[SIV-2]

Functional Investigation of Pteridine Glycosides in Cyanobacteria

Young Shik Park
Department of Microbiology, Inje University, Kimhae 621-749, Korea
E-mail: mbyspark@ijnc.inje.ac.kr

Introduction

Unconjugated pteridines are ubiquitous in nature as cofactors and pigments [1]. Tetrahydrobiopterin (BH4) is the best known as a cofactor for aromatic amino acid hydroxylation and nitric oxide synthesis in higher animals [2]. In cyanobacteria and some microbes [3,4], unconjugated pteridines have been found as glycosidic forms. The pteridine glycosides have various kinds of sugars attached to the side chain at C6 of the pterin ring. Pteridine glycosides are peculiar in their high cellular concentrations [3,4]. In *Synechocystis* sp. PCC 6803 the intracellular concentration of cyanopterin was comparable to that of chlorophyll a [4]. However, the cellular function of pteridine glycosides remained unknown, although earlier studies postulated some roles in photosynthesis [3].

To investigate the putative cellular function of pteridine glycosides, our research has been focused on enzymes which catalyze the transfer of sugar to the pterin ring. The enzymes, named pteridine glycosyltransferases as a novel group of UDP-glycosyltransferases, were considered important as a useful target for gene disruption study in the relevant organisms, because it was presumed that the putative function of pteridine glycosides might be conferred by the sugar moiety. The gene encoding a pteridine glycosyltransferase was cloned for the first time in *Synechococcus* sp. PCC 7942 [5], following purification of the enzyme [6]. The enzyme catalyzes transfer of glucose from UDP-precursor to BH4 and was named UDP-glucose:BH4 α-glucosyltransferase (BGluT). Disruption of BGluT gene in the organism resulted in low level of aglycosidc BH4 production and impaired the cellular growth in photoautotrophic growth conditions, thereby suggesting the role of sugar residue to maintain the high cellular concentration of the compound [5]. On the other hand, elucidation of the primary structure of BGluT led us to find a homologous protein in the translated genome sequences of *Synechocystis* sp. PCC, in which a pteridine glycoside named cyanopterin was identified previously [4]. The encoding gene slr1166 was disrupted in *Synechocystis* sp. PCC 6803 in order to identify the protein function and to investigate the putative role of sugar residue in cyanopterin.

Materials and methods

Growth conditions. Synechocystis sp. PCC 6803 was grown at 30℃ in BG-11 medium in batch culture under continuous white light (100~120 μmol m^{-2} s^{-1}) and air bubbling. Growth was measured by the optical density at 730 nm.

Construction of knockout vectors. The ORF slr1166 was amplified by PCR using a primer pair, 5'-TAGGATCCATGCGCCTACTATTTGTT-3' (forward primer) tailed with *Bam*HI sequence (underlined) and 5'-TTATTCTCGAGTTATTGGTAAAAATTCAT-3' (reverse primer) with *Xho*I sequence (underlined). The 1141 bp DNA product was cloned into the pBS-SK(+) vector. To construct a knockout vector the 2.2 kb kanamycin resistant gene was digested from pHP45 Ω-Km with *Eco*RI and ligated into the corresponding site of slr1166, which was previously cloned in pBS-SK(+) vector. *Synechococcus* cells were transformed following the protocol [6]. Positive colonies were restreaked to single ones with at least five serial transfers to obtain full segregation of the mutation.

Assays of pteridine compounds. Intracellular pteridine compound in cyanobacterial cells was analyzed by HPLC as described previously [7]. Cells harvested by centrifugation were suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and oxidized by acidic iodine solution. HPLC was performed using a Kontron Model 430 system equipped with Inertsil ODS-3 C18 column (5 μm, 150 x 2.3 mm, GL Sci., Japan). Pteridine compounds were eluted isocratically with 10 mM potassium phosphate buffer (pH 6.0) at a flow rate of 1.2 ml/min and monitored at 350/450 nm (excitation/emission) by using a fluorescence detector (HP Model 1046A). Pteridine compounds were purchased from Dr. B. Schircks Lab (Jona, Switzerland).

Results and Discussion

Cyanopterin produced by *Synechocystis* sp. PCC 6803 has a peculiar chemical structure of 6-[1-(4-*O*-methyl-(α-D-glucuronyl)-(1,6)-(β-D-galactosyloxy)methylpterin. Therefore, it was expected that there should be two different pteridine glycosyltransferases catalyzing the transfer of each sugar residues. A homology search of the translated genome sequences of *Synechocystis* revealed a few putative proteins. Among them, the protein encoded by slr1166 was the most homologous one, having a sequence identity of 45% (Fig. 1). The putative protein consisted of 354 amino acid residues having a calculated molecular mass of 38751. Therefore, it was highly probable that slr1166 encodes a pteridine glycosyltransefrase responsible for cyanopterin synthesis. However, homologies of the other putative proteins were not significant enough to show sequence identities of around 10%.

Fig. 1. Pairwise alignment of the translated amino acid sequences of BGluT and slr1166 genes. The protein sequence of BGluT from *Synechococcus* sp. PCC 7942 was aligned with the deduced polypeptide from the ORF slr1166 in *Synechocystis* sp. PCC 6803. Conserved sequences are indicated at four levels (*, :, . and none).

In order to confirm whether the slr1166 gene encodes a genuine pteridine glycosyltransferase and to further investigate the putative role of sugar residues in cyanopterin, the slr1166 gene in *Synechocystis* was disrupted using a knockout plasmid constructed in pBS-SK(+). The knockout vector contained 2.2 kb kanamycin resistant gene cassette in the *Eco*RI site of slr1166 gene. A transformant obtained after several rounds of segregation was analyzed by PCR. The mutant genomic DNA generated only disrupted slr1166 gene (Fig. 2A). The approx. DNA size of 3.3 kb corresponds to the sum of slr1166 and Km genes, indicating insertion of Km gene into the chromosomal slr1166 gene. The transformant was further analyzed for intracellular pterdine compounds (Fig. 2B). The iodine oxidized cellular extract exhibited an unknown pteridine peak eluting at 23 min (Fig. 2BII), instead of cyanopterin eluting at 7 min (Fig. 2BI, peak b). If the putative pteridine glycosyltransferase encoded by slr1166 is responsible for the first sugar transfer, the resulting pteridine compound produced by the mutant should be 6-hydroxymethylpterin. Therefore, the unknown peak was speculated to be a 6-hydroxymethylpterin-galactoside, based on the chemical structure of cyanopterin. In order to identify the pteridine moiety in the compound the acidic cellular extract was heated at 100℃ and then chromatographed (Fig. 2BII). As expected, the original peak disappeared gradually with concomitant increase of a new peak eluting at the position of 6-hydroxymethylpterin. These results clearly indicate the transformant is completely blocked in the second sugar transfer for cyanopterin synthesis and demonstrate in vivo that slr1166 encodes a pteridine glycosyltransferase responsible for the step.

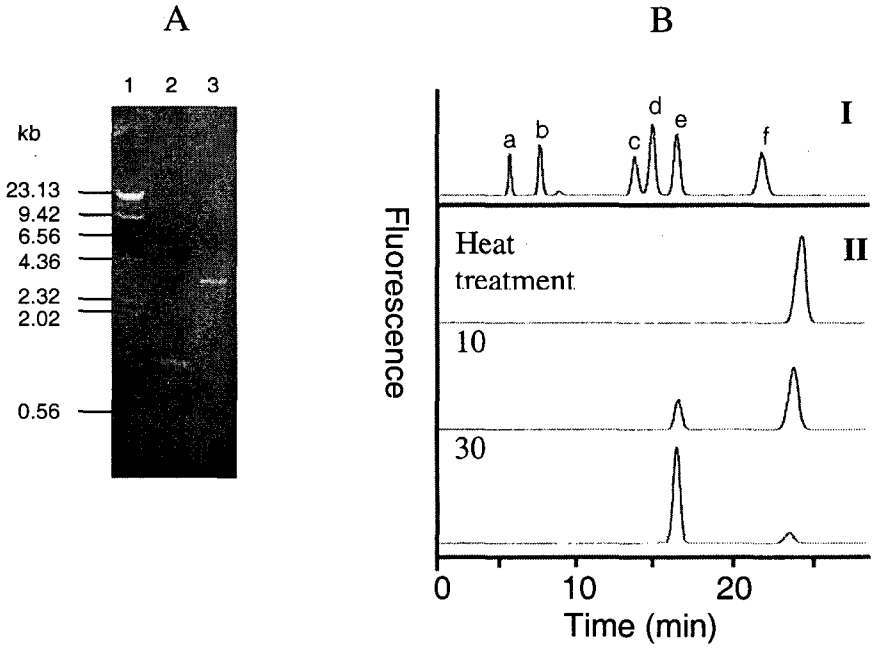

Fig. 2. Identification of gene disruption in the knockout transformant. (A) PCR amplification of slr1166 gene was performed with genomic DNAs of wild type (lane 2) and mutant (lane 3) strains. DNAs were separated on 1% agarose. Lambda-*Hind*III DNA markers (lane 1) are shown on the left. (B) HPLC analysis of intracellular pteridine compound in mutant cells. I: Standard pteridines: a, neopterin; b, cyanopterin; c, pterin; d, biopterin; e, 6-hydroxymethylpterin; f, dictyopterin. II: Pteridine compound in the mutant cells. The cellular extract in acidic iodine solution was heated at 100℃ for the indicated times and separated on C18 column.

The mutant was analyzed for the cell growth and pteridine production in normal photoautotrophic conditions (Fig. 3). The mutant was viable but showed slightly impaired growth. The growth rate measured by absorbance at 730 nm was approximately 80% level of wild type (Fig. 3A). Their chlorophyll a levels and intracellular concentration of pteridines also showed similar patterns. The molar ratios of pteridine against chlorophyll a are plotted in Fig. 3B. The approx. value of wild type was 0.68, while that of the mutant was 0.5, indicating that the yield of 6-hydroxymethylpterin-galactoside in the mutant decreased to 74% level of cyanopterin production in wild type. The level of pteridine production seems to reflect the growth rate, meaning a quantitative role of pteridine glycoside in *Synechocystis*. If the second sugar residue 4-methoxyglucuronic acid is required for any specific function of cyanopterin, the growth rate of the mutant should have been decreased more drastically. Therefore, these results support the previous suggestion [5] that glycosylation of pteridine compound is required for the maintenance of high cellular concentration of the compound, thereby supporting the normal growth of cyanobacterial cells. We presume that the second sugar residue in cyanopterin may have a subsidiary role to implement the cellular concentration of cyanopterin. It might originate from a problem that the pterin moiety of cyanopterin is 6-hydroxymethylpterin not biopterin, which is more common in other pteridine glycosides. The

additional decoration might have been evolved in *Synechocystis* to meet the requirement of high intracellular concentration of pteridne glycoside. In case of BGluT gene disruption in *Synechococcus* sp. PCC 7942 [5], the yield of aglycosidic BH4 in the mutant decreased to approx. 8.3% of wild type in a normalized value to chlorophyll a concentration.

We currently have two mutant strains deficient in sugar transfer to pteridines: BGluT knockout mutant of *Synechococcus* sp. PCC 7942 and slr1166 knockout mutant of *Synechocystis* sp. PCC 6803. Further characterization of them using functional genomics techniques will provide valuable information on the cellular function of pteridine glycosides in cyanobacteria.

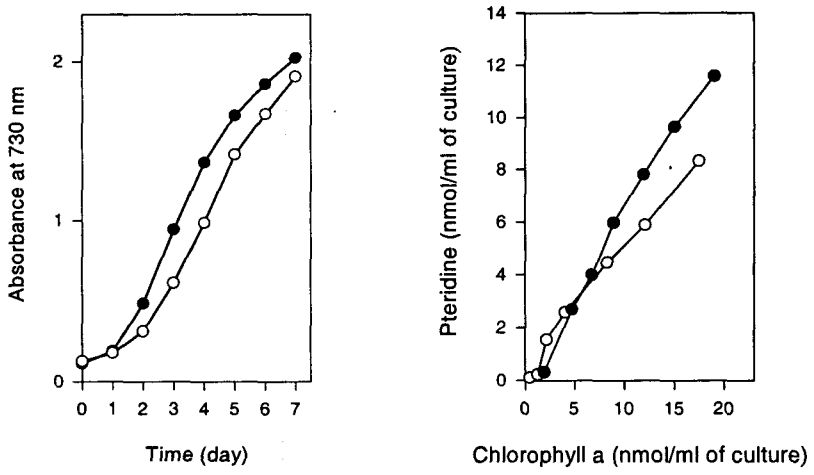

Fig. 3. Comparative analyses of the cell growth and pteridine production in both wild type (closed circle) and the knockout mutant (open circle). (A) Cell growth measured at 730 nm. (B) Intracellular pteridine concentration plotted against the chlorophyll a concentration. 6-hydroxymethylpterin-galactoside from mutant and cyanopterin from wild type were quantified as an equivalent to 6-hydroxymethylpterin. HPLC was performed exactly as described in Fig. 2, except using 5% aqueous methanol as a mobile phase for quantitative assay.

Acknowledgments

This study was supported by Korea Research Foundation Grant (KRF-2001-015-DP0507).

References

[1] Nichol, C.A., Smith, G.K. and Duch, D.S. (1985) Annu. Rev. Biochem..54, 729-764.

[2] Thöny, B., Auerbach, G. and Blau, N. (2000) Biochem. J. 347, 1-16.

[3] Forrest, H.S. and Van Baalen, C. (1970) Annu. Rev. Microbiol. 24, 91-108.

[4] Lee, H.W., Oh, C.H., Geyer, A., Pfleiderer, W. and Park, Y.S. (1999) Biophys. Biochim. Acta 1410, 61-70.

[5] Choi, Y.K., Hwang, Y.K. and Park, Y.S. (2001) FEBS Lett. 502, 73-78.

[6] Chung, H.J., Kim, Y.-A., Kim, Y.J., Choi, Y.K., Hwang, Y.K. and Park, Y.S. (2000) Biochim. Biophys. Acta 1524, 183-188.

[7] Lee, S.W., Lee, H.W., Chung, H.J., Kim, Y.-A., Kim, Y.J., Hahn, Y., Chung, J.H. and Park, Y.S. (1999) FEMS Microbiol. Lett. 176, 169-176.