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Short communication

Molecular cloning and characterization of acyl-CoA binding protein (ACBP) gene from shrimp *Penaeus monodon* exposed to salinity stress

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ABSTRACT

Acyl-CoA binding protein (ACBP), a protein present ubiquitously in wide range of organisms play significant role in transport of acyl groups for macromolecular biosynthesis involved in various functional and regulatory processes. In crustaceans, ACBP has functional role in growth, reproduction and temperature tolerance. In the present study, two suppression subtractive hybridization (SSH) cDNA libraries were performed using gut tissues of shrimp *Penaeus monodon* exposed to low (3ppt) and high (55ppt) salinity stress conditions. SSH library resulted in identification of differentially expressed genes that belonged to various functional classes such as the nucleic acid regulation and replication, defence proteins, allergen protein, signal transduction pathways, apoptosis, energy and metabolism, cell cycle regulation and hypothetical proteins. ACBP was identified as one of the differentially expressed gene in both the SSH libraries of shrimp *P. monodon* subjected to low and high salinity stress. The full-length cDNA of *P. monodon* ACBP gene was isolated and the sequence revealed 273 bp open reading frame encoding 90 amino acids with molecular mass of 10 kDa and pI 6.8. The ORF showed presence of four phosphorylation sites, with absence of signal peptide sequence and glycosylation sites. The deduced amino acid sequence of ACBP exhibited high sequence identity (92%) with ACBP class of protein identified from *Fenneropenaeus chinensis*. Real time PCR analysis of shrimps subjected to 3ppt salinity conditions after 2 weeks revealed an increase in expression of ACBP transcripts, in the gut (28.08-folds), gills (11.71-folds) and in the muscle tissues (1.70-folds). Whereas, shrimps exposed to 55ppt salinity conditions after 2 weeks exhibited increased ACBP transcript levels in the gut (11.95-folds), gills (1.052-folds) and muscle tissues (7.35-folds). The significant increase in expression levels of ACBP in various tissues of shrimps suggests a functional role of this gene in salinity stress tolerance and adaptation.

1. Introduction

Acyl-CoA binding protein (ACBP) has been identified as a highly conserved 10 kDa cytosolic protein. The protein is known to function as a neuropeptide involved in inhibiting of diazepam (DBI)/endozepine (EP) binding to GABA receptor system (Guidotti et al., 1983; Kragelund et al., 1999). ACBP belongs to a multigene family of protein that is conserved from yeast to mammals (Elholm et al., 2000). ACBP is involved in multiple functions such as modulation of fatty acid biosynthesis, enzyme-regulation, regulation of the intracellular acyl-CoA pool, donation of acyl-CoA esters for β-oxidation, vesicular trafficking and gene regulation (Burton et al., 2005). ACBP is suggested to be involved in the transport of newly synthesized acyl-CoA esters from the fatty acid synthetase to acyl-CoA-consuming processes in yeast (Schjerling et al., 1996), as acyl-CoA transporter (Elholm et al., 2000) and in m-calpain regulation process (Melloni et al., 2000) in rats. Different types of ACBPs are reported to be encoded by set of genes in plants *Arabidopsis thaliana* (Xiao and Chye, 2009) such as the membrane-associated proteins ACBP1 and ACBP2 (Chye, 1998; Chye et al., 1999, 2000; Li and Chye, 2003), the extracellularly targeted ACBP3 (Leung et al., 2006) and the cytosolic proteins ACBP4, ACBP5 and ACBP6 (Chen et al., 2008; Xiao et al., 2008). *Arabidopsis* over expressing ACBP2 showed improved tolerance to heavy metal (Cd) stress suggesting the role of ACBP2 in membrane repair (Gao et al., 2008; Gao et al., 2010). Transgenic *A. thaliana* with high expression of ACBP6 along with decrease in phosphatidyl choline and accumulation of phosphatic acid displayed an enhanced freezing tolerance (Chye et al., 1999) and ACBP1 in transgenic *A. thaliana* has been reported to be specifically playing a role in freeze stress (Du et al., 2010). *Saccharomyces cerevisiae* with depleted levels of ACBP resulted in severe distortions in membrane assembly, organization and trafficking suggesting its function in maintenance of cellular integrity (Gaigg et al., 2001).

In invertebrates such as shrimp *Fenneropenaeus chinensis*, studies have shown that ACBP might possess antibacterial activity against *Vibrio anguillarum* (Ren et al., 2009). ACBP was identified

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as differentially expressed gene from white spot syndrome virus resistant shrimp *Litopenaeus vannamei* suggesting its role in shrimp immune response against viral infections (Zhao et al., 2007).

However, the functional role of ACBP in shrimp in response to biotic and abiotic stress remains poorly understood. In the present study, as an approach to identify genes that play critical role in salinity tolerance, suppression subtractive hybridization (SSH) libraries were constructed from *Penaeus monodon* gut tissues. In the present study ACBP has been identified as one of the differentially expressed gene in shrimp subjected to salinity stress. The gene has been cloned and characterized for the first time from shrimp *P. monodon* and the transcription profiles in various tissues during low (3ppt) and high (55ppt) salinity induced stress has been analyzed and its relevance towards salinity stress is discussed.

2. Materials and methods

2.1. Collection of shrimps, salinity conditions and tissue samples

P. monodon shrimps (10–15 g) were procured from the shrimp farms located in Chennai, India. The shrimps (intermolt stage) used in the experiment were divided into three groups of six numbers each and were acclimatized to three different salinity conditions. Low salinity levels (3ppt) were achieved by reducing the salinity of sea-water by 2ppt per day by adding fresh water. High salinity levels (55ppt) were achieved by increasing the salinity by 2ppt per day using brine. The third group of shrimps were maintained in normal sea-water (28ppt) as control group, without altering the salinity conditions. The gills, gut and muscle tissue samples from six shrimps maintained for a period of 2 weeks at low (3ppt), high (55ppt) and 28ppt salinity conditions were collected in RNA*later* (Qiagen, USA) and stored in −80 °C till further use.

2.2. Construction of SSH cDNA library

Two SSH cDNA libraries were constructed separately using gut tissues of the 3ppt and 55ppt salinity induced shrimp samples. Pooled gut tissues (six numbers in each group) collected from the experimental (3ppt or 55ppt) and control shrimp served as tester and driver respectively. The SSH cDNA libraries were constructed following the procedure described in PCR-Select cDNA subtraction kit (Clontech, USA) and as reported previously (Rajesh et al., 2012).

2.3. Screening of SSH cDNA library

The PCR products obtained by amplification of SSH clones were ligated into pGEM-T Easy vector (Promega, USA) and transformed into competent *Escherichia coli* DH5α cells. The recombinant clones were screened by colony PCR for the insert cDNAs with the vector primers (T7 forward and SP6) and sequenced (Scigenom technologies, India). The sequences were assembled using KEGG (Kyoto Encyclopedia of Genes and Genomes) software (www.genome.jp/kegg) and were grouped into contigs and singletons. Sequence analysis by BLASTX, BLASTN or TBLASTX (www.ncbi.nlm.nih.gov/blast) was used to identify differentially expressed genes in cDNA libraries constructed from low and high salinity shrimp groups.

2.4. Amplification and cloning of P. monodon ACBP gene

The ACBP gene amplification of *P. monodon* was carried out using gene specific forward and reverse primers (ACBP-F and ACBP-R) that were designed based on the reported *F. chinensis* ACBP gene (Ren et al., 2009) (Supplementary data, Table 1). The PCR reaction conditions included initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and final extension at 72 °C for 7 min. The cloned ACBP gene was confirmed by sequencing.

2.5. Rapid Amplification of cDNA ends (RACE) of P. monodon ACBP

Total RNA was extracted and mRNA was purified using NucleoSpin RNA II kit and NucleoTrap mRNA mini kit respectively from gut tissues of shrimps following the manufacturer's instructions (Macherey Nagel, Germany). 5′ and 3′ RACE were performed using FirstChoice RLM – RACE kit following manufacturer's instructions (Ambion, USA). The sequences of primers used in RACE are shown in Supplementary data, Table 1.

2.6. Sequence analysis of P. monodon ACBP gene

Nucleotide sequence analysis of *P. monodon* ACBP was performed by BLASTN (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1997) and the sequence was submitted in GenBank database. ACBP sequence was translated using the TRANSLATE tool in Expasy proteomics server (www.expasy.org). The multiple sequence alignment of ACBP protein was performed by CLUSTALW method (Higgins et al., 1992) in Expasy proteomics server (http://www.ebi.ac.uk/tools/msa/clustalw2/). Phylogenetic tree was constructed (PAM250) using MEGA 5 software (Tamura et al., 2011) with the Neighbour-Joining method and bootstrap analysis was performed for 1000 replicates. Protein sequence of *P. monodon* ACBP was analyzed for phosphorylation sites (www.cbs.dtu.dk/services/NetPhos), potential glycosylation sites (http://www.cbs.dtu.dk/services) and signal peptide cleavage site (http://www.cbs.dtu.dk/services/SignalP). The tertiary structure of *P. monodon* ACBP was developed with the Geno3D software tool (http://geno3d-pbil.ibcp.fr) using armadillo ACBP (2FDQ) as template.

2.7. P. monodon ACBP mRNA expression analysis by Real-time PCR

Total RNA extracted using NucleoSpin RNA II kit (Macherey–Nagel, Germany) from gills, gut and muscle tissues of the control, low and high salinity stressed shrimps were converted to cDNA using Protoscript M-MuLV first strand cDNA synthesis kit (New England Biolabs, USA). The cDNA was used to analyze the relative expression of ACBP transcripts by real-time PCR using the Power SYBR green PCR master mix (Applied Biosystems, UK). The gene specific primers for ACBP (ACBP RT-F and ACBP RT-R) were used to generate 89 bp PCR products for real time analysis. The shrimp β-actin gene was amplified with primers (β-actin RT-F and β-actin RT-R) to generate 124 bp product which was used as an endogenous control (Pongsomboon et al., 2009) (Supplementary data, Table 1). The relative quantification results were expressed as the fold change in levels of the gene expression and statistical analysis of the data for comparison between groups was carried out by one-way ANOVA and the values with $p < 0.05$ were considered significant.

3. Results and discussion

The SSH cDNA library generated multiple colonies, representing genes up-regulated in gut tissues of low and high salinity stressed *P. monodon* shrimps. An approximate of 500 clones was obtained from low and high salinity stressed shrimps with the insert size ranging from 200 to 1.5 kb. Clustering of ESTs using KEGG software generated 19 contigs and 32 singletons from low salinity SSH cDNA library; 10 contigs and 39 singletons from high salinity group respectively. SSH clones were grouped into different classes based on the predicted functional category by BLAST analysis (Table 1) (Supplementary data, Fig. 1A and B).

The putative ACBP gene was identified to be differentially regulated in the gut of both low and high salinity SSH libraries. The complete cDNA sequence of ACBP consisted of 273 bp ORF coding for 90 amino acids corresponding to a molecular mass of 10 kDa and pI 6.8 with 83 and 198 bp UTR at the 5′ and 3′ terminal regions respectively. The complete ORF sequence of ACBP from *P. monodon* was submitted in Genbank with accession number (JN572541). *P. monodon* ACBP nucleotide and deduced amino acid sequence obtained in this study is given in Supplementary data, Fig. 2.

BLAST analysis of the putative ACBP isolated from *P. monodon* (JN572541) exhibited 97% and 99% nucleotide and amino acid sequence homology respectively with the reported ACBP sequence from *F. chinensis* (GQ377109). The deduced amino acid sequence of *P. monodon* ACBP exhibited similarity with the ACBP class of proteins from *F. chinensis* (97%), *L. vannamei* (95%), *Drosophila ananassae* (72%), *Mellagris gallopavo* (71%), *Loxodanta africana* (75%), *Taeniopygia guttata* (72%), and *Culex quinquefasciatus* (71%). The multiple sequence analysis of *P. monodon* ACBP protein sequence revealed one amino acid substitution (phenyl alanine for tyrosine) at position 33 in *F. chinensis*. A total of 19 amino acids were conserved among the various ACBP sequences (Supplementary data, Fig. 3). The phylogenetic tree analysis showed the presence of *P. monodon* ACBP and *F. chinensis* ACBP in the same clade (Supplementary data, Fig. 4).

Protein sequence analysis of *P. monodon* ACBP suggested the presence of four phosphorylation sites as analyzed by NetPhos

Table 1
The differentially expressed genes identified in gut tissues of low salinity (3ppt) and high salinity (55ppt) stressed *P. monodon* shrimps.

Putative function	Acc. No.	Size (bp)	*E*-value	No. of clones
Low salinity				
Nucleic acid regulation and replication				
Polymerase	AEL29690	353	4.62e-5	2
Nucleocapsid protein	AFB82712	314	1.32e-6	1
Defence proteins				
Ferritin	AY955373	246	4.00e-49	2
Relish	JQ728539	329	2.96e-52	1
Lysozyme	AY257549	276	7.72e-10	1
Allergen protein				
Allergen Pen m 2	AF479772	621	1.26E-30	1
Signal transduction				
Adenylate cyclase	ABK91814	245	2.90e-15	1
Apoptosis				
Cathepsin B	ABQ10737	318	9.11e-74	1
Energy and metabolism				
Acyl-CoA-binding protein	AEP84099	334	4.08 e-45	3
Serine protease	HQ288088	174	4.20e-4	1
ATP synthase	NP038292	261	2.82e-28	1
Cytochrome c oxidase subunit II	NP038290	295	4.53e-44	1
Na^+/K^+-ATPase alpha subunit	ABD59803	147	5.84e-16	1
Glucose-6-phosphate dehydrogenase	JQ771576	277	7.26e-4	1
Eukaryotic translation initiation factor 3	XR084200	267	7.83e-31	1
Cofilin/actin-depolymerizing factor homolog	XP003701502	492	1.11e-54	1
Glycoprotein	NP941986	222	2.30e-5	1
Hypothetical protein				12
Unknown				18
High salinity				
Nucleic acid regulation and replication				
RNA polymerase	NP941973	359	0.021	1
Transposase	CAL47051	435	0.027	1
Defence proteins				
Immunoglobulin heavy chain variable region	AAK67989	1049	6.6	1
Signal transduction				
Putative senescence-associated protein	ABO20851	431	9e-37	1
Sensor histidine kinase	ZP07294336	511	7.8	1
Protocadherin alpha-3-like	XP003480984	701	0.91	1
Apoptosis				
Endonuclease G	YP008924	221	8.9	1
Cathepsin B	ABQ10737	290	9e-68	1
Energy and metabolism				
Proline-rich extensin-like family protein	AEE82632	120	5.0	1
Cytochrome b	NP038299	583	3e-100	1
Elongation factor 2	ABR01223	1165	0.0	1
Glucuronosyltransferase	NP001170967	448	1e-04	1
Acyl-CoA Binding protein (ACBP)	ACU82846	635	4e-54	2
Fructose 1,6-bisphosphate aldolase	NP001091766	600	2e-60	1
2-isopropylmalate synthase	ZP02963711	359	3.8	1
Cytoskeletal protein				
Collagen alpha-1(XI) chain	NP001077313	383	3.6	1
Membrane protein				
Small oligopeptide transporter, OPT family	XP002340616	142	2.8	1
Amino acid transporter	XP001864053	365	3.8	1
Membrane protein	YP002561656	461	4.0	1
Inner-membrane translocator	YP533555	290	9.3	1
Hypothetical protein				15
Unknown				13

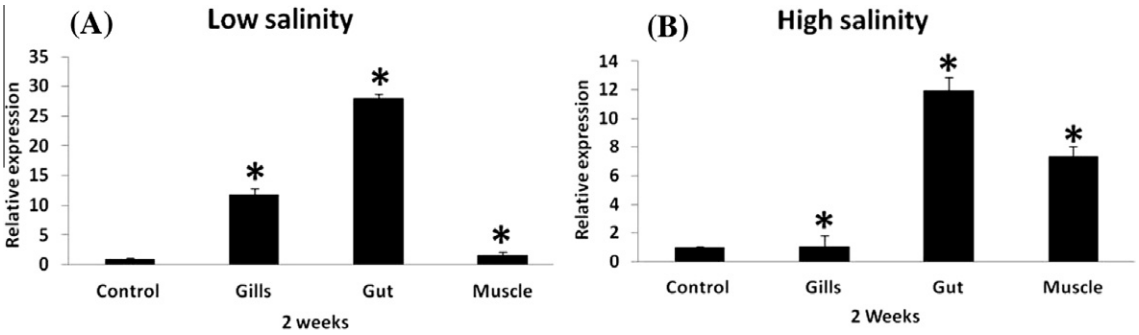

Fig. 1. Real time PCR analysis of putative *P. monodon* ACBP transcript expression in tissues (gills, gut and muscle) at 2 weeks time interval (A) under low salinity stress (B) under high salinity stress. The significant difference ($p < 0.05$) in ACBP expression levels are indicated with asterisks.

2.0 and an absence of potential glycosylation sites. Similar presence of multiple phosphorylation sites has been reported for *F. chinensis* ACBP which is involved in lipid transport (Ren et al., 2009). The protein displayed no signal peptide cleavage site as predicted by SignalP prediction. Similar observation of lack of signal peptide has been reported for the putative *F. chinensis* ACBP (Ren et al., 2009). The absence of signal peptide in *P. monodon* ACBP suggests that this might be cytosolic in nature and is not secreted into circulation as suggested for other shrimp genes lacking signal peptide (Rajesh et al., 2012; Hui et al., 2008).

Secondary structure analysis suggested the presence of conserved domains from amino acid position 3–87 which is a salient feature of ACBP-family of proteins from plants and animals. The *P. monodon* ACBP possess conserved domains for ligand binding similar to that present in the members of ACBP family such as *F. chinensis* (ACU82846), *Bos taurus* (NP001106792) and *Armadillo harderian* Gland (2FDQ) that function in the transport of lipids across membranes. The sequence analysis of ACBP from *P. monodon* revealed high sequence similarity among the shrimp ACBPs and ACBPs from other species suggesting a common ancestral gene among various organisms. The phylogenetic analysis suggested that the *P. monodon* ACBP was closely related to the ACBP gene isolated from *F. chinensis*.

The sequence and structure analysis revealed the presence of 18 acyl-CoA binding pockets and 6 CoA binding sites. The three dimensional structure predictions using Geno3D indicated the protein consisting of 4 alpha helices and 4 coiled structures. The tertiary structure of *P. monodon* ACBP protein was found similar to the ACBP homologue from Armadello ACBP (2FDQ) (Supplementary data, Fig. 5).

ACBP of *P. monodon* shrimp isolated in the present study appears to be a member of the ACBP superfamily of proteins that might have a function in lipid metabolism and transport similar to other homologues of the ACBP family as suggested by Ren et al. (2009) for ACBP isolated from *F. chinensis*. ACBPs belong to a multigene family which constitute different isoforms that could reflect functional diversity ranging from lipid biosynthesis, acyl-CoA transport and defence mechanisms via ethylene or jasmonate pathway etc. (Li et al., 2008).

Adaptation to salinity variations involves alterations in the ionic and osmotic capacity of permeable channels present in tissues such as gills and gut which are the primary osmoregulatory organs in shrimp (Péqueux, 1995). Morphological and biochemical changes in muscles are associated with molt cycle in shrimps (Cesar et al., 2006) and the shrimps exposed to salinity stress exhibit higher molting frequency (Vijayan and Diwan, 1995). The gill, gut and muscle tissues were therefore selected for quantifying the relative ACBP expression levels.

Real time PCR analysis of shrimp samples subjected to low salinity stress conditions at 3ppt revealed a significant increase in the expression of ACBP transcripts after 2 weeks in gills (11.71-folds), gut (28.08-folds) and muscle tissues (1.70-folds). Shrimps exposed to high salinity stress conditions for 2 weeks exhibited a significant increase in ACBP transcript levels in gills (1.05-folds), gut (11.95-folds) and muscle tissues (7.35-folds). The highest ACBP transcript levels from both low (3ppt) and high (55ppt) salinity stressed shrimps were obtained in the gut tissues as compared to gills and muscles tissues of shrimp (Fig. 1).

A higher expression of ACBP in the tissues of salinity stressed (3ppt and 55ppt) shrimps suggest a functional role of this gene during salinity stress. The ACBP gene expression levels in other organisms were found to be regulated and differentially expressed with stress conditions that involved cold or freezing conditions (Chen et al., 2008), bacterial (Ren et al., 2009) and viral infection (Zhao et al., 2007). ACBP is shown to be required for cuticle development and defence against microbial pathogens in plants (Xia et al., 2012). In cotton bollworm, *Helicoverpa armigera*, ACBP was found to be upregulated during larval molting and metamorphosis (Wang et al., 2008). However, ACBP in shrimps need further characterization at molecular and biochemical levels to decipher the functional significance of this protein during salinity stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.01.008.

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