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Molecular analysis and its expression of a pou homeobox protein gene during development and in response to salinity stress from brine shrimp, *Artemia sinica*

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ABSTRACT

Brine shrimps of the genus *Artemia* are aquatic species of economic importance because of their important significance to aquaculture and are used as a model species in physiology and developmental biology. Research on *Artemia* POU homeobox gene function will enhance our understanding of the physiological and developmental processes of POU homeobox gene in animals. Herein, a full-length cDNA encoding an *Artemia* POU homeobox protein gene 1 (*APH-1*) from *Artemia sinica* (designated as *As-APH-1*) was cloned and characterized by a reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end (RACE) method. The *As-APH-1* gene encoded a protein of 388 amino acid polypeptide with a calculated molecular mass of 42.85 kDa and an isoelectric point of 6.90 and the protein belongs to the POU III family. Multiple sequence alignments revealed that *A. sinica* As-APH-1 protein sequence shared a conserved POU homeobox domain with other species. The early and persistent expression of *As-APH-1* in the naupliar stages by semi-quantitative RT-PCR and whole-mount embryonic immunohistochemistry suggest that *As-APH-1* functions very early in the salt gland and may be required continuously in this organ. Later in development, expression of *As-APH-1* begins to dramatically decrease and disappear in salt gland of the sub-adult *Artemia*. In addition, we also discovered that As-APH-1 increased obviously as the salinity increased, indicating that As-APH-1 might be used as a good indicator of salinity stress. In summary, we are the first to identify the *As-APH-1* gene and to determine its gene expression patterns in early embryogenesis stages and in different salinity stress in brine shrimp, *A. sinica*. The result of expression of As-APH-1 affected by salinity changes will provide us further understanding of the underlying mechanisms of osmoregulation in *Artemia* early embryonic development.

1. Introduction

The brine shrimp *Artemia* is used as a model species because it can easily be obtained and maintained in laboratory. It is an aquatic species of economic importance in China because of its important significance to aquaculture as a main feed resource for newborn fish and widely used in much areas ranging from physiology to developmental biology and ecology (Kishida et al., 1998; Jiang et al., 2007; Sugumar and Munuswamy, 2007; Zhou et al., 2008). *Artemia sinica,* a bisexual strain in Yunchen, Shanxi province, China, has been characterized by Cai (1989). *Artemia tibetiana* also has been described by Abatzopoulos et al. (1998) as a new species. Hou et al. (2006) using analysis of DNA sequences of ITS-1region and COI gene showed that *A. tibetiana* from Tibet and *A. sinica* are geographically related by a common location in China, but they belong to different *Artemia* species. *A. sinica* mainly resides in the hyperosmotic brine lakes or salt pools. The natural populations of *A. sinica* in salt pools or brine pits have declined dramatically due to drought of its natural habitats (Wang et al., 2011). A decline in the brine shrimp population could also have a negative impact on the fisheries because brine shrimp play an important role in the diet of the newborn fish. *A. sinica* are also frequently exposed to seasonal salinity changes. Therefore, osmoregulation is an especially fundamental aspect of *Artemia* physiology. *Artemia* cysts hatch in seawater into free-swimming naupliae in 24 to 36 h, and the naupliae will grow out to juveniles and then adults in 1 to 3 weeks depending on the feeding conditions. *Artemia* carried out the process of salt excretion with different organs at different development stages of their life cycle. The salt excretion of naupliar stages depend on a transient salt gland, whereas juveniles or adults depend on the thoracic epipods. At the beginning of juvenile stage, the salt gland is resorbed at the same time as the appearance of the thoracic epidpods (Conte, 1984). However, relatively little knowledge is known about the cellular processes and mechanisms that underlie the formation, development, and function of the brine shrimp osmoregulation organs. Once elucidating the molecular processes involved enhances our understanding

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of how salinity changes in the environment, will affect aquatic crustaceans' survival and geographical distribution range. This is particularly significant in the context of global climate warming and drought.

The three mammalian transcription factors the pituitary-specific Pit-l, the ubiquitous Oct-1, the predominantly B cell-specific Oct-2 and a *Caenorhabditis elegans* transcription factors cell lineage control gene *Unc-86* were originally found to possess a conserved DNA binding region of 150–160 amino acids, and the conserved DNA binding region which was termed as the POU domain (Herr et al., 1988). The POU domain consists of two subdomains, the POU-specific domain and a POU-homeobox domain separated by a short variable linker sequence. POU domain proteins constitute a class of transcription factors and products of developmental control genes that all share a highly conserved DNA-binding domain and are expressed in various critical phases of embryonic development or cellular differentiation (Fukuta et al., 1993; Foygel et al., 2008; Robert-Moreno et al., 2010). The POU family genes are expressed in patterns that vary from exquisitely cell type specific (Finney et al., 1988; Finney and Ruvkun, 1990) to ubiquitous (Sturm et al., 1988) and have been shown to regulate transcription of genes involved in various physiology processes (Ryan and Rosenfeld, 1997). At present, POU family genes are divided into six or more classes (POU-I~—VI), based on the variations of the POU domain, with POU III the largest class (Sumiyama et al., 1996; Cupit et al., 2006).

In mammalian, POU III family members (Brain-1, Brain-2, Brain-4 and Scip) are mainly expressed in the central nervous system (He et al., 1989; Le Moine and Young, 1992; Mathis et al., 1992). In *Arthropoda*, several POU III family members have also been cloned and identified based on their sequence similarities. Cfla/drifter/ventral veinless (vvl) from *Drosophila melanogaster* was first cloned as a neuron-specific regulator binding the dopa decarboxylase gene (Johnson and Hirsh, 1990). Afterwards Cfla/drifter/vvl was observed to be involved in multiple pivotal developmental process: cell fate determination of wing imaginal disks (Certel et al., 2000), migration and differentiation of tracheal cells (Anderson et al., 1996; Zelzer and Shilo, 2000), and neuronal lineage and wiring (Inbal et al., 2003; Komiyama et al., 2003; Certel and Thor, 2004). *POU-M1* from *Bombyx mori* was cloned and identified from the silk gland and was observed to regulate transcription process of the *sericin-1* gene (Fukuta et al., 1993). POU-M2 is found to regulate the expression of the *B. mori DH-PBAN* gene, an insect neuropeptide related to embryonic development (Zhang et al., 2004). Recently, *Har-POU* from *Helicoverpa armigera*, was cloned from the suboesophageal ganglion and is distributed widely in a variety of tissues and has high expression in the central nervous system, trachea and salivary gland (Zhang and Xu, 2009).

In *Artemia* species, to date, only a partial cDNA of *Artemia franciscana APH-1* (named *Af-APH-1*) was cloned and the sequence was acquired in EMBL (Accession no. Y15070). The *Af-APH-1* gene encodes a POU domain family of transcription factors which is observed expression in the larvae salt gland but not in the adult *Artemia* (Chavez et al., 1999). However, the full-length sequence of *APH-1* gene has not been identified in *Artemia* species yet. Here we isolated a full-length cDNAs of *A. sinica* POU-homeoprotein encoding gene, named *As-APH-1*, as the first step to study the function of *Artemia* POU-homeoprotein. We also studied the phylogenetic and sequence analysis of *As-APH-1* and the gene expression patterns in larval salinity stress and in different stages of embryogenesis, in addition to whole mount immunohistochemistry analysis to determine the temporal and spatial expression pattern during early embryogenesis, thereby providing important foundations for further understanding of the underlying mechanisms of osmoregulation in *Artemia*.

2. Materials and methods

2.1. Artemia preparation

A. sinica cysts were purchased from the salt lake of Yun Cheng of Shanxi Province (China). The cysts were kept in axenic seawater at our laboratory following the procedure described by Li et al. (2011). The polypides at different developmental stages (0, 10, 15, 20, 40 h and 4 days; 5, 40 h and 4 days) were raised and frozen in view of extracting RNA or were fixed in paraformaldehyde for immunohistochemistry experiments.

2.2. Total RNA extraction and reverse transcription

All tissues were homogenized in the Trizol Reagent (TaKaRa, Dalian, China), and total RNA at different developmental stages (0, 10, 15, 20, 40 h and 4 days) was isolated in accordance with the manufacturer's instruction, and then was digested with RNase-free DNase I (Promega, Madison, WI, USA) to remove genomic DNA contamination. A total of 5 μg RNA was quantified by UV spectrophotometer (DU-640; Beckman, Fullerton, CA, USA), and then was reverse-transcribed using MMLV reverse transcriptase and an oligo (dT) primer (TaKaRa) in accordance with the manufacturer's manual.

2.3. Cloning of full-length cDNA of As-APH-1

Using the cDNA prepared above as a template, highly conserved region of *As-APH-1* was amplified with primers (As-APH-1F and As-APH-1R, Table 1) designed by Primer Premier 5.0 based on reported POU homeobox protein gene sequence alignment from other species. The PCR amplification was performed as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C 1 min, and a final 10 min of elongation at 72 °C. The resultant PCR products were recovered and cloned into PMD18-T Vector (TaKaRa, Dalian, China) and sequenced. The full-length cDNA for *As-APH-1* was obtained by 3′ and 5′ rapid amplification of cDNA ends (RACE) using 3′-RACE and 5′-RACE Kits (TaKaRa) according to the kit manufacturer's instruction, respectively. Total RNA (5 μg) was reversely transcribed with the MMLV reverse transcriptase to obtain the first-strand cDNA. The 3′ and 5′ termination fragments of *As-APH-1* were both amplified by nested PCR with 3′-RACE primers (3′ As-APH-1-1 and 3′ As-APH-1-2) and 5′-RACE primers (5′ As-APH-1-1 and 5′ As-APH-1-2, Table 1) which were designed from the highly conserved region of the *As-APH-1* cDNA fragment mentioned above, respectively.

2.4. Bioinformatics

Bioinformatics analyses were performed with programs in ExPaSy (http://www.expasy.org) and NCBI (http://www.ncbi.nlm.nih.gov). The molecular weight and pI were calculated with Compute pI/Mw (http://web.expasy.org/compute_pi/). Multiple sequence alignment in nucleotides or proteins was performed using ClustalW (http://www.ebi.ac.uk/clustalw/). The conserved domains were predicted with SMART (http://smart.embl-heidelberg.de). The protein intracellular localization and sorting signals were predicted by PSort II (http://psort.hgc.jp/). The homology-modeling was performed by SWISS-MODEL (http://swissmodel.expasy.org/). Subsequently, a phylogenetic tree was constructed by the neighbor-joining (NJ) method with the MEGA 4.0 software and displayed with Treeview software. The reliability of the tree was measured by bootstrap analysis with 1000 replicates.

Table 1
Sequences of oligonucleotide primers.

Name	Sequence (5′–3′)
As-APH-1-F	ACCACAACCAGCAGATTTAG
As-APH-1-R	TGAGGCACTCGCAAGTTTCC
5′ As-APH-1-1	TTTGGCAAACCCTTCTAAGTC
5′ As-APH-1-2	TTACCGCATTATGCCAAG
3′ As-APH-1-1	GGTGGTTCGAGTTTGTAATAG
3′ As-APH-1-2	CATTGAAGTGTCAGTGAAAGGTGC
RT-As-APH-1-F	ACCACAACCAGCAGATTTAG
RT-As-APH-1-R	GGAAACTTGCGAGTGCCTCA
β-actin-F	GGTCGTGACTTGACGGACTATCT
β-actin-R	AGCGGTTGCCATTTCTTGTT