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Cloning and expression of the sorbitol dehydrogenase gene during embryonic development and temperature stress in *Artemia sinica*

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ABSTRACT

Sorbitol dehydrogenase (SDH) catalyzes the interconversion of polyols and ketoses, using zinc and NAD^+ as cofactors. SDH converts sorbitol into fructose and plays an important role in the sorbitol metabolic pathway and in the early embryonic development of many invertebrates. Sorbitol usually accumulates in diapause embryos of insects to protect the embryos from frostbite, which indicates the vital function of SDH in the diapause and diapause-termination stages of embryo development. In this study, a 1311-bp full-length cDNA of *As-sdh*, including a 28-bp 5′ UTR and a 59-bp 3′ UTR, was cloned from *Artemia sinica*. This gene encodes 348 amino-acid proteins. Bioinformatic analysis revealed that this gene is highly conserved in arthropods. The expression patterns of *As-sdh* were investigated during different stages of embryonic development using real-time PCR and in situ hybridization. *As-sdh* was expressed at relatively high levels during the 0 h embryonic stage, and transcript levels were quite high in 5- and 7-day-old embryos. In situ hybridization analysis showed that *As-sdh* is expressed in a widely dispersed pattern before incubation but is mainly concentrated on the body surface and the inner wall of the alimentary tract after the nauplius stage. Our results suggest that *As-sdh* is integral to the process of diapause and diapause termination in *A. sinica*.

1. Introduction

Artemia sinica, a small crustacean widely used in commercial aquaculture, is a valuable biological resource due to its high unsaturated fatty acid and protein content (Abatzopoulos et al., 2002). *A. sinica* is easy to acquire, and its diapause cysts are easy to transport and preserve. These attributes, along with the relatively short life cycle and simple culturing conditions of this crustacean, make *A. sinica* an excellent model system for studies of genetics, development and evolution (Zheng et al., 2011). Recently, cDNAs encoding sorbitol dehydrogenase (*sdh*) have been cloned from rat (Karlsson et al., 1991), silkworm (Niimi et al., 1993), *Bacillus* (Ng et al., 1992), and yeast (Sarthy et al., 1994). Multiple sequence alignment analysis of the encoded protein sequences revealed a highly conserved structure, which indicates that this enzyme may have an important physiological function. Diapause is a specific stage of embryonic development in *A. sinica*. During diapause, embryo development is paused at the gastrula stage to enable the organism to survive under extreme conditions (Liang et al., 1997). In the diapause stage, embryos remain dormant until they are stimulated by suitable environmental factors to resume development to the nauplius stage (Liang and MacRae, 1999). Sorbitol often accumulates in the resting eggs of most diapause-mandatory animals, such as *Bombyx* (Toshinobu et al., 1990), flesh flies (Michaud and Denlinger, 2007), and *A. sinica*, because the water absorbing ability of sorbitol makes it an excellent cryoprotectant (Wang et al., 2005). Indeed, *sdh* is vital to the process of diapause termination.

SDH is a member of zinc-dependent alcohol dehydrogenase-like (ADH) family. Unlike other members (Jornvall et al., 1993), the holoenzyme of SDH is a tetramer (Banfield et al., 2001), which comprises four identical subunits, each with one catalytic zinc atom (Eklund et al., 1985; Karlsson et al., 1989, 1995; Maret, 1996). This coenzyme-binding motif, which consists of seven residues of the N-terminus located between two β-strands, has a classical Rossmann fold structure (Pauly et al., 2003), that allows all four subunits of the enzyme to reversibly combine with NAD(H) (Kenneth and Ruiqiong, 1992). The catalytic zinc atom in each subunit is associated with Cys, His, Glu, and a water molecule. In human SDH, these residues include Cys43, His68 and Glu150 (Maret, 1996). Although Glu155 in human SDH is not thought to be a zinc-binding site, mutation of this residue results in the loss of catalytic activity (Karlsson et al., 1995).

Abbreviations: ADH, alcohol dehydrogenase; *As-sdh*, sorbitol dehydrogenase gene of *Artemia sinica*; *As*-SDH, sorbitol dehydrogenase of *Artemia sinica*; DEPC, diethylpyrocarbonate; DIG, digoxigenin; NJ, neighbor-joining; ORF, open reading frame; pI, isoelectric pointand; SDH, sorbitol dehydrogenase; *sdh*, sorbitol dehydrogenase; UTR, untranslated region.

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The process by which SDH catalyzes the interconversion of polyols and ketoses begins when the zinc atom associates with the three amino acid residues and a water molecule; this process requires NAD^+ as a cofactor. Next, the oxygen atoms of C_1 and C_2 in the sorbitol carbon chain combine with the zinc atom, releasing Glu150 and converting the zinc to $zinc^{5+}$. In addition, C_3–C_5 of the carbon chain convert to a fructose conformation. The hydroxyl group of C_2 loses a proton, triggering a tandem reaction after the water molecule provides a hydrogen bond for Gly155 in the oxidase and Gly150 in the SDH/NAD^+ complex. The hydrogen of the hydroxyl group of C_2 releases an electron by reducing NAD^+ to NADH. Finally, the reaction is completed when the hydroxyl group of C_2 is oxidized to form keto carbonyl (Pauly et al., 2003).

While the piperazine pyrimidines are the only class of in vivo SDH inhibitors (SDIs) (Geissen et al., 1992), a class of potent in vitro SDIs was identified through their interaction with catalytic zinc (Lindstad and Mckinley-Mckee, 1996). The identification of the prototypical SDI CP-166,572 by Geissen (Geissen et al., 1994) made it possible for more potent SDIs to be synthesized (Chu-Moyer et al., 2002). In addition, this discovery led to the proposal that SDH could be inhibited by directly chelating the catalytic zinc (Mylari et al., 2001). The prodrug SDI-157 increases the activity of CP-166,572 by inducing a chemical change in its pyrimidine ring (Pauly et al., 2003). CP-166,572 is noncompetitive with sorbitol, NAD^+ or NADH in the inhibition procedure, but competes with fructose by binding to the SDH/NADH complex in sheep (Geissen et al., 1994) and human SDH (Pauly et al., 2003).

To acclimate to cold weather, insect diapause eggs often contain sorbitol, in addition to glycerol. NAD-SDH activity is not induced in overwintering eggs of *Bombyx* exposed to temperature of 0 °C (Toshinobu et al., 1990), and therefore sorbitol is not utilized. And the sorbitol accumulation, by overloading glucose from glycogen (Joanisse and Storey, 1995), could also be examined in the resting eggs of flesh flies (Michaud and Denlinger, 2007). Therefore, the storage of sorbitol is a direct response to extremely cold temperatures (Storey and Storey, 1983). Whether sorbitol accumulates or not based on the activity of *sdh*, which was not induced in the overwintering eggs. In diapause *Bombyx* eggs, *sdh* is normally expressed at a very low level. However, when the eggs are transferred from approximately 0 °C to 25 °C, NAD-SDH activity strongly increases (Toshinobu et al., 1990); this is also true for diapause eggs of *Drosophila* (Bischoff, 1978). However, it is unclear whether the same phenomena also occur in *A. sinica*. As the hatchability of *A. sinica* is vital to its value, fully understanding the mechanism underlying diapause termination in this small crustacean would greatly improve *A. sinica* aquiculture.

The functions of SDH have been studied in many species, but little is known about the post-diapause and diapause terminations of *A. sinica*. In this study, we focused on the molecular characterization and expression patterns of *As-sdh* gene during development, and the role of *As-sdh* in the temperature–stress response.

2. Methods and materials

2.1. Animal preparation

A. sinica cysts were collected from the salt lake of Yuncheng in Shanxi Province (China) and were hatched in filtered fresh seawater in the laboratory. The cysts were incubated for 30 min (designated 0 h), and samples of roughly 50 mg were collected at 5, 10, 20 and 40 h, and 3, 5, and 7 days (gastrula stage (0 h), diapause termination stage; embryonic stage (5 and 10 h), the embryo returns to the normal rate of development; nauplius stage (15, 20, and 40 h); metanauplius stage (3 and 5 days) alimentary system is formed which allows individuals to gain nutritions from surroundings; sub-adult (7 days) segmentation stage). Adult brine shrimp cultured at 30 °C for 48 h were employed as the control group in the low-temperature assay, and adult *Artemia* in the experimental group were maintained at 25 °C, 20 °C, 15 °C, 10 °C or 5 °C.

2.2. Cloning of full-length As-sdh cDNA

Total RNA from each sample was extracted using the TRIzol-A + (Tiangen, Beijing, China) method, and oligo (dT) primer and MLV reverse transcriptase (Takara, Dalian, China) were used for reverse transcription. Primer Premier 5.0 was used for primer design, based on the *sdh* of *Artemia franciscana*, and the forward (5′-TGCGAAGAGGTGAGGT-3′) and reverse (5′-TGCAAGCAGGGTGTAAT-3′) primers were synthesized (Takara, Dalian, China). An *As-sdh* fragment of 332 bp was obtained. The full-length cDNA of *As-sdh* was obtained using 3′ RACE core set ver. 2.0 (Takara, Dalian, China) and the SMART RACE cDNA amplification kit

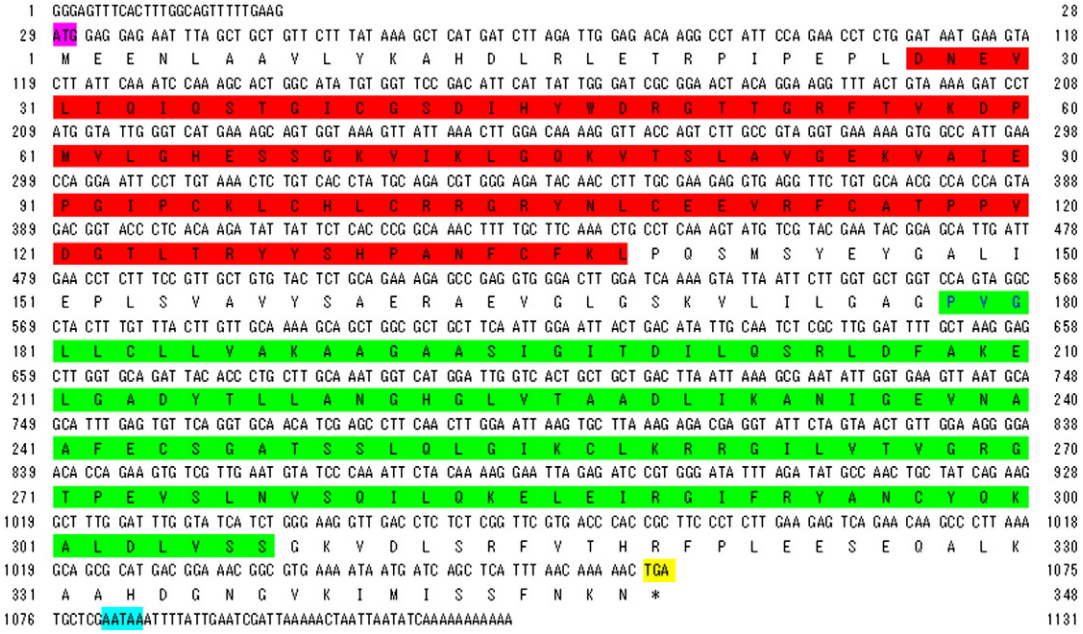

Fig. 1. Sequence analysis of the cDNA and predicted peptide sequences of *As-sdh*. The start codon is indicated in purple; the stop codon is indicated in yellow; the ADH_N domain is indicated in red; the ADN_zinc_N domain is indicated in green; and the polyadenylation site (AATAAA) is indicated in blue.