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Research article

Biosynthetic pathways of glycinebetaine in *Thalassiosira pseudonana*; functional characterization of enzyme catalyzing three-step methylation of glycine

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ABSTRACT

Betaine (trimethylglycine) is an important compatible solute that accumulates in response to abiotic stresses such as drought and salinity. Biosynthetic pathways of betaine have been extensively studied, but it remains to be clarified on algae. A diatom *Thalassiosira pseudonana* CCMP1335 is an important component of marine ecosystems. Here we show that the genome sequence of *Thalassiosira* suggests the presence of two biosynthetic pathways for betaine, via three step methylation of glycine and via two step oxidation of choline. The choline oxidation via choline dehydrogenase was suggested and its sequential characteristics were analyzed. A candidate gene *TpORF1* for glycine methylation encodes a protein consisted of 574 amino acids with two putative tandem repeat methyltransferase domains. The TpORF1 was expressed in *E. coli*, and the purified protein was shown to synthesize betaine via three step methylation of glycine and designated as TpGSDMT. The proteins containing C-terminal half or N-terminal half were expressed in *E. coli* and exhibited the methyl transferase activities with different substrate specificity for glycine, sarcosine and dimethylglycine. Upregulation of *TpGSDMT* transcription and betaine levels were observed at high salinity, suggesting the importance of TpGSDMT for salt tolerance in *T. pseudonana* cells.

1. Introduction

Glycine betaine (trimethylglycine), here after betaine, is an important compatible solute that accumulates under abiotic stress conditions such as drought, salinity, extreme temperatures, UV radiation and heavy metals. Four biosynthetic pathways for betaine are known (Rontein et al., 2002; Chen and Murata, 2011; Takabe et al., 2015); three are oxidation of choline and one is the methylation of glycine. Most well-known pathways of betaine are two step oxidation of choline in plants, animals and bacteria (choline → betaine aldehyde → betaine). The first step enzyme is choline monooxygenase (CMO) in plants and choline dehydrogenase (CDH) in animals and bacteria (Rathinasabapathi et al., 1997; Salvi and Gadda, 2013). The second step is catalyzed by the same enzyme betaine aldehyde dehydrogenase (BADH) in all these organisms. Some bacteria synthesize GB by single enzyme, choline oxidase (COX), without BADH. The second biosynthetic pathway is the three-step methylation of glycine (glycine → sarcosine → dimethylglycine → betaine) which is known in some bacteria. In anaerobic phototrophic sulfur bacterium *Ectothiorhodospira halochloris* (Nyyssla et al., 2000; Takabe et al., 2015), two enzymes EcGSMT and EcSDMT are involved in betaine synthesis. Here, EcGSMT catalyzes the AdoMet dependent methyltransferase (MT) reactions of glycine and sarcosine whereas EcSDMT catalyzes the MT reactions of sarcosine and dimethylglycine. In a halotolerant cyanobacterium *Aphanothece halophytica*, two MTs named as ApGSMT and ApDMT were involved in three methylation steps of betaine synthesis (Waditee et al., 2003). The importance of ApGSMT and ApDMT for the construction of salt tolerance plants were demonstrated (Waditee et al., 2005; Waditee-Sirisattha et al., 2012).

In a halophilic methanoarchaeon *Methanohalophilus portucalensis* strain FDF1^T, three kinds of MTs (MpGSDMT, MpGSMT and MpSDMT) were reported (Lai et al., 2006; Lai and Lai, 2011). Here, the enzyme MpGSDMT catalyzed the MT reactions of glycine, sarcosine and dimethylglycine. In the above, all MTs except MpGSDMT, have 270–280 amino acids residues. The MpGSDMT exists as 240 kDa tetramer of 52 kDa protein, but its gene is unknown (Lai et al., 2006). Presence of

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; ASW, artificial sea water; CDH, choline dehydrogenase; CMO, choline monooxygenase; DMSP, dimethyl surfoniopropionate; MT, methyltransferase; GSDMT, glycine sarcosine dimethylglycine methyltransferase

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long size MT with two putative tandem repeat methyltransferase domains has been reported in aerobic heterotrophic eubacterium *Actinopolyspora halophila* (Nyyssla et al., 2000). The protein consists of 565 amino acids and exhibited the sarcosine MT (SMT) and dimethylglycine MT (DMT), but no glycine MT (GMT) activity. Since the truncated C-terminal polypeptide (282–565) showed the weak GMT activity (Nyyssla et al., 2000), here this full length protein was also designated as AcGSDMT.

Diatoms are unicellular and photosynthetic eukaryotic algae distributed in marine and freshwater systems all over the world (Alverson et al., 2011). Effects of salinity on the growth of some *Talassiosira* have been reported (Vrieling et al., 2007; Garcia et al., 2012), but not on the growth of *Talassiosira pseudonana* CCMP1335. A diatom *T. pseudonana* accumulates both betaine and DMSP, and intercellular contents of these compounds are regulated by temperature and carbon dioxide (Spielmeyer and Pohnert, 2012). However, biosynthetic pathways of betaine in marine algae including diatoms have not been reported. We searched the genome sequence of *T. pseudonana* (Armbrust et al., 2004) and found the candidate genes for betaine synthesis via both glycine methylation and choline oxidation. Biochemical approaches were conducted on the glycine methylation pathway and revealed that the single enzyme catalyzed all three step methylation reactions to synthesize betaine. Primary structure of this MT protein consisted of two tandem MT domains, and each domain showed different substrate specificities.

2. Materials and methods

2.1. Culture conditions

T. pseudonana CCMP1335 in this study was obtained from National centre for Marine Algae and Microbiota (Bigelow, USA). *T. psuedonana* cells were cultured in artificial sea water (ASW) containing 2% of Provasoli's enriched seawater (Summers et al., 1998) under continuous illuminated condition (120 μmol m^{-2} s^{-1}) at 18 °C. For investigation of salinity effects, the concentration of NaCl was changed from 0.453 M (x 0.1) to 0.906 M (x 2.0). *E. coli* DH5α and *E. coli* BL21 (DE3) cells were grown at 37 °C or 25 °C in LB medium containing appropriate antibiotics. Optical densities were measured at 420 and 620 nm for *T. psuedonana* and *E. coli*, respectively.

2.2. Extraction of compatible solute from T. pseudonana cells

T. pseudonana cells were collected by centrifugation at 10,000 × g for 10 min, suspended in 200 μl of 100% methanol, and then centrifuged again at 22,000 × g for 20 min. The resulting supernatants were dried up by rotary evaporator (TAITEC, Saitama, Japan), and then dissolved in 200 μl of water. After addition of 200 μl chloroform, the mixture was vigorously shaken and centrifuged at 22,000 × g for 10 min. The upper aqueous phase was used to measure DMSP and betaine by a time-of-flight mass spectroscopy (TOF-MS) (Kratos AXIMA-CFR, Shimadzu, Kyoto, Japan) (Waditee-Sirisattha et al., 2012). For measurement, 1 μl of the extract, 1 μl of matrix (10 g/L solution of sinapinic acid in 2:3 acetonitrile: 0.1% v/v trifluoroacetic acid), and 1 μL of internal standard solution were mixed, dried, and then applied to the instrument. For internal controls, the stable isotope compounds, d_{11}-betaine was used.

2.3. Isolation, expression, and purification of MTs

The putative MT gene was isolated from the cDNA of *T. pseudonana* by PCR using specific primer sets (Table.S1). The cDNA was obtained by using RNeasy plant mini kit (QIAGEN, Hilden, Germany). PCR reactions were initiated with KOD plus neo polymerase (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Obtained DNA fragments were added deoxyriboadenocine (dA) using with Mighty TA-cloning Kit for PrimeSTAR (Takara, Otsu, Japan), cloned into pMD20-T vector (Takara), and then transformed into *E. coli* DH5α. Resulting plasmid, pCR-ORF1, was purified by Miniprep kit (QIAGEN) and then sequenced. After digestion with restriction enzymes *Bam*HI and *Not*I, the resulting DNA fragment containing ORF was ligated into the expression vector pGEX6P-1 (GE Healthcare Life Science, Little Chalfont, United Kingdom). Resulting plasmid, pGEX-ORF1, was transformed into *E-coli.* BL21 (DE3). *E. coli* cells containing expression vectors were grown in 1 ml of LB medium with 50 μg/ml of ampicillin at 37 °C for overnight. These cultures were added to 200 ml of LB medium with 50 μg/ml of ampicillin, and subcultured for several hours. When the optical density reached to ~0.5, the cultures were cooled down to 25 °C, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM. After culturing 15 h, the cells were collected by centrifugation at 7000 × g for 20 min, and suspended in 5 ml of Buffer A (50 mM Tris-HCl, pH 8.0, 5 mM DTT, 1 mM $MgCl_2$) containing 1% Triton-X. The cells were broken by sonication (output: 7, on time: 30s, off time: 30 s, total on time: 4 min, model VP-5s, TITEC, Saitama, Japan), and the supernatants after centrifugation at 22,000 × g for 15 min were used for protein purification step.

For purification of GST-fused protein from pGEX6P-1 based system, 100 μl (bed volume) of Glutathione Sepharose 4 B beads (GE Healthcare Life Science) equilibrated with Buffer A was added to the supernatants, and then incubated at 4 °C for 30 min. After incubation, the beads were collected and washed with 1 ml of Buffer A three times, and then 1 unit of PreScission protease (GE Healthcare Life Science) was added into the beads suspension in 100 μl of Buffer A. After incubation at 4 °C for overnight, the purified proteins were collected and determined their concentration.

2.4. Measurement of methyltransferase activities

Methyltransferase activities were measured as previously described with a slight modification (Waditee et al., 2003). The reaction mixture consisted of 25 μl of 25 mM substrate (glycine, sarcosine, dimethylglycine, alanine, and methionine as methyl acceptors), 25 μl of buffer B (125 mM Tris-HCl, pH 8.0, 5 mM DTT, 1 mM $MgCl_2$), 25 μl of 4 mM AdoMet (10 nCi of *S*-adenosyl-L-[*methyl*-^{14}C]methionine), and 25 μl of enzyme solution. The reaction was started by the addition of enzyme, and after incubation for 30 or 60 min at 25 °C, the reaction was stopped by addition of charcoal suspension (133 g/l in 0.1 M acetic acid). Then the mixtures were incubated on ice at 10 min. After centrifugation at 10,000 × g for 10 min, 75 μl of supernatants were removed and mixed with 100 μl of Insta-Gel Plus cocktail (PerkinElmer, MA, USA). The resulting mixture was subjected to a liquid scintillation counter 3200C (ALOKA, Tokyo, Japan) to measurement the amount of ^{14}C in methyl acceptors. Blank values obtained in the absence of enzyme were subtracted. The enzyme activities were calculated as nanomoles of methyl groups transferred per min. The pH in the reaction mixture was adjusted by the following buffers, 125 mM potassium phosphate (pH 6.2 and 7.0), 125 mM Tris-HCl (pH 8.0 and 8.8), and 125 mM bicarbonate (pH 10.0). For measurement activities in *T. pseudonana* extracts, the soluble protein extracts were used. *T. pseudonana* cells collected as described above were suspended in Buffer A, and then cells were broken by sonication (output: 7, on time: 15s, off time: 15 s, total on time: 1 min). After centrifugation at 22,000 × g for 15 min, the supernatant was used for measurement of methyltransferase activities as soluble protein extracts. For the measurements of kinetic parameters, the concentrations of methyl acceptors were changed between 0 and 12.5 mM. Methyl donor was changed between 0 and 2.5 mM. The reaction products of methyltransferases were identified by TOF-MS.

2.5. Gel filtration chromatography

Gel filtration analyses were carried out at 4 °C using an AKTA prime liquid chromatography system (GE Healthcare Life Science, Little Chalfont, United Kingdom). The purified methyltransferases or soluble