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Identification of the *glycerol kinase* gene and its role in diapause embryo restart and early embryo development of *Artemia sinica*

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ABSTRACT

Glycerol kinase (GK) catalyzes the rate-limiting step in glycerol utilization by transferring a phosphate from ATP to glycerol, yielding glycerol 3-phosphate, which is an important intermediate for both energy metabolism and glycerolipid production. *Artemia sinica* has an unusual diapause process under stress conditions of high salinity, low temperature and lack of food. In the process, diapause embryos of *A. sinica* (brine shrimp) accumulate high concentrations of glycerol as a cryoprotectant to prevent low temperature damage to embryos. Upon embryo restart, glycerol is converted into glucose and other carbohydrates. Therefore, GK plays an important role in the diapause embryo restart process. However, the role of GK in diapause termination of embryo development in *A. sinica* remains unknown.

In the present study, a 2096 bp full-length cDNA of *gk* from *A. sinica* (*As-gk*) was obtained, encoding putative 551 amino acids, 60.6 kDa protein. As a crucial enzyme in glycerol uptake and metabolism, GK has been conserved structurally and functionally during evolution. The expression pattern of *As-gk* was investigated by quantitative real-time PCR and Western blotting. Expression locations of *As-gk* were analyzed using in situ hybridization. *As-gk* was widely distributed in the early embryo and several main parts of *Artemia* after differentiation. The expression of *As*-GK was also induced by stresses such as cold exposure and high salinity. This initial research into the expression pattern and stress response of GK in *Artemia* provides a sound basis for further understanding of the function and regulation of genes in early embryonic development in *A. sinica* and the stress response.

1. Introduction

Artemia sinica is a small crustacean distributed worldwide in hypersaline waters. It has been used economically as a main food resource for newly born fish and crustacean species in aquaculture because of its high protein and unsaturated fatty acid content in the nauplii. *Artemia* has a relatively short process of embryo development. It is easy to obtain and feed, which makes it a perfect animal model for experimental research of genetics, evolution, molecular biology, developmental biology, ecology and other life science fields (Abatzopoulos et al., 2002). *Artemia* has an unusual diapause process under conditions of high salinity and low temperature stress (MacRae, 2010). During this process, diapause embryos accumulate high concentrations of glycerol as cryoprotectant to prevent low temperature damage to the embryos (Michaud and Denlinger, 2007). After diapause termination, glycerol is converted into glucose and other carbohydrates (Kihara et al., 2009). Diapause may be terminated under suitable conditions and embryos resume development. Previous studies demonstrated that glycerol kinase plays a vital role in embryo diapause termination of *Bombyx mori* (Kihara et al., 2009); however, the role of GK in glycerol metabolic process of diapause embryo restart and early embryonic development of *A. sinica* remains unknown.

Glycerol is an important intermediate of energy metabolism that plays fundamental roles in several vital physiological processes. Glycerol metabolism provides a central link between sugar and fatty acid catabolism (Yeh et al., 2009). Glycerol kinase (ATP: glycerol 3-phosphotransferase, GK) was first isolated and studied by Lin et al. in the 1960s and was extensively investigated in *Escherichia coli* (Lin, 1976). It catalyzes the rate-limiting step in glycerol utilization by transferring a phosphate from ATP to glycerol, thus yielding glycerol

Abbreviations: GK, glycerol kinase; *As-gk*, glycerol kinase gene of *Artemia sinica*; G3P, glycerol 3-phosphate; FBP, fructose-1,6-bisphosphate; PTS, phosphotransferase system; ISH, in situ hybridization; NJ, neighbor-joining; DIG, Digoxigenin; PBS, phosphate-buffered saline; DEPC, diethylpyrocarbonate; RT-PCR, real-time PCR; LSD, least square difference; ORF, open reading frame; PI, isoelectric point; GKD, glycerol kinase deficiency.

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3-phosphate (G3P) in the cytoplasm (Aragon et al., 2008). As the phosphorylated form of glycerol, G3P can be converted to dihydroxyacetone phosphate by G3P dehydrogenase and fed into the glycolysis or gluconeogenesis pathways, according to the metabolic status of the cell (Schnick et al., 2009). Therefore, GK enables glycerol derived from fats or glycerides to enter the glycolytic/gluconeogenesis pathway and facilitate external glycerol to participate in cellular metabolism. Meanwhile, it is also an obligatory step in glycerolipid production (Agosto and McCabe, 2006).

GK has been found throughout all three kingdoms of living organisms, from bacteria to humans. As a crucial enzyme in glycerol uptake and metabolism, it has been conserved both structurally and functionally during evolution (Koga et al., 1998). GK belongs to the FGGY family of carbohydrate kinases, a sub-family hierarchy of the NBD_sugar-kinase_HSP70_actin superfamily (Pawlyk and Pettigrew, 2001). This family predominantly comprises GK and similar carbohydrate kinases, including rhamnulokinase (RhuK), xylulokinase (XK), gluconokinase (GntK), ribulokinase (RBK) and fuculokinase (FK). They show a high degree of structural similarity, even though they share little sequence similarity (Bork et al., 1992; Holmes et al., 1993). The FGGY proteins contain two large domains: the N-terminal domain that adopts a ribonuclease H-like fold, and the structurally related C-terminal domain (Agosto and McCabe, 2006). The N-terminal domain is primarily involved in substrate binding, while the C-terminal domain is mainly responsible for ATP binding. They are separated by a deep cleft that forms the active site (Pettigrew et al., 1996). The high affinity ATP binding site of GK is created only by a substrate-induced conformational change. GKs from different species may exist in different oligomeric states. The enzyme exists at physiological concentrations in equilibrium between functional dimers and tetramers (Riel and Paulus, 1978; Feese1 et al., 1998). The tetrameric protein is composed of identical subunits, but it can be dissociated into dimers at low protein concentrations (Riel and Paulus, 1978; Thorner and Paulus, 1971). GK is a regulatory enzyme that was shown to be subject to feedback regulation by the glycolytic intermediate, fructose-1,6-bisphosphate (FBP), in a noncompetitive manner with respect to both substrates (Thorner and Paulus, 1973; Huang et al., 1998). FBP is said to bind to and stabilize only the tetrameric form, decreasing the dimer–dimer dissociation dramatically. IIA^{Glc}, glycose phosphotransferase system (PTS) phosphocarrier protein, has been identified as another allosteric effector whose effect is independent of GK concentration over a wide range (Novotny et al., 1985; Mao et al., 1999).

A lower metabolic rate is a vital survival strategy and common feature of diapause. Previous research showed that proteins related to carbohydrate and energy metabolism make up the largest proportion of the identified proteins in *A. sinica* undergoing diapause. This result further indicated that proteins, especially metabolic enzymes, may be the key factors in diapause regulation (Qiu and MacRae, 2007). In mammals, GK is already known as a key enzyme for the utilization of glycerol (Agosto and McCabe, 2006). There have been reports that GK also functions as a rate-limiting enzyme in insect diapause (Kihara et al., 2009). However, its corresponding role in crustacean metabolic regulation remains undetermined.

In the present study, the *gk* gene from *A. sinica* was cloned and its expression levels during early embryonic development and in response to salinity/temperature stress were analyzed by real-time PCR. In addition *As*-GK was expressed in *E. coli* by a prokaryotic expression plasmid, pET-30a. Meanwhile, the expression pattern of GK and the location of its gene expression were investigated using Western blotting and in situ hybridization (ISH), respectively. Our aims are to further understand the role of the glycerol metabolic process during diapause embryo restart and early embryonic development of *A. sinica*.

Table 1
Oligonucleotide primers used in this study.

Primer	Sequence (5′–3′)	Direction
*As-gk*F	CTGGGAAAAACTAAACGACC	Forward
*As-gk*R	GCTTGTATCTGAAGGAGGA	Reverse
3′*As-gk*(outer)	GAAGGTTCTGTAGCCGTAGC	Forward
3′*As-gk*(inner)	TATTTTGTGCCCGCCTTCTC	Forward
5′*As-gk*	GCGGGCACAAAATAAACGCCACCAGAAT	Reverse
ISH-*gk*F	TGCGAATAGCAGTGGACGA	Forward
ISH-*gk*R	CACACATCTGACCAAGAAGCG	Reverse
RT-*gk*F	GCAATAGTTTGGTGCGATAA	Forward
RT-*gk*R	AGCAACAGGTAAGCCACAAT	Reverse
ORF-*gk*F	CGGAATTCATGGGAGACGCCTTA	Forward
ORF-*gk*R	CCGCTCGAGTCACTGGGAAGCA	Reverse
β-actinF	AGCGGTTGCCATTTATTGTT	Forward
β-actinR	GGTCGTGACTTGACGGACTATAT	Reverse

2. Materials and methods

2.1. Animal preparation

A. sinica cysts were harvested from the salt lake of Yuncheng, Shanxi Province (China) and stored at −20 °C in the dark until use. The cysts were hatched in filtered seawater (salinity 28‰) under laboratory conditions: 28 °C, at an illumination intensity of 1000 lx.

The development of *A. sinica* consists of five main stages: the embryo, nauplius, metanauplius, pseudoadult and the adult stages. In this experiment, 0–10 h corresponded to the cyst stage; 15–20 h corresponded to the nauplius stage; 40 h corresponded to the metanauplius stage; 3 and 5 d corresponded to the pseudoadult stage; after 5 d corresponded to the adult stage. Animal samples of roughly 50 mg were collected each time point (0, 5, 10, 15, 20 and 40 h; 3, 5 and 7 d, adult) at different periods of development for subsequent experiments.

2.2. Cloning of full-length gk cDNA

To prepare a cDNA template for PCR amplifications, total RNA was extracted using TRIzol-A^+ (Tiangen, Beijing, China), followed by reverse-transcription with an oligo (dT) primer and MLV reverse transcriptase (TaKaRa, Dalian, China). Specific primers (*As-gk*F, *As-gk*R, Table 1) were designed using primer Premier 5.0 (Premier), based on the partial sequence of *Artemia franciscana gk* and synthesized by TaKaRa. The PCR reaction conditions were as follows: an initial incubation at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min; with a final incubation at 72 °C for 10 min. PCR products were separated on 1.0% agarose/TAE gels and sequenced by TaKaRa. A 359 bp fragment of *As-gk* was obtained.

Subsequently, the full-length cDNA sequence of *As-gk* was obtained by 5′–3′ rapid amplification of cDNA ends (RACE) using the 3′RACE Core Set Ver.2.0 (TaKaRa) and the SMART™ RACE cDNA Amplification Kit (Clontech, Dalian, China), respectively. All the reaction processes of RACE were carried out according to the manufacturers' instructions. Gene-specific primers of 3′RACE (3′*As-gk*, Table 1) and 5′RACE (5′*As*-gk, Table 1) were designed based on the amplified 359 bp gene fragment of *As-gk* mentioned above. The RACE-PCR products were purified with a Gel Extraction Kit (TaKaRa), ligated into a pMD19-T vector (TaKaRa), transformed into *E. coli* strain DH5α and then sequenced by TaKaRa. The 3′ and 5′ termination fragments were spliced together using DNAman 6.0.3.48 (Lynnon Biosoft) to obtain the full-length

Fig. 1. Sequence analysis of *Artemia sinica* GK. Sequence of *A. sinica gk* cDNA and the deduced protein sequence. The start and stop codons are shown in green and pink, respectively. Carbohydrate kinase FGGY N-terminal domain is indicated by a straight blue line, with the C-terminal domain defined by a wavy green line. The red letters with asterisks show conserved sites in GK.