

## Characterization and Function of a Gene Pc 14-3-3 Isoform from Red Crayfish, *Procambarus clarkii*

Baojian Zhu\*, Daojun Wang, Tao Peng, Lei Wang, Guoqing Wei and Chaoliang Liu\*

College of Life Sciences, Anhui Agricultural University, 130 Changjiang West Road, Hefei 230036, P.R. China

**Abstract.** 14-3-3 proteins are a family of conserved regulatory molecules participating in the coordination and regulation of many cellular processes. In this study, we first report the 14-3-3 gene, named as Pc-14-3-3, in the crayfish *Procambarus clarkii*. The Pc-14-3-3 gene encodes a polypeptide of 247 amino acids and the deduced 14-3-3 protein sequence reveals the higher percent identity with its homologues from crustaceans. Real-time quantitative reverse transcription-PCR (qRT-PCR) analysis shows that Pc-14-3-3 expression is higher in hepatopancreas than that in other examined tissues. In addition, prokaryotic expression and purification of the Pc-14-3-3 protein were performed. SDS-PAGE and western blot analysis demonstrated that a 30 KD recombinant protein was successfully expressed in *E. coli* cells. The titer of the antibody against recombinant Pc-14-3-3 protein was about 1: 3100 which was determined by ELISA. The expression of Pc-14-3-3 was significantly up-regulated in hepatopancreas after eyestalk ablation or ecdysone induction, which was confirmed by qRT-PCR and western blot analysis. These results suggest that Pc-14-3-3 plays a role in the development process of *P. clarkii*.

**Key words:** Eyestalk ablation, ecdysone induction, hepatopancreas, gene expression.

### INTRODUCTION

**P**<sub>c</sub> 14-3-3 protein was first identified in 1967 and named based on its particular migration pattern on DEAE-cellulose chromatography and gel electrophoresis (Moore and Perez, 1967). 14-3-3 proteins are a family of highly conserved acidic proteins found in all eukaryotes with molecular weights of about 30 kDa (Fu *et al.*, 2000; Ferl *et al.*, 2002) and can form both homo- and heterodimers (Benzinger *et al.*, 2005; Wilker *et al.*, 2005). Through binding interactions with hundreds of diverse proteins (Yaffe *et al.*, 1997; Obsil and Obsilova, 2011), 14-3-3 proteins participate in a wide range of cellular processes including cell cycle regulation, apoptosis, metabolism, transcriptional regulation of gene expression, development and the DNA damage response (Fu *et al.*, 2000; Tzivion and Avruch, 2002; Bridges and Moorhead, 2004; Mackintosh, 2004; Gardino and Yaffe, 2011). Although the molecular and cellular functions of 14-3-3 proteins have been well studied (Aitken, 2006), the mechanisms of the 14-3-3 protein function still

remain elusive (Darling *et al.*, 2005).

Up to now, 14-3-3s had been extensively studied in various organisms (Wang and Shakes, 1996; Rosenquist *et al.*, 2001; Gardino *et al.*, 2006). However, only few 14-3-3 proteins had been identified from crustaceans such as *Penaeus monodon* (Kaeodee *et al.*, 2011), *Litopenaeus vannamei* (Wanna *et al.*, 2012), *Scylla paramamosain* (Shu *et al.*, 2012) and *Fenneropenaeus merguensis* (AEW70348.1) and their functions remains to be further discovered. In this study, the cloning, expression and biological function analysis of 14-3-3 gene from the red crayfish *P. clarkii* were performed.

### MATERIALS AND METHODS

#### *Experimental animals*

Crayfishes (about 10g and 7-8cm each) were collected from the market and fed in tanks containing continuously flowing water, which had been filtered through a Brimak/carbon filtration unit (Silverline Ltd.). Ten individuals were randomly divided into two groups, one group for eyestalk ablation (bilateral ablation) and the other group for ecdysone injection (0.5µg/g). Muscle, heart, stomach, gut, gill and hepatopancreas were collected from *P. clarkii* and immediately frozen in liquid

\* Corresponding authors: Baojian Zhu (zhubaojian@ahau.edu.cn) and Chaoliang Liu (cyschx@163.com)

nitrogen for RNA extraction.

#### *RNA extraction and cloning of Pc-14-3-3 gene*

The hepatopancreas were collected for RNA extraction by Trizol™ Reagent (Invitrogen) according to the instructions. Using 500 ng of total RNA per sample to generate first strand cDNA with TURScript cDNA Synthesize Kit (Aidlab Biotech Co. Ltd., Beijing). The primers F1 and R1 (restriction enzyme sites are underlined) were designed to amplify the mature peptide coding Pc-14-3-3 gene which has been obtained from the SSH library constructed in our laboratory. PCR was performed according to the protocol as following: an initial delay at 94°C for 5min, 35 cycles of denaturation at 94°C for 35 s and annealing at 54°C for 35 s. PCR product was resolved in 1% agarose gel by electrophoresis, then cloned into the PGEM T easy cloning vector (Promega, USA) after DNA purification and sequenced at Invitrogen, Shanghai.

#### *Protein expression, purification and antibody preparation*

PCR products were digested with restriction enzymes (*Bam* HI and *Xho* I), then ligated to pET28a vector (Novagen, USA). The resulting recombinant plasmids pET28a-Pc-14-3-3 were confirmed by DNA sequencing and then transformed into *Escherichia coli* BL21 (DE3) (Novagen, USA) for protein expression. The recombinant fusion protein was analyzed by 12% SDS-PAGE. Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography (Qiagen, Germany) was used to purify the recombinant proteins according to the instructions. Quantification for total protein was performed by the Bradford method (Bradford, 1976). The New Zealand White rabbits were immunized with 100 µg of purified proteins homogenized in complete Freund's adjuvant for three times at two-week intervals. A boost injection in incomplete Freund's adjuvant was given for another week. Rabbit serum was collected seven days after the last immunization. Antiserum was prepared according to the method described (Harlow and Lane, 1999). Monoclonal anti-6-His antibody (Qiagen, Germany) was used to verify protein expression and their sizing.

#### *Enzyme-linked immunosorbent assay (ELISA)*

The titer of antibody from the immunized rabbits was determined by enzyme-linked immunosorbent assay. Briefly, the optimum concentration of recombinant proteins (diluted in 0.05M carbonate buffer) were coated onto a 96-well plate (Nune, Denmark) and then incubated overnight at 4°C with blocking solution (5% skimmed milk powder in phosphate-buffered saline). The plates were washed three times with PBST (phosphate-buffered saline containing 0.1% Tween 20). Next, rabbit serum (primary antibody) of different dilutions were added to each well and incubated for 1h at 37°C. After washing with PBST, the plates were incubated with 100 µl of a 1:1000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase for 30 min at room temperature. The plates were washed again and 100 µl of TMB/H<sub>2</sub>O<sub>2</sub> substrate was added to each well. Optical density was measured at 450nm with Elix 800 Universal micro-plate reader (Biotek Instruments).

#### *Western blotting*

Proteins were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma, USA) by an electrophoretic transfer system (Bio-Rad). Membranes were blocked with PBST containing 1% BSA for 2 h at room temperature. Membranes were washed with PBST and subsequently incubated with primary antibodies for 2h at room temperature. After washing with PBST, membranes were incubated with anti-rabbit IgG antibody for 1h at room temperature. The antibodies used in this experiment were diluted (1:1000) with PBST. Detection was done with horseradish peroxidase-3,3'-diaminobenzidine (HRP-DAB) Detection Kit (Qiagen, Germany). Quantification for total protein was performed by the Bradford method.

#### *Real-time PCR*

Muscle, heart, stomach, gut, gill and hepatopancreas were sampled for RNA extraction using Trizol following the manufacturer's instructions. Five micrograms of total RNA was reverse transcribed into cDNA with M-MLV reverse

transcriptase (Invitrogen, USA). Real-time RT PCR was carried out to measure the expression levels of Pc-14-3-3 in various tissues or after different treatments. Gene specific primers F2 and R2 (shown in Table 1) were designed by Primer 5.0 software based on the known sequences. The PCR reaction was performed in a 25  $\mu$ l volume with a SYBR Premix Ex Taq™ Kit (Takara, Japan), 2 $\mu$ M of each specific primer, 2 $\mu$ l of cDNA in iCycler iQ™ thermocycler (Bio-Rad), using the following procedure: initial denaturation at 95°C for 2min; followed by 40 cycles of amplification (95°C for 20 s and 55°C for 35 s). The continuous fluorescence acquisition (55–95°C with an increase of 0.5°C per 10 s) was used for melting curve analysis. The relative expression level of Pc-14-3-3 was calculated according to the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001) and actin gene (shown in Table 1) was used as a housekeeping gene. All data are represented as mean $\pm$ S.E. Differences between samples were analyzed by Student's *t*-test and it was considered statistically significant when *p*-values were less than 0.05.

**Table I.- Primers used for PCR in this study**

| Primer No. | Primer sequence (5'-3')    |
|------------|----------------------------|
| F1         | CAGGATCCATGTCCGTCGATAAGGAG |
| R1         | AGTCTCGAGTTAGTTGTCCGCCCT   |
| F2         | CATACCAGGATGCCTTTGAGAT     |
| R2         | TCCACAGCGTCAGGTTGTCTC      |
| Actin F    | TCATCACCAACTGGGACGAC       |
| Actin R    | GAAACCTCCATAGACGGGGAC      |

## RESULTS

### *Sequence analysis of Pc-14-3-3*

A cDNA fragment of 744 bp encoding Pc-14-3-3 was obtained from SSH library constructed by us and the deduced amino sequence was shown in Figure 1A. This sequence (GenBank No. JQ284433) encodes 176 amino acid residues and contains the conserved structure of 14-3-3 zeta proteins. Theoretical isoelectric point and molecular weight for the protein are 4.9 and 28.1 kDa, respectively. Comparison of Pc-14-3-3 in overall sequence with 14-3-3 zeta proteins from *Caenorhabditis elegans*,

*Drosophila melanogaster*, *Danio rerio*, *Mus musculus* and *Homo sapiens* revealed a similarity ranging from 80 to 89% (Fig. 1A). Phylogenetic analysis showed the Pc-14-3-3 was highly homologous to those 14-3-3s from crustaceans (Fig. 1B).

### *Protein expression, antibody preparation and western blot analysis*

A recombinant protein with a molecular weight of 30 kDa from *E. coli* BL21(DE3) was detected by SDS-PAGE (Fig. 2A). Western blotting of recombinant proteins using the anti-His-tag antibody suggested that a consensus protein of 30 kDa was detected (Fig. 2B). No immunoreactive band was revealed in the control group (before induction). This result indicated that recombinant Pc-14-3-3 protein was successfully obtained from *E. coli* BL21 (DE3) cells. The titer of anti-Pc-14-3-3 antibody (about 1: 3100) against rabbit was determined by ELISA.

### *Expression of Pc-14-3-3 in various tissues*

Real-time RT-PCR was performed to determine the expression levels of Pc-14-3-3 in different tissues. Expression level of actin gene was used for normalization. The result showed that the Pc-14-3-3 was ubiquitously expressed in all examined tissues and higher expression levels in hepatopancreas and muscle were determined (Fig.3).

### *Effects of eyestalk ablation and ecdysone induction on the expression of Pc-14-3-3*

To investigate the effects of eyestalk ablation or ecdysone injection on the expression levels of Pc-14-3-3 in hepatopancreas, the real time PCR and western blotting were performed. Samples were prepared at 0, 2, 4, 6, 8 and 10 h after treatments, respectively. The result revealed that the expression of Pc-14-3-3 was obviously increased from 6 to 10 h after eyestalk ablation and reached the maximum at 10 h (Figs. 4A, B). Similarly, ecdysone obviously induced the expression of Pc-14-3-3 from 4 to 8 h after treatments (Figs. 5A, B). These results suggest that eyestalk ablation or ecdysone can induce the activation of Pc-14-3-3.

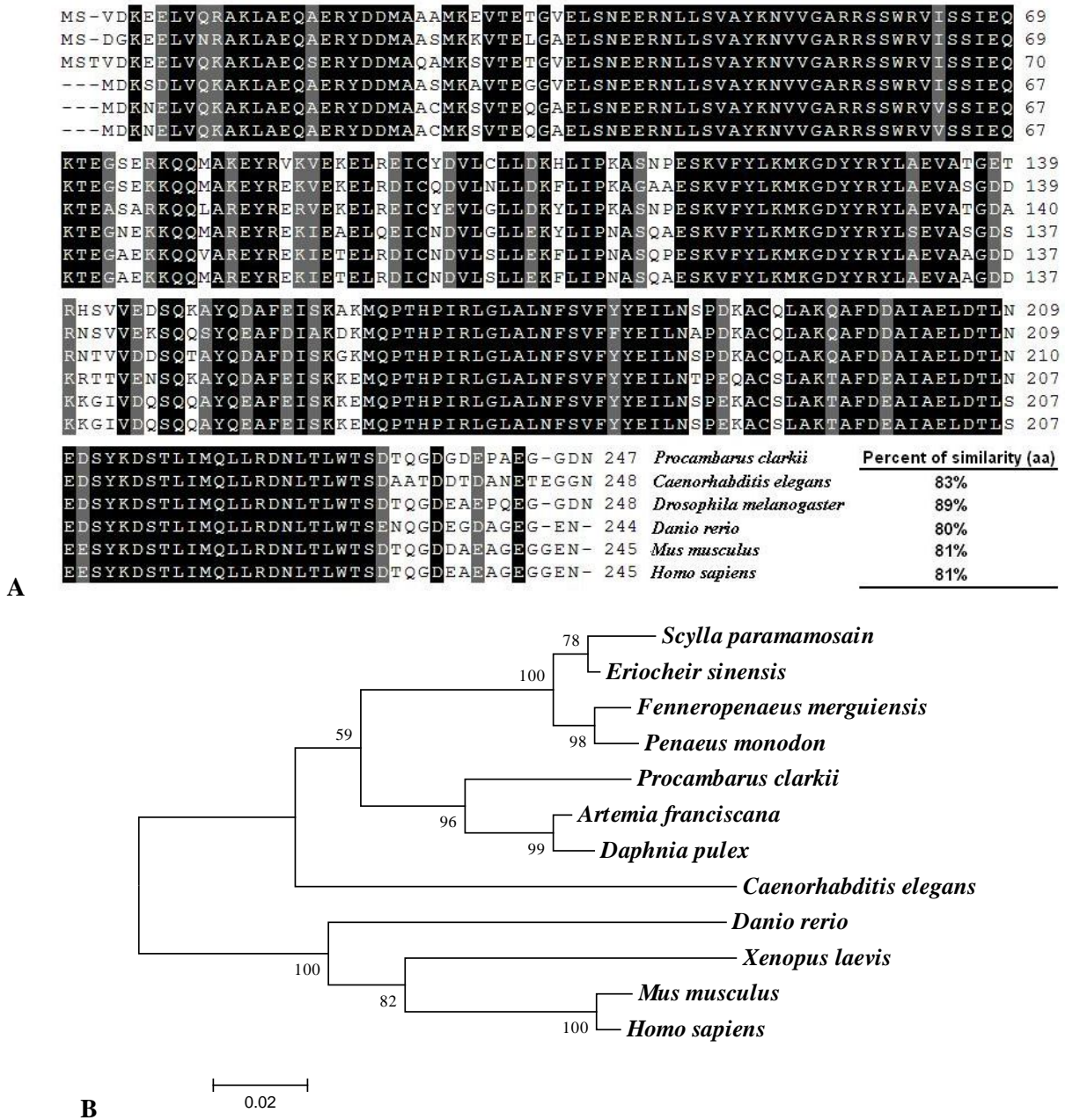


Fig. 1. **A**, Alignment of PC-14-3-3 with 14-3-3 zeta proteins from various organisms. Identical and similar amino acids are presented in black and gray, respectively. Comparisons of similarity in full-length amino acid sequence between 14-3-3 zeta proteins are shown in the lower corner. **B**) Phylogenetic analysis was performed by MEGA program based on the 14-3-3 amino acid sequences from various animals. The phylogenetic tree was constructed using the neighbor-joining algorithm method and bootstrap values (1000 repetitions) of the branches are indicated. The origins and accession numbers of the 14-3-3 zeta sequences are: *Drosophila melanogaster*, NP\_476885; *Mus musculus*, BAA11751; *Homo sapiens*, NP\_003397; *Caenorhabditis elegans*, NP\_509939; *Scylla paramamosain*, AFD33362; *Fenneropenaeus merguensis*, ADI87601; *Penaeus monodon*, AAY56092; *Eriocheir sinensis*, JF681948; *Danio rerio*, NP\_001076267; *Artemia franciscana*, ABX80390; *Daphnia pulex*, EFX74245; *Xenopus laevis*, Q91896.

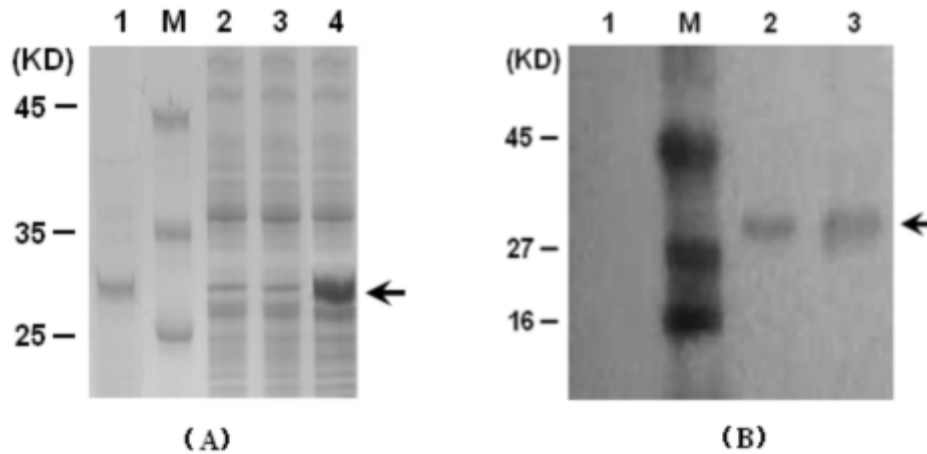


Fig. 2 (A) Analysis of recombinant Pc-14-3-3 protein on 12% SDS-PAGE gels. The gels were revealed by Coomassie blue R-250 staining. Bacterial proteins were collected after 4 h of IPTG induction. Lane 1, purified recombinant protein; Lane 2, *E. coli* BL21(DE3); Lane 3, before induction; Lane 4, after induction by 1mM IPTG; M, molecular weight marker. (B) Western blotting of purified recombinant proteins using anti His-tag antibody. Lane 1, before induction; Lane 2, after induction; Lane 3, purified recombinant proteins. A 30 kDa protein was detected by western blotting. No immunoreactive band was found in the control group.

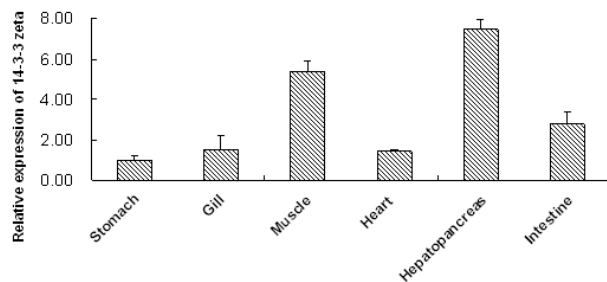


Fig. 3. Expression level of Pc-14-3-3 in various tissues. The expression of Pc-14-3-3 was determined by real-time RT-PCR. Expression levels were assessed using actin gene for normalization. The data were analyzed by Student's *t*-test and presented as Mean $\pm$ SE of independent experiments done in triplicates.

## DISCUSSION

In this study, a 14-3-3 gene has been identified from *P. clarkii* and analysis of deduced amino acid sequence shows that the Pc-14-3-3 is a member of 14-3-3 zeta isoforms (Yaffe and Elia, 2001). Based on homologous alignment and phylogenetic analysis, the Pc-14-3-3 protein was highly homologous to those of invertebrates, this is consistent with the conclusion that the 14-3-3 genes

have a long evolutionary history. Although multiple isoforms of 14-3-3 proteins had been found in animals (Mhawech, 2005; Aitken, 2006), the information about 14-3-3s in crustaceans was limited.

Compared with the other examined tissues, the expression levels of Pc-14-3-3 were higher in hepatopancreas and muscle, this result was similar to that in *S. paramamosain* (Shu *et al.*, 2012). Most 14-3-3 isoforms bind the partners with similar affinity, however, some isoforms have specific effects (Aitken, 2002; Van Hemert *et al.*, 2004). Among these isoforms, 14-3-3 zeta had been confirmed to be involved in the response to salinity stress (Kaeodee *et al.*, 2011; Shu *et al.*, 2012) and virus infection (Chongsatja *et al.*, 2007; Wang *et al.*, 2007). To investigate the role of Pc-14-3-3 in the physiological process, the expression of Pc-14-3-3 was determined after eyestalk ablation or ecdysone injection. In our experiments, eyestalk ablation and ecdysone stimulation have the similar effects on the expression of Pc-14-3-3. It has been traditionally accepted that ablation of the eyestalks leads to enhanced ecdysteroid secretion and elevates molting (Keller and Schmid; 1979; Chang and Bruce, 1980). However, whether or not this gene plays a role in the molting process of *P. clarkii* is still unclear. Although a 14-3-3 zeta isoform has been identified

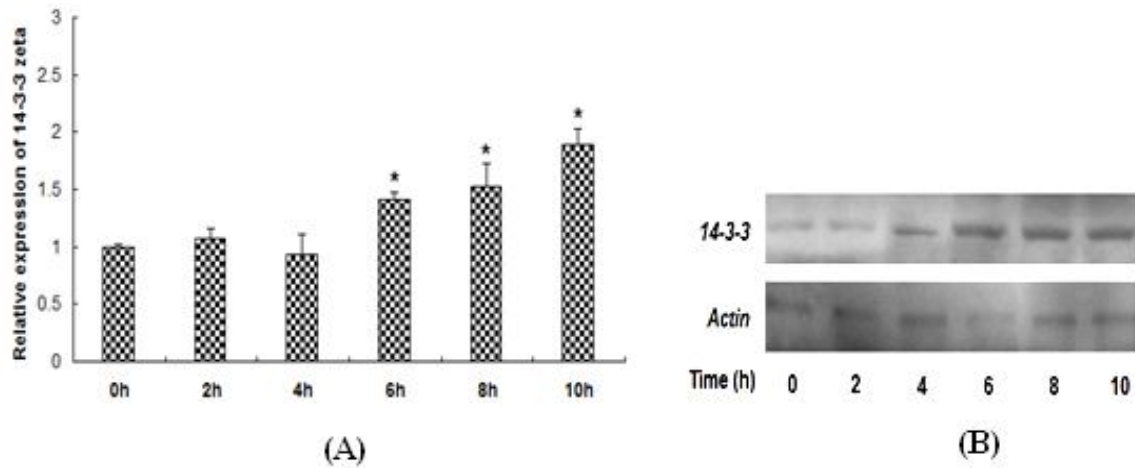


Fig.4 Validation of the expression of Pc-14-3-3 after eyestalk ablation. Hepatopancreas were collected from *P. clarkii* at 0, 2, 4, 6, 8 and 10 h after treatments, respectively. (A) Expression levels were assessed by real-time RT-PCR. The group without eyestalk ablation was used as a control. The values were presented as mean $\pm$ S.E. of independent experiments done in triplicates and analyzed by Student's *t*-test, \* $P \leq 0.05$  when compared to control values. (B) The expression of Pc-14-3-3 was determined by western blotting using actin for normalization.

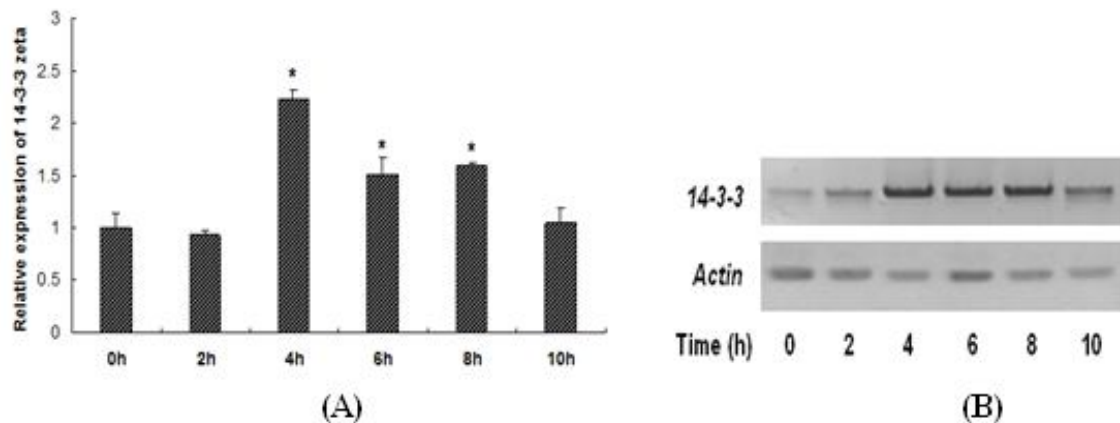


Fig.5 The expression of Pc-14-3-3 in hepatopancreas after ecdysone induction. (A) The groups without ecdysone induction or treated with sterile water injection were used as controls. (B) The expression of Pc-14-3-3 was determined by western blotting and actin was used for normalization.

from *P. clarkii*, the information about other 14-3-3 isoforms and their physiological functions remain the subjects of further investigation.

#### ACKNOWLEDGEMENTS

This work was supported by Research Fund for the Doctoral Program of Higher Education of China (20123418120003), Natural Science Foundations of Anhui Province of China (10040606Q21).

#### REFERENCES

- AITKEN, A., 2006. 14-3-3 proteins: a historic overview. *Semin. Cancer Biol.*, **16**:162–172.
- AITKEN, A., 2002. Functional specificity in 14-3-3 isoform interactions through dimer formation and phosphorylation: Chromosome location of mammalian isoforms and variants. *Pl. Mol. Biol.*, **50**: 993–1010.
- BENZINGER, A., POPOWICZ, G.M., JOY, J.K., MAJUMDAR, S., HOLAK, T.A. AND HERMEKING, H., 2005. The crystal structure of the non-liganded 14-3-3 sigma protein: insights into determinants of isoform specific

- ligand binding and dimerization. *Cell Res.*, **15**:219–27.
- BRADFORD, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72**:248–254.
- BRIDGES, D. AND MOORHEAD, G.B., 2004. 14-3-3 proteins: a number of functions for a numbered protein. *Sci. STKE*, **242**:re10.
- CHANG, E.S. AND BRUCE, M.J., 1980. Ecdysteroid titers of juvenile lobsters following molt induction. *J. exp. Zool.*, **214**:157–160.
- CHONGSATJA, P.O., BOURCHOOKARN, A., LO, C.F., THONGBOONKERD, V. AND KRITTANAI, C., 2007. Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura syndrome virus infection. *Proteomics*, **7**: 3592–3601.
- DARLING, D.L., YINGLING, J. AND WYNshaw-BORIS, A., 2005. Role of 14-3-3 proteins in eukaryotic signaling and development. *Curr. Top. Dev. Biol.*, **68**:281-315.
- FERL, R.J., MANAK, M.S. AND REYEA, M.F., 2002. The 14-3-3s. *Genome Biol.*, **3**: 3010.1–3010.7
- FU, H., SUBRAMANIAN, R.R. AND MASTERS, S.C., 2000. 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.*, **40**:617–647.
- GARDINO, A.K., SMERDON, S.J. AND YAFFE, M.B., 2006. Structural determinants of 14-3-3 binding specificities and regulation of subcellular localization of 14-3-3-ligand complexes: a comparison of the X-ray crystal structures of all human 14-3-3 isoforms. *Semin. Cancer Biol.*, **16**:173–182.
- GARDINO, A.K. AND YAFFE, M.B., 2011. 14-3-3 proteins as signaling integration points for cell cycle control and apoptosis. *Semin. Cell Dev. Biol.*, **22**: 688–695.
- HARLOW, E. AND LANE, D., 1999. *Using antibodies: A laboratory manual*. Cold Spring Harbor Laboratory Press.
- KAEODEE, M., PONGSOMBOON, S. AND TASSANAKAJON, A., 2011. Expression analysis and response of *Penaeus monodon* 14-3-3 genes to salinity stress. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **159**: 244-251.
- KELLER, R. AND SCHMID, E., 1979. In vitro secretion of ecdysteroids by Y-organs and lack of secretion by mandibular organs of the crayfish during molt induction. *J. Comp. Physiol.*, **130**: 347–353.
- LIVAK, K.J. AND SCHMITTGEN, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2-[Delta] [Delta] CT method. *Methods*, **25**: 402-408.
- MACKINTOSH, C., 2004. Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *Biochem. J.*, **381**:329–342.
- MHAWECH, P., 2005. 14-3-3 proteins—an update. *Cell Res.*, **15**: 228-236.
- MOORE, B.W. AND PEREZ, V.J., 1967. Specific acidic proteins of the nervous system. In: *Physiological and biochemical aspects of nervous integration* (ed. F.D. Carlson). The Marine Biological Laboratory, Prentice-Hall, Inc., Woods Hole, MA, pp. 343–359.
- ROSENQUIST, M., ALSTERFJORD, M., LARSSON, C. AND SOMMARIN, M., 2001. Data mining the Arabidopsis genome reveals fifteen 14-3-3 genes. Expression is demonstrated for two out of five novel genes. *Pl. Physiol.*, **127**:142–149.
- SHU, M.A., ZHANG, L.T., XU, B.P., HU, H.J. AND GUO, X.L., 2012. The full length cDNA cloning and expression profile of 14-3-3 gene from the mud crab (*Scylla paramamosain*). *J. Fish. China*, **36**: 1193-1200.
- OBSIL, T. AND OBSILOVA, V., 2011. Structural basis of 14-3-3 protein functions. *Semin. Cell Dev. Biol.*, **22**: 663–672.
- TZIVION, G. AND AVRUCH, J., 2002. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *J. Biol. Chem.*, **277**:3061–3064.
- VAN HEMERT, M.J., NIEMANTSVERDRIET, M., SCHMIDT, T., BACKENDORF, C. AND SPAINK, H.P., 2004. Isoform-specific differences in rapid nucleocytoplasmic shuttling cause distinct subcellular distributions of 14-3-3 $\sigma$  and 14-3-3 $\zeta$ . *J. Cell Sci.*, **117**: 411-1420.
- WANG, H.C., WANG, H.C., LEU, J.H. KOU, G.H., WANG, A.H. AND LO, C.F., 2007. Protein expression profiling of the shrimp cellular response to white spot syndrome virus infection. *Dev. Comp. Immunol.*, **31**: 672–686.
- WANG, W. AND SHAKES, D.C., 1996. Molecular evolution of the 14-3-3 protein family. *J. mol. Evol.*, **43**:384–398.
- WANNA, W., THIPWONG, J., MAHAKAEW, W. AND PHONGDARA, A., 2012. Identification and expression analysis of two splice variants of the 14-3-3 epsilon from *Litopenaeus Vannamei* during WSSV infections. *Mol. Biol. Rep.*, **39**:5487-5493.
- WILKER, E.W., GRANT, R.A., ARTIM, S.C. AND YAFFE, M.B., 2005. A structural basis for 14-3-3 sigma functional specificity. *J. Biol. Chem.*, **280**:18891–18898.
- YAFFE, M.B. AND ELIA, A.E., 2001. Phosphoserine/threonine-binding domains. *Curr. Opin. Cell Biol.*, **13**:131–138.
- YAFFE, M.B., RITTINGER, K., VOLINIA, S., CARON, P.R., AITKEN, A., LEFFERS, H., GAMBLIN, S.J., SMERDON, S.J. AND CANTLEY, L.C., 1997. The structural basis for 14-3-3: phosphopeptide binding specificity. *Cell*, **91**:961-971.

(Received 1 November 2013, revised 5 December 2013)