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Full Length Research Paper

Isolation of an ATP synthase cDNA from *Sinonovacula constricta* and its mRNA expression by thermal stress

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ATP synthase is a key enzyme in the cellular energy metabolism. In the present study, cDNA of ATP synthase subunit from *Sinonovacula constricta* (ScATPase) was isolated and characterized by cDNA library and RACE appraoaches. The full-length cDNA of ScATPase was of 737 bp, consisting of a 5'-untranslated region (UTR) of 48 bp, a 3'-UTR of 248 bp and a complete open-reading frame of 441 bp, encoding a polypeptide of 146 amino acid residues with preidicted molecular mass of 15.4 kDa and theoretical isoelectric point of 9.33. BLASTp and phylogenentic analysis revealed that, the deduced amino acid of ScATPase shared higher identity with the ATP synthase subunit C subunit from other species, indicating it should be a novel member of ATPase C family. Quantitative real-time polymerase chain reaction (RT-PCR) analysis indicated that, the expression of ScATPase could be induced by the thermal stress in three different tissues. These results strongly suggest that, ScATPase was an acute protein involved in temperature challenge in *S. constricta*.

Key words: *Sinonovacula constricta,* ATP synthase, quantitative real-time PCR, thermal stress.

INTRODUCTION

ATP is a multifunctional nucleotide used in cells as a coenzyme to transport chemical energy for metabolism (Oleg et al., 2008). The bulk of ATP is provided by the final step of the oxidative phosphorylation process catalyzed by ATP synthase. ATP synthase is present in subcellular structure of mitochondria and chloroplasts as a multi-subunit enzyme complex, which composed of a membrane embedded hydrophobic sector F_0 and a hydrophilic extrinsic sector F_1 (Boyer, 1997; Fillingame et al., 2000; Yoshida et al., 2001; Capaldi and Aggeler, 2002). F_0 is involved in proton translocation across the membrane. The simplest F_0 form contains three subunits in stoichiometry $ab_2c_{10–15}$, where the $c_{10–15}$ subunits (also called ATP synthase subunit C) form a ring called *c*-ring. The simplest form of the catalytic site F_1 contains five subunits in stoichiometry $\alpha_3\beta_3\gamma\delta$ (Manuela et al., 2008).

Previous studies on ATP synthase have mainly focused on its structure and function (Nakamoto et al., 1999; Weber, 2007; Wittig and Schagger., 2008). Rare knowledge is available about its molecular characteristics and expression patterns, especially in invertebrates. The main objectives of the present study were: (1) to clone the full-length cDNA of ATPase from *Sinonovacula constricta*; (2) to investigate the temporal expression profile of ScATPase transcript in muscle, visceral mass and mantle after thermal stress.

MATERIALS AND METHODS

Experimental animals

Sinonovacula constricta (7 to 8 g in weight) were purchased from a commercial fish market in Zhejiang, China. They were maintained in

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500 L tank equipped with air-lift circulating water at 28°C in our laboratory.

Clone and sequence of ScATPase

The SMART cDNA library of *S. constricta* was constructed by our laboratory. Random sequencing of the library using T3 primer yielded 1220 successful sequencing reactions. BLAST analysis of the EST sequences revealed that an EST of 326 bp was highly similar to the known ATPase sequences. This sequence was then selected for further cloning of full-length cDNA of ATP synthase subunit of *S. constricta* (ScATPase). Two gene specific primers, ATP-3: 5'-AGGCGAATCCAAGGACAGC -3' and ATP-5: 5'-TCAAGCGGAAGCAGGACG-3, were first designed based on the above EST. The polymerase chain reaction (PCR) reactions were carried out in 25 µL reaction mix containing 2.5 µL 10 × buffer, 2.0 µL of each of the dNTPs (2.5 mM), 2.0 µL Mg^{2+} (25 mM), 1.0 µL each of the primer pairs (gene specific primer and vector primer) (10 µM), 0.2 µL Taq polymerase, 1.0 µL cDNA library and 15.3 µL PCR-grade water. After denaturation at 94°C for 5 min, amplification was performed for 35 three-step cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min, followed by 72°C for 5 min. The PCR products were gel-purified and sequenced. The sequencing results were assembled and subjected to cluster analysis.

Sequence analysis

The obtained full-length cDNA of ScATPase were analyzed by the BLAST algorithm at National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). MITOPROT (http://ihg.gsf.de/ihg/mitoprot.html) was selected to predict mitochondrial targeting sequences of ScATPase. Phylogenetic tree was constructed according to amino acid sequences of the selected ATPase genes using the neighbor-joining method in program Mega 3.1(http://www.megasoftware.net/). The bootstrap trials were replicated 1000 times to derive the confidence value for the phylogeny analysis.

Temperature treatment

S. constrictas were assigned into three groups (50 individuals for each group) and acclimated for a week before commencement of the experiment. For thermal stress experiment, the razor clam was kept at 37°C, 28°C (control group) and at 4° C, respectively. The tissues of muscle, mantle and visceral mass were randomly sampled at 6 and 12 h. Samples were immediately frozen in liquid nitrogen and stored at –80°C for mRNA extraction.

Temporal expression profile of ScATPase mRNA after thermal stress

The expression profiles of ScATPase transcript in three tissues after thermal stress were measured by quantitative real time PCR in RG-6000 real-time PCR detection system and each amplification was carried out in triplicate. Two sets of gene specific primers (5'-CAGGCTGCCAGATTTATTGG-3' and 5'-GCTGCTTCAGGGATGGGTTT -3') were used to amplify a 124 bp fragment of ScATPase. β-actin gene specific primers (5'-AGTTGCCGCTCTTGTCGTGG-3' and 5'-TGCTCTGGGC TTCATCTCCG -3') were used to obtain a 170 bp fragment and served as the internal control to verify the successful reverse transcription and to calibrate the cDNA template. The reaction was performed in a total volume of 20 µL containing 10 µL of 2×SYBR Green Master Mix (Applied Biosystems), 4 µL of the diluted cDNA mix, 0.25 µL of each primer (10 µM), 5.5 µL of DEPC-treated water. The thermal profile for real time PCR was 50°C for 2 min and 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1min. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of ScATPase. The Ct for the target amplified ScATPase and the Ct for the internal control β-actin were determined for each sample. Difference in the Ct for the target and the internal control, called ΔCT, was calculated to normalize the differences in the amount of template and the efficiency of the RT-PCR. The ΔCT value for control group was used as the reference sample, called the calibrator. The ΔCT for each sample was subtracted from the ΔCT of the calibrator; the difference was called ΔΔCT. The expression level of ScATPase could be calculated by $2^{-\Delta\Delta CT}$, and the value stood for an n-fold difference relative to the calibrator. All data were given in terms of relative mRNA expression as means ± S.D. The data were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed t-test. Differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.01$

RESULTS AND DISCUSSION

Sequence analysis of the cDNA encoding ScATPase

A SMART cDNA library was constructed from the whole bodies of *S. constricta*, using SMART™ cDNA library construction kit (Clontech). Random sequencing of the library using T3 primer yielded 1220 successful sequencing reactions. BLAST analysis ((http://www.ncbi.nlm.nih.gov/blast)) of the EST sequences revealed that, an EST of 326 bp was highly similar to the known ATPase sequences. This sequence was then selected for further cloning of full-length cDNA of ATPase gene from *S. constricta*. The 3'end and 5'end of ScATPase were cloned with gene specific primers and vector primers with cDNA library as template. In the end, a 737 bp fragment representing the full-length cDNA of ScATPase was cloned and deposited in Genbank with accession number GQ906973. The complete nucleotide and the deduced amino acid sequence are shown in Figure 1.

The ScATPase cDNA consisted of a 5'-untranslated region (UTR) of 48 bp, a 3'-UTR of 248 bp followed by a polyadenylation signal AATAAA, a polyadenylation tail and a complete open-reading frame of 441 bp encoding a polypeptide of 146 amino acid residues with predicted molecular mass of 15.4 kDa and theoretical isoelectric point of 9.33. MITOPROT (http://ihg.gsf.de/ihg/mitoprot.html) analysis indicated that, ScATPase could be exported to mitochondria matrix with the possibility of 75.47% and the cleavage site was located between the amino acid of AKQ and DID. Our results are consistent with popular viewpoint that, ATPase was thought to be localized exclusively to mitochondria. However, the protein had also been identified on the plasma membrane

Figure 1. Complete cDNA sequence of ScATPase and its deduced amino acid sequence. Nucleotides were numbered from the first base at the 5'end. The canonical polyadenylation signal-sequence was italic and underlined. The asterisk indicated the stop codon. The domain for ATP synthase C was underlined.

of several cell types in recent years, including hepatocytes where it was the HDL receptor and on endothelial cells where it acted as the angiostatin receptor.

BLASTP analysis revealed that, the deduced amino acid sequence of ScATPase shared 61, 61 and 60% identity with ATPase from *Haliotis diversicolor* (ABY87376), *Salmo salar* (ACI66488) and *Danio rerio* (AAH71368), respectively. These conserved characteristics informed us that, ScATPase belonged to a novel member of ATP synthase family. A NJ evolutionary tree for different types of ATPase subunit was constructed based on the amino acid sequence (Figure 2). In the phylogenetic tree, A-type, B-type and C-type ATPase were clustered independently and formed three sister groups. ScATPase was identified in C-type ATPase subgroup, further indicating that the identity of ScATPase belonged to ATPase C subunit family.

Regulation of ScATPase expression by thermal stress

Quantitative real-time PCR was employed to analyze ScATPase expression in response to different temperatures (37, 28 and 4℃) in three tissues of muscle, mantle and visceral mass. The mRNA expression levels of ScATPase were calculated and normalized to β-actin by comparing the CT difference (ΔΔCT). The statistical results were subjected to one-way variance analysis (ANOVA) and *p* values less than 0.05 was considered as significant difference. The amplification specificity was determined by analyzing the melting curves. Only one peak presented in the melting curves for both ScATPase and β-actin genes, suggesting that the amplifications were specific (data not shown). The results are shown in Figure 3. The ScATPase expression was significantly increased after temperature elevation to 37℃ during the first 6 h and

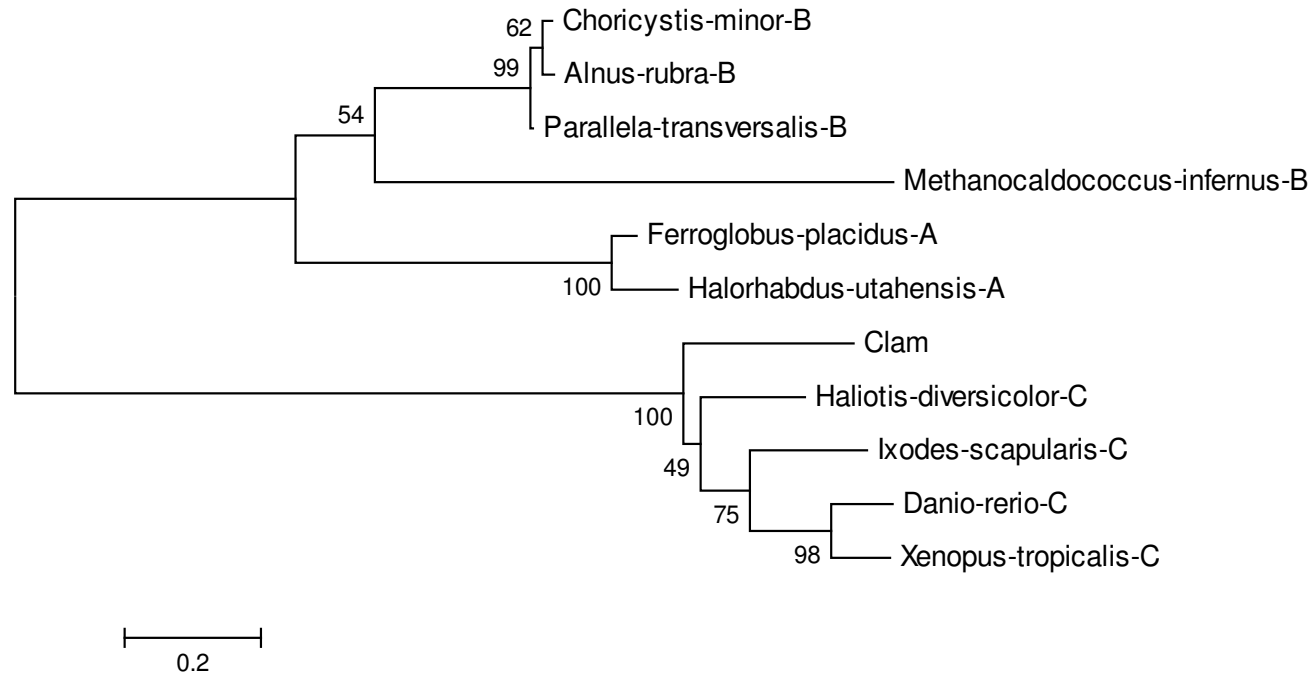

Figure 2. Consensus neighbour-joining tree based on the sequences of different types of ATPase with Mega3.1 software package (http://www.megasoftware.net/) (Kumar et al., 2004) and clustal X (1.81). The numbers at the forks indicated the bootstrap. The detail information for the used sequences were as follows: Haliotis-diversicolor-C (ABY87376); Ixodes-scapularis-C (AAY66884); Danio-rerio-C (NP-957470); Xenopus-tropicalis-C (NP-001005087); Parallela-transversalis-B (ADR63863); Choricystis-minor-B (ADL41368); Methanocaldococcus-infernus-B (ADG13121); Alnus-rubra-B (AF132889); Ferroglobus-placidus-A (ADC66428); Halorhabdus-utahensis-A (ACV11613).

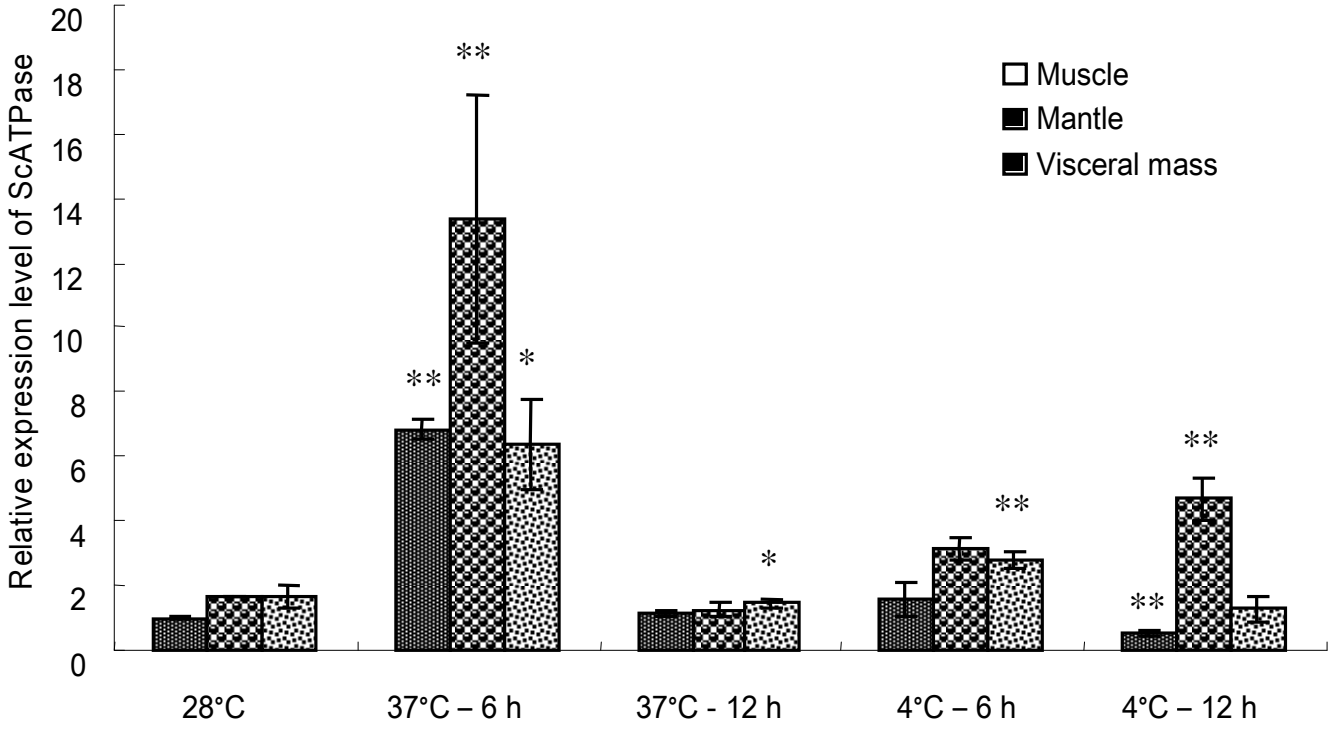

Figure 3. The relative expression profile of ScATPase mRNA at different tissues after thermal stress by quantitative real-time PCR. Asterisks (*) indicate significant difference ($P < 0.05$). Double asterisks (**) indicate extremely significant difference ($P < 0.01$)

reached to 6.85-, 13.36- and 1.41-fold compared with the control group in tissues of muscle, mantle and visceral mass. With time progressed, the expression level of ScATPase was dropped back to the original level at 12 h. By contrast, lower temperature had no obvious effect on the expression of ScATPase mRNA in the tissues of muscle and visceral mass in all time points; but for the tissue of mantle, the increased expression level of ScATPase was detected at 12 h after temperature reduced. The peak expression was 4.70-fold increase compared with control group (Figure 3). The significant change of ScATPase expression in these tissues together indicated that, protein was involved in the thermal stress of razor clam.

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