[Original]

Gene Expression of Trehalase during Post-dormant Development of the Brine Shrimp, *Artemia*: Comparison of the Two Species

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Abstract : Based on a homology screening approach, two degenerate oligonucleotides were employed as primers in a polymerase chain reaction to amplify a fragment of DNA encoding trehalase with a template of cDNA derived from embryos of American *Artemia*. Sequence analysis revealed that the fragment was composed of 228 bp comprising 76 amino acids, and highly homologous to trehalases of *Tenebrio molitor* (mealworm beetle), rabbit, *Caenorhabditis elegans*, *Bombyx mori* (silkworm) and *Escherichia coli* *treA* and *treF* (58 – 38%, in order of description). This fragment was used as a hybridization probe. A Northern blot analysis on American *Artemia* showed three transcripts of 5.0, 2.7 and 2.2 kb, and the two larger transcripts were also detected in Chinese *Artemia*. The developmental profile of the gene expression and the trehalase activity suggest that the transcripts of 5.0 and (or) 2.7 kb in both *Artemia* may be directly or indirectly related to translation of the trehalase. A Southern blot analysis on both *Artemia* suggested the existence of two highly homologous genes or one gene having an intron within the region where the probe binds in their haploid genome.

Key words : trehalase, gene expression, brine shrimp, *Artemia*.

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Introduction

The brine shrimp, *Artemia*, is known as a cryptobiotic organism, which enters an encysted state of dormancy at the gastrula stage embryo. The dormant cyst contains trehalose, accounting for 15% of its dry weight [1]. Such an amount of trehalose is thought to be necessary for the organism to be tolerant against cold, desiccation and vacuum during its dormancy.

The dried encysted embryo resumes development on rehydration and adequate salinity under an aerobic condition, coinciding with a decrease in the

content of trehalose accompanied by a corresponding increase in the level of glycogen and glycerol. Thus, the trehalose is thought to be a respiratory substrate [2, 3].

The enzymatic hydrolysis of the trehalose is due to the activity of the trehalase (α , α -trehalose glucohydrolase, EC 3.2.1.28) and it was investigated in detail during the course of post-dormant development of American *Artemia* [4]. The trehalase activity of American *Artemia* is unchanged until the emergence of the stage E-1 prenauplius (at 6 hr of the culture), followed by a 10 fold increase in its activity coinciding with the peak of the emergence of the stage E-2 prenauplius (at 12 hr of the culture). The level of the trehalase activity decreased thereafter, and half of the maximal activity remained in the nauplius incubated for 30 hr.

To elucidate in greater detail the biosynthesis and molecular nature of the trehalase, we obtained a cDNA fragment of *Artemia* trehalase and investigated the developmental change of trehalase gene expression during the post-dormant stage. A Southern blot analysis was also carried out. We compared these data between American and Chinese *Artemia*.

Materials and Methods

Animals

Two species of *Artemia* were used in our experiments. The dried dormant cysts of *Artemia* from the Great Salt Lake in Utah, USA and from China were purchased from Japan Pet Drugs Co. (Tokyo and Los Angeles, CA). Rearing conditions of the dormant cysts of American *Artemia* have been described elsewhere [4]. Chinese *Artemia* was incubated in the same way except for using 3% NaCl instead of 2% NaCl as for the American one.

Enzyme Assay

Trehalase activity was measured as described previously [4].

Oligonucleotides as Primers for Polymerase Chain Reaction (PCR)

Based on a homology screening of the C-terminal region of the known trehalases of rabbit small intestine [5], *Tenebrio molitor* (mealworm beetle) [6], *Bombyx mori* (silkworm) [7, 8], *Caenorhabditis elegans* [9], and *Escherichia coli treA* [10], two degenerate oligonucleotides were designed to amplify a supposedly conserved C-terminal region of *Artemia* trehalase. The sense primer and the antisense primer used are shown in Fig. 1.

the resulting product was again amplified under the same conditions because of its small quantity. The amplification was performed with a DNA thermal cycler (Perkin Elmer Cetus). The resulting products were separated on a 3% agarose (NuSieve® GTG Agarose, TaKaRa) gel, and a 228 bp fragment was isolated. This fragment was subcloned into the *EcoR* V site of pBluescript SK (–) (Stratagene).

Sequencing and Analysis of the cDNA Fragment

DNA sequences of 5 inserts were determined by the dideoxy chain termination method [12] using the Thermo Sequenase fluorescent labeled cycle sequencing kit (Amersham) adapted to a 373 DNA Sequencer (Applied Biosystems). All inserts were sequenced completely on both strands, and they all proved to be identical except for one base at their center region, resulting in a change of one amino acid (Fig. 1). The amino acid sequence deduced from the fragment of trehalase cDNA was compared with amino acid sequence data base SWISS-PROT using the FASTA program.

Northern Blot

All of the RNA was extracted from both *Artemia* whole embryos and larvae at different culture times as described above in the PCR Amplification section. For blot analysis, about 20 μ g of aliquot from the total RNA were electrophoresed on a 1% agarose gel with formaldehyde, transferred to Hybond-N⁺ nylon membrane (Amersham) [11], and fixed by baking at 120°C for 25 min. As a hybridization probe, the subcloned PCR fragment was labeled with digoxigenin in PCR containing 2 μ M each of definite primers based on the nucleotide sequence analysis, 160 μ M each of dATP, dCTP, dGTP, 104 μ M dTTP, 56 μ M digoxigenin-11-dUTP (Boehringer Mannheim), 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1 unit of *TaKaRa Taq* (TaKaRa). The blotted RNAs were hybridized with the labeled probe at 68°C and washed at 65°C, and the probe hybridized was detected by the antibody (Anti-Digoxigenin-AP, Fab fragment, Boehringer Mannheim) and chemiluminescent substrate (CSPD®, Boehringer Mannheim) [13].

Southern Blot

Genomic DNA was prepared from more than 48-hr cultured nauplii of both *Artemia* by the method of Yamada *et al.* [14]. Aliquots of 8 μ g of the DNA were digested separately with 27 units of *EcoR* I, *Hind* III, and *Bam*H I for 15 hr, at 37°C. The digests were separated on a 0.7% agarose gel and blotted onto a Hybond-N⁺ nylon membrane [15]. All the following procedures necessary for hybridization and detection were performed as mentioned above

in the Northern Blot section.

Results and Discussion

Characterization of the cDNA fragment of *Artemia* trehalase

Sequence analysis showed that the obtained cDNA fragment was composed of 228 bp. It was deduced to encode 76 amino acids. A computer-assisted research revealed no significant similarity in the fragment to any other protein except for trehalases of several species (Fig. 1). The rate of identical residues with *T. molitor* (mealworm beetle) [6], rabbit [5], *C. elegans* [9] *B. mori* (silkworm) [7, 8], and *E. coli treA* [10] and *treF* [16] were 57.9–37.7% (Fig. 1). We could not compare the fragment with that of human trehalase, because N- and C-terminal regions of the human trehalase have not yet been isolated [17]. The obtained fragment had less similarity with trehalases of *Candida albicans* (33.3%) [18], *Kluyveromyces lactis* (33.3%) [19] and *Saccharomyces cerevisiae* (yeast) *YBR0106* (*NTH2*) (33.3%) [20, 21, 22] and *NTH1* (31.3%) [23, 24], especially in the region where the primer Y might bind. The sequence of the fragment resembles those of higher eukaryotes and bacteria, but differs somewhat from fungi. These results indicate that the cDNA fragment really encodes the C-terminal region of the trehalase of *Artemia*, one of the crustacean.

Analysis of trehalase gene expression

Results of Northern blot analysis on the total RNA from whole embryos and larvae from both *Artemia* are shown in Fig. 2. The probe clearly hybridized to RNAs of 2.7 and 2.2 kb at 0 hr of the culture on American *Artemia*, followed by the appearance of a 5.0 kb RNA from 6 hr to 30 hr, maximized at 12 hr. The smallest RNA diminished early in the post-dormant development. Chinese *Artemia* expressed two RNAs of 2.7 and 5.0 kb, which were detected from 6 hr and 30 hr of the culture, respectively (Fig. 2). The contents of the 18 S rRNA from both species increased gradually during their developments (Fig. 2).

The change in the activity of the trehalase during the post-dormant development, as described in the Introduction, suggests the significance of the 5.0 kb transcript in American *Artemia*.

The activity of the trehalase in the development of Chinese *Artemia* was unchanged until 10 hr of the culture, and gradually increased, reaching a plateau of 5 fold increase in its activity between 30–40 hr of the culture (data not shown). The difference in the developmental profile of the trehalase activity between American and Chinese *Artemia* might be due to the low hatchability and poor synchronism of Chinese *Artemia* [4]. The developmental

Fig. 2. Developmental profile of *Artemia* trehalase gene expression. Northern blot analysis and the ethidium bromide-stained 18 S rRNA of American (A-1, A-2) and Chinese (B) *Artemia* are shown. RNAs extracted from both *Artemia* whole embryos and larvae at different times in culture were hybridized with the 228 bp probe labeled with digoxigenin and chemiluminously detected by the antibody. (A-1) and (A-2) show results obtained by a shorter exposure and a longer exposure, respectively. Digoxigenin-labeled molecular markers (RNA Molecular Weight Marker I, Boehringer) were used on the gel.

profile of the trehalase activity in Chinese *Artemia* well correlated with the appearance of the 2.7 kb transcript in Chinese *Artemia*.

The molecular mass of the enzyme trehalase was reported to be 70 kDa in both *Artemia*, and Endoglycosidase H digestion of the trehalase resulted in a molecular mass of 66 kDa on SDS-PAGE [4]. This means that mRNA for the trehalase may be equal to or longer than 2 kb. All of the transcripts detected were in agreement with this condition.

Newly synthesized transcripts in the post-dormant development of American *Artemia* were the 5.0 kb RNA, and the RNAs of 2.7 and 5.0 kb in Chinese *Artemia*. The size of the larger transcript in Chinese *Artemia*, which was detected rather later, corresponded to that of the newly synthesized RNA in American *Artemia*. The size of the smaller transcript in Chinese *Artemia* corresponded to the larger transcript already present at 0 hr of the culture on American *Artemia*. These suggest a possibility for both *Artemia* that the 5.0 kb RNA is a precursor mRNA, which is usually undetectable but detectable around the maximal level of transcription, and that the 2.7 kb RNA, which is relatively stable, is the mature mRNA molecule for the trehalase. However, a great deal of the 2.7 kb transcript at 0 hr of the culture on American *Artemia* is

incomprehensible.

Reported sizes of mRNA for trehalase, numbers of encoded amino acids and molecular mass of the mature trehalase are as follows: human, 2 kb, 75 kDa [17]; rabbit, 1.9 kb, 578 aa, 65 kDa [5, 25]; *T. molitor*, 2.0 kb, 555 aa, 62 kDa [6, 26]; *B. mori*, 3.0 kb, 579 aa, 70 kDa [7, 8]; *Escherichia coli treA*, 565 aa, 58 kDa [10]; *E. coli treF*, 549 aa, 64 kDa [16]; *Artemia*, 70 kDa [4].

The role of the transcript of 2.2 kb at 0 hr of the culture on American *Artemia* is not clear at present. It is plausible that it might be a degradation product of the transcripts of 5.0 and 2.7 kb or a processed transcript which has other functions than trehalase.

Southern Blot Analysis

Genomic DNAs from whole nauplii of both *Artemia* were digested with

Fig. 3. Southern blot analysis of trehalase gene. DNA from American (A) and Chinese (B) *Artemia* nauplii digested with *EcoR* I (1), *Hind* III (2), and *BamH* I (3) restriction enzyme was hybridized with the 228 bp probe labeled with digoxigenin, and chemiluminously detected by the antibody. Digoxigenin-labeled molecular markers (DNA Molecular Marker VII, Boehringer) were used on the gel.

EcoR I, *Hind* III, and *Bam*H I restriction enzyme, respectively, and used in a Southern blot analysis to examine the complexity of the gene. The probe carries one *Hind* III cleavage site (Fig. 1), but no sites for *EcoR* I and *Bam*H I. As shown in Fig. 3, *EcoR* I and *Hind* III digestions gave two and three DNA fragments, respectively, on both *Artemia*. *Bam*H I gave one fragment on American *Artemia* and two fragments on the Chinese one. These results are consistent with the idea for both *Artemia* that the trehalase gene is unique and has one intron which is on a region where the probe binds, or two highly homologous trehalase genes exist. Quotations described in the next paragraph are not contrary to the probability of the above idea and give perspective to the complexity of the trehalase gene.

The genetic and biochemical analyses of *Drosophira* trehalase indicated that soluble and membrane bound trehalases are manifestations of a single protein encoded by a single gene [27]. A single copy of the trehalase gene was reported to be present in the haploid genome of *B. mori*, expressing two types of mRNA, each of which is for soluble and membrane bound trehalase [7, 8]. The Southern blot analysis of rabbit genomic DNA suggested that there exist(s) three genes or one gene with two introns in its haploid genome [5]. In *S. cerevisiae* (yeast), the neutral trehalase gene named *NTH1* was reported to be on chromosome IV and a putative trehalase-encoding gene named *YBR0106* (*NTH2*) was also reported to be on chromosome II. The amino acid sequence of the latter gene showed 77% identity with *NTH1* [20, 21, 22]. Periplasmic trehalase is known to be homologous to cytoplasmic trehalase in *E. coli*, and they are coded by *treA* [10] and *treF* [16], respectively.

Experiments designed to isolate full length cDNA encoding *Artemia* trehalase are now under study. The results and discussion described above gave us perspectives for the coming experiments. Isolation of cDNA corresponding to *Artemia* trehalase will provide further insight into the biosynthesis and molecular nature of the enzyme as well as the phylogenetic origin of the enzyme and the regulation of the expression of the gene.

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アルテミアの発生におけるトレハラーゼ遺伝子の発現： 2種のアルテミアにおける比較

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要 旨： ホモロジーに基づいてプライマーを設計し、アメリカ産アルテミア胚の cDNA を鋳型として PCR を行い、トレハラーゼ cDNA の断片を増幅した。得られた断片は 228bp で、76 個のアミノ酸からなり、ゴミムシダマシ、ウサギ、*C. elegans*、カイコおよび大腸菌のトレハラーゼに高い類似性 (58–38%) を認めた。この断片をプローブとしてノーザンブロットしたところ、アメリカ産アルテミアでは 5.0, 2.7 および 2.2 kb の転写産物を、中国産では前 2 者のみを検出した。トレハラーゼ活性と転写産物の発生における変化を両アルテミアで比較検討した結果、5.0 および 2.7 kb の産物がトレハラーゼの翻訳に直接または間接的に関与していると考えられた。サザンブロットの結果、両アルテミアにハプロイドゲノムあたり 2 コピーの遺伝子が存在するか、またはプローブ結合部位にイントロンのある 1 コピーのトレハラーゼ遺伝子があると考えられた。

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