



Molecular cloning and functional analysis of the fatty acid-binding protein (*Sp-FABP*) gene in the mud crab (*Scylla paramamosain*)

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Abstract

Intracellular fatty acid-binding proteins (FABPs) are multifunctional cytosolic lipid-binding proteins found in vertebrates and invertebrates. In this work, we used RACE to obtain a full-length cDNA of *Sp-FABP* from the mud crab *Scylla paramamosain*. The open reading frame of the full length cDNA (886 bp) encoded a 136 amino acid polypeptide that showed high homology with related genes from other species. Real-time quantitative PCR identified variable levels of *Sp-FABP* transcripts in epidermis, eyestalk, gill, heart, hemocytes, hepatopancreas, muscle, ovary, stomach and thoracic ganglia. In ovaries, *Sp-FABP* expression increased gradually from stage I to stage IV of development and decreased in stage V. *Sp-FABP* transcripts in the hepatopancreas and hemocytes were up-regulated after a bacterial challenge with *Vibrio alginolyticus*. These results suggest that *Sp-FABP* may be involved in the growth, reproduction and immunity of the mud crab.

Keywords: fatty acid-binding protein, immunity, ovary development, real-time quantitative PCR, *Scylla paramamosain*.

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Introduction

Fatty acid-binding proteins (FABPs) are small (14-15 kDa), ubiquitous, multigenic cytosolic proteins that bind non-covalently to hydrophobic ligands, mainly fatty acids (FAs) (Esteves and Ehrlich, 2006). Apart from functioning as energy sources, FAs can act as signaling molecules (Sumida *et al.*, 1993; Graber *et al.*, 1994; Nunez, 1997) and regulate Na⁺, K⁺, Ca²⁺ and Cl⁻ ion channels (Ordway *et al.*, 1991; Kang and Leaf, 1996; Xiao *et al.*, 1997; Liu *et al.*, 2001). FAs also have a role in gene transcription, especially genes that encode proteins involved in lipid metabolism (DeWille and Farmer 1993; Martin *et al.*, 1997; Clarke 2000; Louet *et al.*, 2001). FABPs are therefore indirectly involved in biological responses mediated by FAs.

Since the isolation of the first invertebrate FABP from the desert locust, *Schistocerca gregaria*, by Haunerl and Chisholm (1990), a growing number of FABPs have been identified in invertebrates. In vertebrates and invertebrates, FABPs have a wide range of crucial biological roles, including the regulation of cellular lipid homeostasis, cell growth and differentiation, cellular signaling, gene transcription and cytoprotection (Zimmerman and Veerkamp, 2002). Studies in knockout mice have confirmed the importance of FABPs in the uptake and transport of long-chain fatty acids and their interaction with other transport systems and enzymes (Coburn *et al.*, 2000). Moreover, studies

with the Chinese mitten crab *Eriocheir sinensis* have shown that *Es-FABP* expression levels vary with the stage of ovarian development (Gong *et al.*, 2010).

FABPs may have a role in the immune reactions of invertebrates and vertebrates. Sm14, the first platyhelminth FABP isolated from the parasite *Schistosoma mansoni* (Moser *et al.*, 1991), is a highly immunogenic peptide that offers important protection against experimental infections in cattle and other animals (Tendler *et al.*, 1996). Homologous proteins such as Sj-FABPc from *Schistosoma japonicum* (Becker *et al.*, 1994), Fh15 from *Fasciola hepatica* (Rodríguez-Pérez *et al.*, 1992) and FgFABP from *Fasciola gigantica* (Estuningsih *et al.*, 1997) also provide protection from challenge with infectious agents. In crustaceans, FABPs are known to be correlated with immunity. In *Litopenaeus vannamei* (Zhao *et al.*, 2007), *Penaeus stylirostris* (Dhar *et al.*, 2003), *Procambarus clarkii* (Zeng and Lu, 2009) and *Fenneropenaeus chinensis* (Wang *et al.*, 2008; Ren *et al.*, 2009) the expression levels of FABPs were up-regulated after a challenge with infectious agents.

Mud crabs (*Scylla* spp.) are a group of four commercially important Portunid species that are found in intertidal and subtidal, sheltered, soft-sediment habitats, particularly mangroves, throughout the Indo-Pacific region (Le Vay *et al.*, 2008). In this report, we provide the first description of the cDNA structure, phylogenetic relationships and tissue distribution of an intracellular FABP from the mud crab *Scylla paramamosain*. The levels of *Sp-FABP* expression

in different stages of ovarian development and after microbial infection were also examined.

Material and Methods

Tissue preparation

Healthy adult female crabs were purchased from a local market in Xiamen, Fujian Province, China. Samples from ten tissues (epidermis, eyestalk, gills, heart, hemocytes, hepatopancreas, muscle, ovary, stomach and thoracic ganglia) were collected. The ovarian samples were collected based on the classification of Shanguan and Liu (1991) for ovarian developmental stages I (undeveloped), II (early-developing), III (developing), IV (nearly ripe) and V (ripe). All tissues were immediately frozen in liquid nitrogen and stored at -80 °C until nucleic acid extraction.

For the immune challenge, *S. paramamosain* crabs from Dongshan farm in Zhangzhou, Fujian Province, China, were injected with *Vibrio alginolyticus* (1×10^7 CFU/mL; 20 µL) at the base of the right fourth pleopod (Cheng *et al.*, 2004). Control crabs were injected with an equal volume of sterile saline solution. A total of 24 crabs per group were used, with three crabs for each time interval. At 0 (basal), 3, 6, 12, 24, 48, 72 and 96 h post-injection the hepatopancreas and hemocytes were collected from individuals injected with saline (control) or *V. alginolyticus* and preserved with RNAsafer stabilizer reagent (TaKaRa, Japan).

Nucleic acid extraction

RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA concentration and quality were assessed spectrophotometrically based on the absorbance of 260 nm (NanoDrop Technologies, Inc., USA) and by agarose gel electrophore-

sis, respectively. Total RNA was reverse transcribed using a PrimeScript RT-PCR kit with oligo (dT)₁₈ primers (TaKaRa, Japan).

Full-length cDNA cloning

To clone the cDNA, *FABP* sequences were downloaded from NCBI and aligned using ClustalX. A pair of degenerate primers, FABPF1 and FABPR1 (Table 1), was designed based on the conserved regions. The PCR was done in an ABI 2720 Thermal Cycler in a total volume of 25 µL containing 2.5 µL of 10x PCR buffer (containing Mg²⁺), 2.0 µL of dNTP mix (2.5 mM each), 1 µL of each primer (10 µM), 2 µL of cDNA (500 ng/µL), 0.125 µL of *Taq* polymerase (5 U/µL; TaKaRa), and 16.375 µL of RNase-free water. The PCR conditions were as follows: 94 °C for 5 min, 32 cycles of 94 °C for 30 s, 46 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR products were assessed visually after electrophoresis on 1.2% agarose gels and those of appropriate size were purified, ligated into a pMD19-T Vector (TaKaRa) and then transformed in *Escherichia coli* by overnight culture. Positive clones with inserts of the predicted size were sequenced using the primers M13-47 and RV-M (Table 1) at Sangon Biotech Co., Ltd (Shanghai, China).

The 3' and 5' end fragments were completed by 3' and 5' rapid amplification of cDNA ends (RACE) with a 3', 5' full RACE kit (TaKaRa). Specific primers based on the initial sequence (FABP3RACE and FABP5RACE), together with a 3' outer primer and a 5' outer primer (Table 1), were used in the PCRs. The full length of *Sp-FABP* was assembled by piecing together the 3' and 5' ends and the initial sequence. The sequence of the full-length cDNA was verified by using a pair of specific primers (FABPF2

Table 1 - Primers used in this study.

Primer name	Primer sequence (5'-3')	Application
FABPF1	TCBGARAACCTTYGAYGAKTTC	cDNA cloning
FABPR1	AVACRAYRTCATCDACYTTRC	cDNA cloning
M13-47	CGCCAGGGTTTTCCAGTCACG	Sequencing
RV-M	GAGCGGATAACAATTTACACA	Sequencing
FABP3RACE	GCGAGGAGTTCGAGGAGACC	3RACE
FABP5RACE	GGTCTCCTCGAACTCCTCGC	5RACE
3Outerprimer	TACCGTCGTTCCACTAGTGATTT	3RACE
5Outerprimer	CATGGCTACATGCTGACAGCCTA	5RACE
FABPF2	ACCAGTCGCGGGGAAGCCA	Full-length confirmation
FABPR2	TTAGAATGTGGACATTTAATATATAAAGGTTA	Full-length confirmation
FABPF3	CACACTTCACAGACCTTC	qRT-PCR
FABPR3	CACAATGCCATCCTCTAC	qRT-PCR
Actin F	GAGCGAGAAATCGTTCGTGAC	Internal control
Actin R	GGAAGGAAGGCTGGAAGAGAG	Internal control

and FABPR2; Table 1) designed based on the preliminary sequencing results.

Homology and phylogenetic analysis

The *Sp-FABP* nucleotide and deduced amino acid sequences were compared to those reported for other organisms using the BLAST algorithm at the National Center for Biotechnology Information. The amino acid sequences of FABP from *S. paramamosain* and representative taxa were retrieved from NCBI GenBank and analyzed using ClustalX software. The open reading frame (ORF) of the cloned *Sp-FABP* cDNA was determined with the ORF Finder, and SignalP 4.0 software was used to identify the putative signal peptide. Hydrophobic regions were predicted with Protscal. A neighbor-joining (NJ) phylogenetic tree was constructed using MEGA software v. 5.0 based on 1000 bootstraps.

Real-time quantitative PCR analysis

Total RNA levels of various mud crab tissues, of ovaries at different stages of development and of the hepatopancreas and hemocytes after bacterial challenge were examined by real-time quantitative PCR (qRT-PCR). The final volume of each qRT-PCR was 20 μ L and contained 10 μ L of 2 x SYBR Premix Ex *Taq* (TaKaRa), 1 μ L of diluted cDNA template, 0.5 μ L of each primer (10 mM FABPF3 and FABPR3; Table 1) and 8 μ L of PCR-grade water. A β -actin fragment was amplified using the primer pair Actin F and Actin R (Table 1) and served as an internal control (Huang *et al.*, 2012). The cDNA template PCR conditions were as follows: 95 $^{\circ}$ C for 30 s, 50 cycles of 95 $^{\circ}$ C for 10 s, 60 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 20 s and a final extension at 72 $^{\circ}$ C for 10 min. All samples were run in triplicate and the *Sp-FABP* expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative CT method. The results were expressed as the mean \pm SD (standard deviation) of triplicate determinations and shown as the n-fold difference relative to β -actin. Statistical comparisons were done using Students *t*-test and a value of $p < 0.05$ indicated significance.

Results

Cloning and identification of *Sp-FABP* cDNA

A full-length (885 bp) *FABP* cDNA (*Sp-FABP*) was isolated from the ovaries of female mud crabs (GenBank: JQ824129). The sequence of the *Sp-FABP* gene contained an ORF of 411 bp (including the stop codon), with 5' and 3' untranslated regions of 67 bp and 407 bp, respectively (Figure 1). A single polyadenylation signal (ATTTAAA) was observed 856 bp upstream of the 12 bp poly (A) tail. The ORF coded for a polypeptide of 136 amino acids, with a calculated molecular mass of 15, 381.67 Da and an isoelectric point of 5.55. Analysis of the *Sp-FABP* cDNA sequence using ClustalX revealed significant similarity to

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1          accagtcgcggggaagccaggc
23  cgtcgagcagtatagcaccgccacgccatccaactcaacgaaa
68  atggccaagatcgtgggaaagtacaagatggactcatccgagggc
   M A K I V G K Y K M D S S E G   15
113  ttcgatgatttcatgaaggccttgggtgtgggaatggtgatgagg
   F D D F M K A L G V G M V M R   30
158  aagatgggcaacgccgccacgcccacggaggatcacgctggat
   K M G N A A T P T V E I T L D   45
203  gacggcacctacactcttaagaccgtcaccacctcaagaccacc
   D G T Y T L K T V T T F K T T   60
248  gaaatcaagttcaagctggcgaggagttcaggagaccactgct
   E I K F K L G E E F E E T T A   75
293  gatggcgcaccgtgaagtcaccatcaccctggacgggcaacaaa
   D G R T V K S T I T L D G N K   90
338  ttgatacacaaccagggtgggagacaaggagaagaagaaggat
   L I H N Q V G D K E K K E K D   105
383  tccgtgctcaccctgaattcactgacacagagatgctcatggaa
   S V L T R E F T D T E M L M E   120
428  tgcaagttgatgatattgtgtgtaaactgtctacaagcgcag
   C K V D D I V C K R V Y K R Q   135
473  gattagaggacagcagcagtggtgtcactggttatgcaagagtga
   D *                               136
518  gcagcagacaattgcaagtcctcttcagcttgaatgaaggaagg
563  ttattgtagaatttcttttagtatcatattaattttgttattgca
608  ttattttattttgattaaattatagtttttcataatttactgat
653  cttgtatttcattgaagctgaattcttttctcaaaagattccaaa
698  cctaattgactaaaagtgaagctcattttgttagttgaactcattt
743  cctttgataattatcagatactgttcttctctttttgagaatact
788  gttcttgattcttaagaaccaaaagttaatgcatgatcatattaa
833  aggaattttgtaacctttatatATTTAAAtgtccacattctaaaa
   aaaaaaaaa

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Figure 1 - Complete cDNA sequence (GenBank: JQ824129) and deduced amino acid sequence for *Sp-FABP*. The stop codon is marked by an asterisk. The polyadenylation signal (ATTTAAA) is enclosed by a black rectangle.

the sequences of other *FABPs* included in the NCBI database. No signal peptide was identified by the SignalP 4.0 Server.

Homology and phylogenetic analysis of *Sp-FABP*

ClustalX alignment of the deduced amino acid sequence with other related sequences revealed a high degree of similarity: 85% identity with the shrimp *Penaeus monodon*, 83% identity with the Chinese mitten crab *E. sinensis* and 60% identity with the ant *Acromyrmex echinator* (Figure 2).

An NJ phylogenetic tree was constructed based on reported FABP sequences using MEGA5.0 software (Figure 3). The reliability of the branching was tested by bootstrap resampling (with 1000 pseudo-replicates). Two distinct sister groups were observed, with a tree topology that agreed with traditional taxonomic relationships. The first group contained invertebrate FABPs (from *E. sinensis*, *S. paramamosain*, *Penaeus monodon*, *Litopenaeus vannamei* and *Apis mellifera*) while the second group contained vertebrate FABPs (*Danio rerio*, *Salmo salar*, *Gallus gallus*, *Homo sapiens* and *Sus scrofa*).

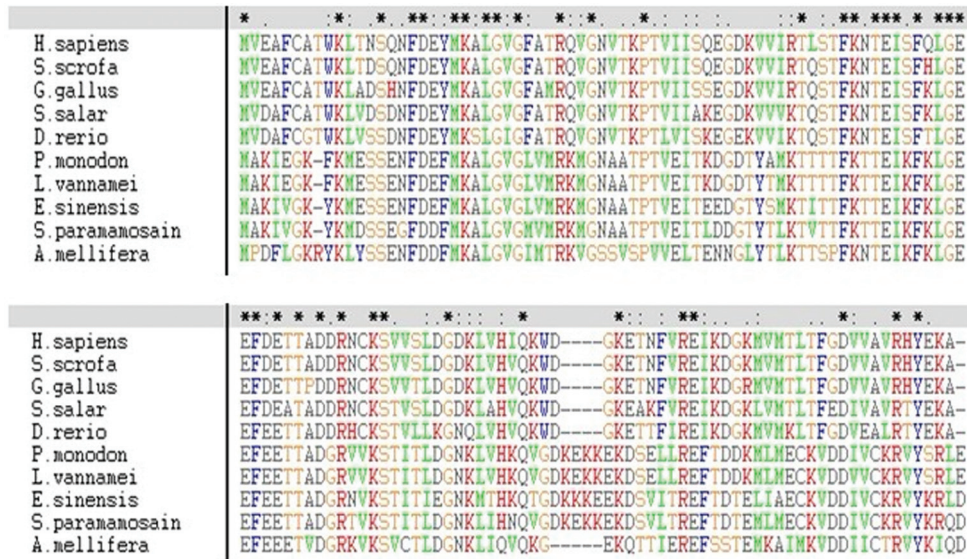


Figure 2 - ClustalX alignment of vertebrate and invertebrate FABP amino acid sequences. Alignment shows the following sequences (GenBank accession numbers in parentheses): *Apis mellifera* (NP_001011630.1), *Danio rerio* (NP_999972.1), *Eriocheir sinensis* (ADM64456.1), *Gallus gallus* (NP_990639.1), *Homo sapiens* (AAB87141.1), *Litopenaeus vannamei* (ADK66280.1), *Penaeus monodon* (ABE77154.1), *Salmo salar* (NP_001135371.1), *Scylla paramamosain* (JQ824129) and *Sus scrofa* (NP_001020400.1).

A homology model of Sp-FABP predicted using the SWISS-MODEL database revealed conservation of the tertiary structure, with the 10 anti-parallel β-strands forming a barrel and a clamshell-like structure.

Sp-FABP expression in different tissues and in ovaries at various reproductive stages

qRT-PCR was used to investigate the distribution of *Sp-FABP* mRNA in different tissues and to assess the expression of this gene in different female reproductive stages. *Sp-FABP* showed variable levels of expression in a wide variety of tissues, including epidermis, eyestalk, gill, heart, hepatopancreas, hemocytes, muscle, ovary, stomach

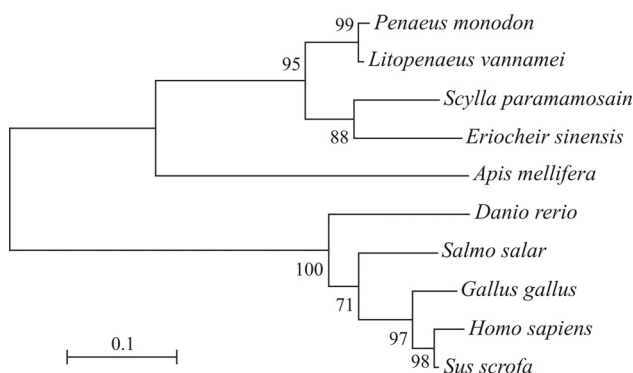


Figure 3 - Neighbor-joining phylogenetic tree of representative vertebrate and invertebrate FABP amino acid sequences. Bootstrap values supporting the branch points are expressed as the percentage of 1000 replicates. The following organisms with FABPs were included in the analysis: *Apis mellifera*, *Danio rerio*, *Eriocheir sinensis*, *Gallus gallus*, *Homo sapiens*, *Litopenaeus vannamei*, *Penaeus monodon*, *Salmo salar*, *Scylla paramamosain* and *Sus scrofa*. See Figure 2 legend for GenBank accession numbers.

and thoracic ganglia (Figure 4). *Sp-FABP* transcripts were constitutively expressed in mud crab ovary, although the level of expression varied with the stage of ovarian maturation. The expression of *Sp-FABP* increased from reproductive stage I to stage IV, when it reached a peak, and then decreased significantly at stage V (Figure 5).

Sp-FABP expression in hepatopancreas and hemocytes after a bacterial challenge

To gain insight into the involvement of FABP in the crab immune response, the expression profiles of *Sp-FABP* were assessed by qRT-PCR after a bacterial challenge. The hepatopancreas showed an increase in the level of *Sp-FABP* transcripts at all time intervals after the bacterial challenge, especially at 3 h; after 3 h, the expression of *Sp-FABP* gradually decreased, but the levels were still higher than in the control group (Figure 6). In hemocytes,

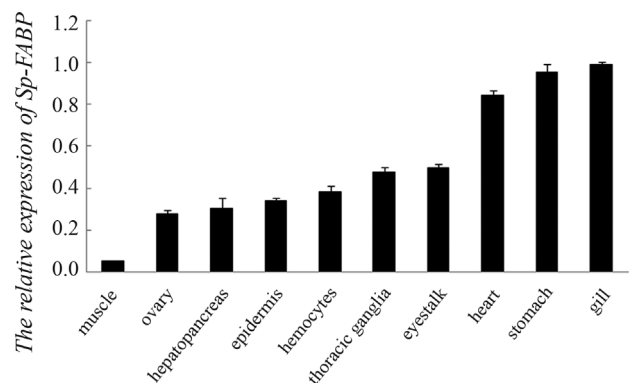


Figure 4 - Tissue distribution of *Sp-FABP* expression as determined by qRT-PCR. The columns are the mean ± SD (n = 3).

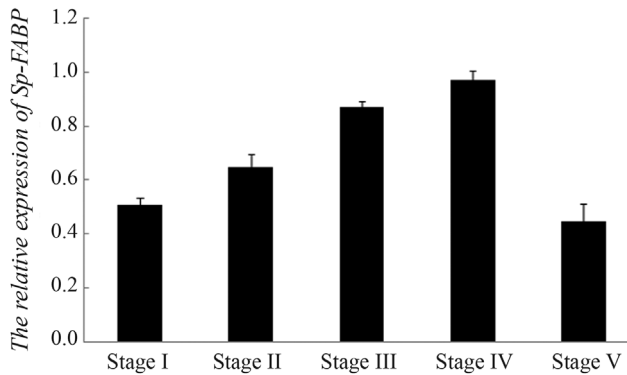


Figure 5 - *Sp-FABP* expression in different stages of ovarian development, as determined by qRT-PCR. The columns are the mean \pm SD (n = 3).

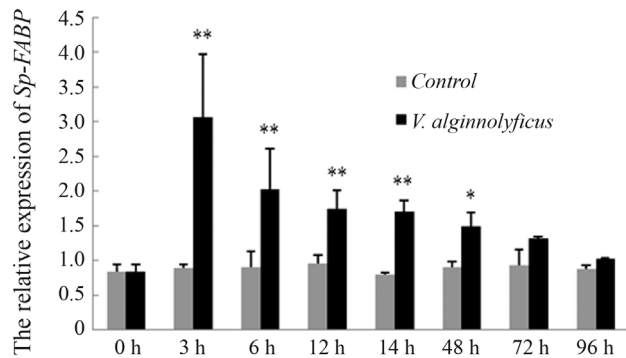


Figure 6 - Hepatopancreatic *Sp-FABP* expression after a bacterial challenge with *V. alginolyticus*, as determined by qRT-PCR. The relative abundance of the *Sp-FABP* transcripts is expressed as the mean \pm SD (n = 3). *p < 0.05 and **p < 0.01 compared to the corresponding controls.

there was a slight increase in the level of *Sp-FABP* transcripts at 3, 6 and 12 h post-challenge and a marked increase at 24, 48, 72 and 96 h post-challenge, with a peak at 72 h (Figure 7).

Discussion

FABPs belong to a large family of ubiquitous, low-molecular-mass, small cytosolic lipid-binding proteins responsible for the non-covalent binding of hydrophobic ligands, primarily fatty acids (Zimmerman and Veerkamp, 2002). The biological roles of these proteins include a wide range of processes such as the transport, cellular uptake and cytoplasmic use of FAs, and FA-mediated regulation of gene expression (Esteves and Ehrlich, 2006). FABPs have been extensively studied in vertebrates whereas considerably less is known about these proteins in invertebrates.

In the current study, the full-length *Sp-FABP* cDNA encoded a putative FABP of 136 amino acids with a theoretical molecular mass similar to that of other FABPs

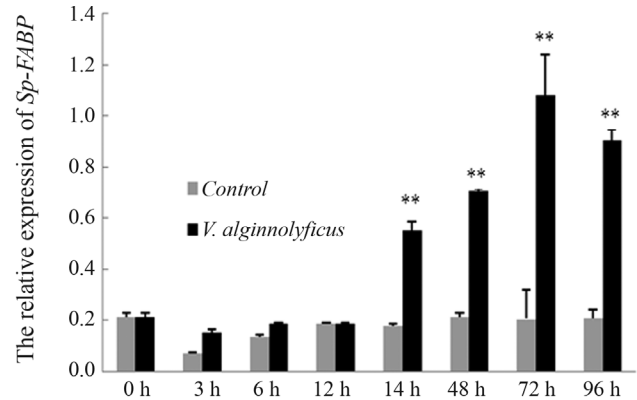


Figure 7 - *Sp-FABP* expression in hemocytes after a bacterial challenge with *V. alginolyticus*, as determined by qRT-PCR. The relative abundance of the *Sp-FABP* transcripts is expressed as mean \pm SD (n = 3). *p < 0.05 and **p < 0.01 compared to the corresponding controls.

(127-136 amino acids) (Chen *et al.*, 2006). The ClustalX alignment of *Sp-FABP* and nine other reported vertebrate and invertebrate FABP sequences revealed high identity (63-85%) among invertebrate sequences. Three-dimensional homology modeling revealed that several key tertiary structures of *Sp-FABP* were similar to those of vertebrate FABPs, such as the ten anti-parallel β -strands, their resultant barrel with a clamshell-like structure, and the barrel cap consisting of a pair of α -helices that enclose the cavity of the FABP lipid-binding site (Chmurzynska, 2006). These results confirmed the identity of the cloned *Sp-FABP*. Phylogenetic analysis revealed two distinct sister groups within the NJ tree: group 1 contained invertebrate FABPs whereas group 2 contained vertebrate FABPs. Evolutionarily, *Sp-FABP* is highly homologous to other FABPs and all FABPs are remarkably conserved.

Variable levels of *Sp-FABP* transcripts were detected in epidermis, eyestalk, gill, heart, hemocytes, hepatopancreas, muscle, ovary, stomach and thoracic ganglia. The presence of *FABP* in all of the tissues examined suggested that *FABP* was ubiquitous and indispensable to crustacean growth. The expression levels were remarkably high in gill and stomach, which may be important for the following reasons. In crustaceans, the gill is a multifunctional organ, responsible for respiratory gas exchange, hemolymph acid-base and osmo-ionic regulation, and the excretion of nitrogenous metabolites (Freire *et al.*, 2008), while the stomach is an important organ of the digestive system. Hence, *Sp-FABP* may be involved in the maintenance and adjustment of essential physiological and pathological processes in the gill and stomach.

Ovarian expression of *Sp-FABP* depended on the stage of ovarian maturation, with the level of expression increasing from stage I to stage IV (peak expression occurred in this stage) followed by a significant decrease in stage V, in agreement with a previous study (Gong *et al.*, 2010). During development, the ovary accumulates substantial

amounts of nutritional substrates, especially FAs (Milla-mena, 1989; Shangguan and Liu, 1991; Mourente *et al.*, 1994; Cheng *et al.*, 2001). FAs accelerate the expression of FABP (Kaikaus *et al.*, 1993a,b) because the tissue FABP content is associated with the rate of FA uptake and utilization (Ockner *et al.*, 1980; Glatz and Vusse, 1990; van Breda *et al.*, 1992). The variation in *Sp-FABP* expression levels with developmental stage suggests that FABP may be associated with ovarian maturation in the mud crab.

Although FABPs have been implicated in invertebrate immunity, there has not yet been any clear demonstration of a link between these two phenomena. In crustaceans, the hepatopancreas not only initiates the humoral immune response but also contains highly specialized cells and phagocytes that function in the cellular immune response (Pan *et al.*, 2005). The invertebrate innate immune system provides the major defense mechanisms against pathogenic agents and the immune responses occur mainly in hemolymph (Somboonwiwat *et al.*, 2006). The hepatopancreas and hemocytes are presumably the primary sites for the production of immune response factors. To investigate the relationship between FABP and the immune response, we initially injected bacteria into crabs and examined the expression of *Sp-FABP* after the challenge. *Sp-FABP* expression was up-regulated in hepatopancreas and hemocytes after challenge with *V. alginolyticus*, indicating that *Sp-FABP* is inducible and is involved in the immune response. Transcripts of *Sp-FABP* reached a peak at 3 h and then gradually decreased in hepatopancreas, while in hemocytes *Sp-FABP* expression was significantly up-regulated at 24, 48, 72 and 96 h post-challenge, with a peak at 72 h. Together, these findings indicate that *V. alginolyticus* stimulated *FABP* expression as part of the host defense against infection, with the response being acute in hepatopancreas and gradual but persistent in hemocytes.

Crustacean diseases have received increasing attention because pathogens such as bacteria and viruses can adversely affect the commercial breeding of these species. FABP may also be involved in hemocyte production since hemocyte numbers are higher in crayfish *Pacifastacus leniusculus* and shrimp *P. monodon* (Söderhäll, 2006). The precise role and mechanism of *FABP* in the immune response to a bacterial challenge remains to be determined. Such information would improve our understanding of processes linked to cellular damage and repair in crabs, and help in the development of more effective methods for mitigating crab diseases.

In conclusion, the results of this study provide evidence for a role of *Sp-FABP* in lipid metabolism during ovarian development and in the defense response after a bacterial challenge.

Acknowledgments

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Internet Resources

- ORF Finder, <http://www.ncbi.nlm.nih.gov/gorf> (June 5, 2012).
- SignalP 4.0 software, <http://www.cbs.dtu.dk/services/SignalP> (June 5, 2012).

Protscal, <http://www.expasy.ch/tools/protscale.html> (June 5, 2012).
MEGA software ver. 5.0, <http://www.megasoftware.net> (June 5, 2012).
SWISS-MODEL database, <http://swissmodel.expasy.org> (June 5, 2012).

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