used as a control housekeeping gene to standardize the amount of cDNA template in each reaction.

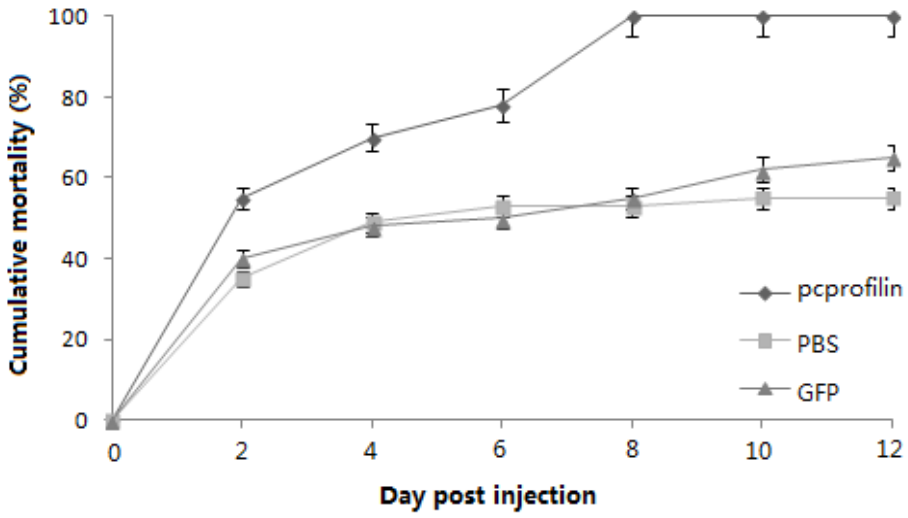

Fig. 2 Cumulative mortality of pc-*profilin* silenced crayfish. Crayfish were injected twice with 5 ug of pc-*profilin* or GFP dsRNAs. Control groups were injected with PBS. Crayfish mortality was recorded twice each day for 6 days. The cumulative mortality (%) in each experimental group (8-12 crayfish/group) is presented as the mean±standard deviation, and is derived from triplicate independent experiments.

3.2. Cumulative mortality assay

To test whether pc-profilin is necessary for crayfish to survive, crayfish (~10 g) were injected twice with pc-profilin dsRNA (~5 ug). Mortality was then scored 2 days after second injection and twice a day thereafter (Fig. 2). The data

shows that crayfish injected with pc-*profilin*-dsRNA attained 100% cumulative mortality within 192 h post injection, whereas crayfish injected with GFP dsRNA attained a total cumulative mortality of ~ 60%, which was not significantly different from that of the control (PBS injected crayfish). The high mortality rate of the pc-profilin knowdown crayfish suggest that profilin is necessary for crayfish survive and plays an important role in vital activity.

3.3. Effect of pc-*profilin*-dsRNA on ovarian development

To determine whether suppression of the pc-*profilin* gene transcript levels by RNAi knowdown would result in a reduction of the process of ovarian development, ovary tissues was collected from the knowdown crayfish and subjected to a Vg transcripts expression level assay using real-time RT-qPCR. The percentage of silencing effect was calculated by subtracting the relative expression of pc-*profilin*-dsRNA treated group from the PBS group. Significant reduction in pc-*profilin* transcripts was observed in the knockdown group on day 2, day 4 and day 6 posts the second injection compared with its constant expression in the two control groups (Fig. 3). The results indicated that the injection of pc-*profilin*-dsRNA into crayfish resulted in a silencing effect of Vitellogenin transcripts level (56.7%) 2 day post injection, more effect (72.5%) in 4 days post injection and maximum level (83.2%) was observed 6 days post injection.

During female maturation in crustacean, Vg is the precursor of vitellin (Vn), and is synthesized by the ovary and/or extra-ovarian tissues. Extra-ovarian Vg is transported through the hemolymph to the ovary and is taken up into the oocytes by receptor-mediated endocytosis [13]. Our results showed that knowdown of the pc-*profilin* transcript led to a decreased level of Vg in ovary, suggesting a possible role in synthesization or transportation of Vg in the ovarian development, and is likely to plays an important role in gonad development as well as sexual maturity in *P. clarkii*.

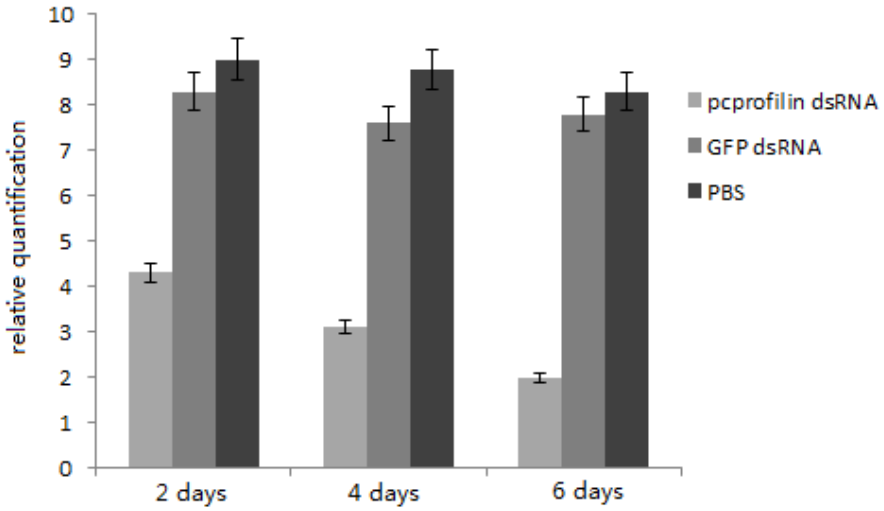

Fig. 3 Real-time RT-qPCR assay for the transcript level of *P. clarkii* Vitellogenin mRNA in pc-*profilin* silenced crayfish. Control crayfish was injected with GFP dsRNA or just PBS. Values are expressed as mean±S.D. from 3 different samples. Quantitative real-time PCR employed the relative quantification (RQ) ΔΔCt method.

Specific mRNA degradation mediated by dsRNA interference is a powerful strategy of suppressing gene expression. In current studies, dsRNA silencing was successfully conducted in studies on antiviral and moulting characteristics, ovarian development and muscle contraction of animals, such as *Litopenaeus vannamei*, *P. monodon*, *Penaeus chinensis*, *Pacifastacus leniusculus*, *Marsupenaeus japonicus* and *P. clarkii* [14-19]. Our previous data shows that profiling is likely to involve in each stage of ovarian development in crayfish. This study, the application of pc-profilin-dsRNA into crayfish, which resulted in the prevention of the Vg transcript level, confirmed this supposition. However, the knockdown of profilin is lethal to crayfish. These results, including the previous report on profilin, reveal the important role in the ovarian development and maturation in *P. clarkii*. However, ovarian development is a complex biochemical process and very little is known about the genetic control of its molecular mechanism in crustaceans. Thus, continued effort to identify and characterize the genes involved, directly and indirectly, in the profilin-Vg signaling pathway is essential for an understanding of ovarian maturation.

Acknowledgements

This study was supported by the Special Fund for Agro-scientific Research in the Public Interest (201003070), Central Public-interest Scientific Institution Basal Research Fund (2013JBFM09), and the Natural Science Foundation of Jiangsu Province, China (Grant no. BK2012090).

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