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Purification and characterization of UDP-glucose:tetrahydrobiopterin glucosyltransferase from *Synechococcus* sp. PCC 7942

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Abstract

Tetrahydrobiopterin (BH4)-glucoside was identified from *Synechococcus* sp. PCC 7942 by HPLC analysis and the enzymatic activity of a glycosyltransferase producing the compound from UDP-glucose and BH4. The novel enzyme, named UDP-glucose:BH4 glucosyltransferase, has been purified 846-fold from the cytosolic fraction of *Synechococcus* sp. PCC 7942 to apparent homogeneity on SDS-PAGE. The native enzyme exists as a monomer having a molecular mass of 39.2 kDa on SDS-PAGE. The enzyme was active over a broad range of pH from 6.5 to 10.5 but most active at pH 10.0. The enzyme required Mn^{2+} for maximal activity. Optimum temperature was 42°C. Apparent K_m values for BH4 and UDP-glucose were determined as 4.3 μM and 188 μM, respectively, and V_{max} values were 16.1 and 15.1 pmol min^{-1} mg^{-1}, respectively. The N-terminal amino acid sequence was Thr-Ala-His-Arg-Phe-Lys-Phe-Val-Ser-Thr-Pro-Val-Gly-, sharing high homology with the predicted N-terminal sequence of an unidentified open reading frame slr1166 determined in the genome of *Synechocystis* sp. PCC 6803, which is known to produce a pteridine glycoside cyanopterin.

Keywords: Biopterin glucoside; Tetrahydrobiopterin; Pteridine glycosyltransferase

1. Introduction

Pteridine glycosides, which consist of unconjugated pteridines and various kinds of sugars attached to the side chain at C-6 of the pterin ring, are abundant in cyanobacteria [1–6]. However, their physiological function has remained unknown since their first discovery in the 1950s [1], in contrast to some unconjugated pteridines which have an essential function as cofactors in several kinds of enzymes [7]. Tetrahydrobiopterin (BH4) is the best known for aromatic amino acid hydroxylation [8] and nitric oxide synthesis in higher animals [9,10]. Molybdopterin, which is ubiquitous from bacteria to human, is essential for aldehyde oxidase, nitrate reductase, sulfite oxidase, and others [7].

Cyanobacteria are notorious for the presence of pteridine glycosides, although some were found in a few prokaryotes such as the anaerobic photosynthetic bacteria *Chlorobium tepidum* [11] and *Chlorobium limicola* [12] and a chemoautotrophic archaebacterium *Sulfolobus solfataricus* [13]. Besides, cyanobacteria have been studied much for the function related to light and nitrogen fixation. Therefore, we believe that the physiological function of pteridine glycosides in cyanobacteria deserves in-depth investigation.

Recently we identified a novel form of pteridine glycoside [6], cyanopterin, in a genome sequenced cyanobacterium *Synechocystis* sp. PCC 6803 [14] and subsequently some genes encoding enzymes involved in the early steps of the synthesis [15]. However, the biosynthesis of cyanopterin, which has the chemical structure 6-[(1-(4-*O*-methyl-α-D-glucuronyl)-(1,6)-(β-D-galactosyloxy)methylpterin, has not been completed because of a failure in identifying the enzyme(s) responsible for the transfer of sugars to the pterin ring. The enzyme, named pteridine glycosyltransferase as a novel group of glycosyltransferases, was considered important not only for establishing the biosynthesis, but also for understanding the functional role of cyanopterin or its sugar moiety, based on the speculation that the putative function of pteridine glycosides is conferred by the sugar attached to the pterin moiety.

Abbreviations: Biopterin, 6-(L-*erythro*-1′-2′-dihydroxypropyl)pterin; BH4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; cyanopterin, 6-[1-(4-*O*-methyl-α-D-glucuronyl)-(1,6)-(β-D-galactosyloxy)methylpterin; BGluT, UDP-glucose:tetrahydrobiopterin glucosyltransferase

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To date, a pteridine glycosyltransferase catalyzing the transfer of *N*-acetylglucosamine to BH4 has only been purified from *C. limicola* [16], and no sequence information is available for application to the genome of *Synechocystis* sp. PCC 6803. As an alternative to solve the problem encountered in the strain, we decided to develop another cyanobacterium as a model organism for our future research. *Synechococcus* sp. PCC 7942 was selected as a suitable one, because it is one of the well-characterized cyanobacteria and has been developed much for genetic manipulation. As expected, a pteridine glycoside was observed in high amounts in the strain, and this finding has driven us to investigate the biochemistry of the compound. In this study, we report the identification of BH4-glucoside in *Synechococcus* sp. PCC 7942 and the purification of a pteridine glycosyltransferase, which catalyzes the synthesis of the compound. The enzyme was also characterized to show the properties.

2. Materials and methods

2.1. Culture conditions

Synechococcus sp. PCC 7942, kindly donated by T. Omata (Kyoto University, Japan), was grown photoautotrophically at 30°C in BG11 medium under continuous white light (100–120 μmol m^{-2} s^{-1}) while bubbling with air.

2.2. Chemicals

All chemicals used were of reagent grade. Pteridine compounds were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). Sephadex G-100, phenyl-Sepharose, DEAE-Sepharose, methotrexate-agarose, and UDP-sugar derivatives were purchased from Sigma. Inertsil ODS-3 C18 analytical column was from GL Sci. (Japan). Mono-Q HR (5/5) and Superose 6 HR were from Pharmacia Biotech.

2.3. Intracellular pterin analysis

Cells harvested by centrifugation were suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and mixed with an equal volume of acidic iodine solution (2% KI/1% I_2 in 1 N HCl). The mixture was incubated for 1 h at room temperature in the dark and centrifuged to remove precipitate. The supernatant was mixed with a one tenth volume of 5% ascorbic acid to reduce excess iodine and assayed for oxidized pteridines by HPLC (see below).

2.4. Enzyme assay

Unless noted otherwise, the standard reaction mixture consisted of 50 mM sodium phosphate, pH 7.5, 10 mM $MgCl_2$, 0.2% ascorbic acid, 50 μM BH4, 500 μM UDP-glucose, and enzyme solution. The reaction was carried out for 30 min at 37°C. The standard assay condition was applied to the characterization of the purified enzyme. During purification, on the other hand, the reaction was performed in 50 mM sodium cacodylate buffer, pH 6.6, and continued for 1 h at 37°C based on the result obtained with desalted ammonium sulfate precipitate of *Synechococcus* sp. PCC 7942 as an enzyme source. The reaction mixture was oxidized by addition of an equal volume of acidic iodine solution for 1 h in the dark. After centrifugation, the supernatant was mixed with 5% ascorbic acid and subjected to HPLC. The amount of produced biopterin-glucoside was quantified as an equivalent to biopterin. One unit of the enzyme was defined as the activity to produce 1 μmol of biopterin-glucoside in 1 min using the described conditions.

HPLC was performed on a Kontron Model 430 equipped with a Rheodyne loop of 20 μl, an Inertsil ODS-3 C18 column (5 μm, 150×2.3 mm, GL Sci.), and a HP Model 1046A fluorescence detector. For fluorescence detection wavelengths were set at 350 nm for excitation and at 450 nm for emission. To quantify biopterin-glucoside aliquots or reaction mixtures were injected into the column in water at a flow rate of 1.2 ml min^{-1}. For qualitative identification of pteridines 10 mM sodium phosphate (pH 6.0) was used as a mobile phase as described previously [15].

2.5. Enzyme purification

Harvested cells (25 g wet weight) were suspended in 100 ml of 20 mM Tris-HCl, pH 8.0, 0.5 mM PMSF, 1 mM EDTA, and 0.4 mg per ml of lysozyme. The suspension was incubated for 1 h at room temperature and sonicated with Vibra Cell (Sonics and Materials, USA). The homogenate was centrifuged for 30 min at 15 000×*g* to discard the precipitate. Saturated ammonium sulfate solution was added to the supernatant to collect the precipitate obtained at 25–50%. The precipitate was dissolved in buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 12 mM β-mercaptoethanol, 10% glycerol) and dialyzed overnight against the same buffer. The dialyzed solution was applied to a column (2.5×100 cm) of Sephadex G-100 preequilibrated with buffer A and chromatographed with the same buffer at a flow rate of 20 ml h^{-1}. The active fractions were mixed with saturated ammonium sulfate solution to a final concentration of 30% and loaded on a column (1×6.4 cm) of phenyl-Sepharose equilibrated previously with buffer A containing 30% ammonium sulfate. The column was washed with the same buffer and subsequently with 5% ammonium sulfate in buffer A until the absorbance at 280 nm became zero. Proteins were eluted with buffer A without ammonium sulfate. The active fractions were combined and then concentrated and desalted by ultrafiltration through a YM30 membrane in a stirred

pressure cell (Amicon). The enzyme solution was applied to a DEAE-Sepharose column (2.5×6.1 cm) equilibrated with buffer A and eluted with a linear gradient of 0–500 mM NaCl in a total volume of 200 ml. The combined active fractions were concentrated and desalted by ultrafiltration and applied to a methotrexate-agarose column (1×6.4 cm) equilibrated with buffer A. After washing with buffer A, the enzyme was eluted with a linear gradient of 0–500 mM NaCl in 100 ml of buffer A. The active fractions combined were concentrated and desalted through ultrafiltration. The enzyme was further purified by a FPLC system equipped with a Mono-Q (HR 5/5) column (1 ml) equilibrated with buffer A. The enzyme was eluted with a linear gradient of 0–350 mM NaCl in 20 ml of buffer A at a flow rate of 1 ml min^{-1}. The active fractions were analyzed for purity by SDS-PAGE. The purified fraction was dialyzed against buffer A and stored at −70°C until used for characterization. All the procedures described above were carried out at 4°C in a cold room. Protein was measured by the method of Bradford [17], using bovine serum albumin as a standard.

2.6. Determination of molecular mass

The molecular mass of the purified enzyme was determined by gel permeation chromatography on a Superose 6 HR column (1×30 cm). The column was equilibrated with 20 mM Tris-HCl, pH 8.0, and then calibrated with aldolase (158 kDa), alcohol dehydrogenase (120 kDa), bovine serum albumin (66 kDa) and ribonuclease A (13.7 kDa). For the determination of the subunit molecular mass, SDS-PAGE was carried out with a 12.5% polyacrylamide gel under reducing condition.

2.7. N-Terminal amino acid sequencing

The purified protein was resolved by SDS-PAGE, transferred to a PVDF membrane in CAPS buffer (pH 10.8), and stained by Coomassie brilliant blue R-250. Sequencing was performed on a Procise 491A automatic sequencer (Applied Biosystems) at the Korea Basic Science Institute (Taejon, Korea).

3. Results and discussion

3.1. Identification of intracellular BH4-glucoside

HPLC analysis of the iodine oxidized cell extract of *Synechococcus* sp. PCC 7942 demonstrated the presence of high amounts of a pteridine compound in the cell. A large fluorescent peak is clearly shown in the first chromatogram of Fig. 1B at the retention time distinguishable from those of common pteridines shown in Fig. 1A. As the pteridine compound was presumed to be a glycoside, the iodine oxidized cell extract was subjected to heat treatment to cleave the attached sugar and then analyzed by HPLC. As shown in Fig. 1B, a new peak appeared at the corresponding position of biopterin and increased in intensity depending on the duration of heat treatment, while the original peak decreased concomitantly (Fig. 1B). The new peak was confirmed by co-injection with the authentic biopterin (data not shown). The result suggested that the pteridine glycoside found in *Synechococcus* sp. PCC 7942 is a biopterin-glycoside, which presumably exists as a re-

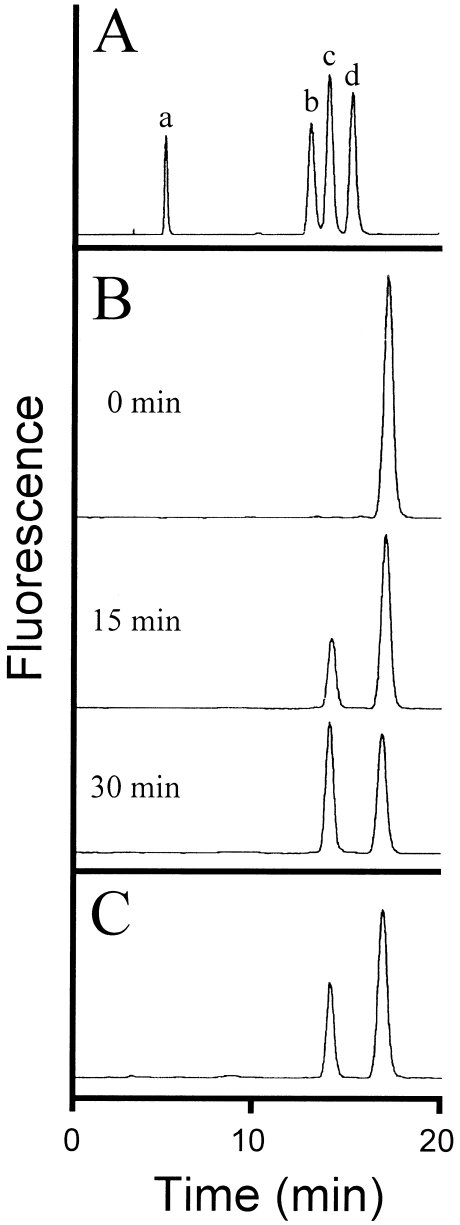

Fig. 1. HPLC identification of biopterin-glucoside in *Synechococcus* sp. PCC 7942 and BGluT activity catalyzing the synthesis of the reduced form. (A) Standard pteridines: a, neopterin; b, pterin; c, biopterin; d, 6-hydroxymethylpterin. (B) Heat treatment of cellular extract in acidic iodine solution. The extract was heated at 100°C for the indicated times and subjected to HPLC. (C) BGluT activity demonstrated in vitro with BH4 and UDP-glucose. Pteridines were eluted isocratically with 10 mM sodium phosphate, pH 6.0, at a flow rate of 1.2 ml min^{-1} and detected with a fluorescence detector (350/450 nm).

Table 1
Summary of the purification of BGluT from *Synechococcus* sp. PCC 7942

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Ammonium sulfate fractionation	370	138.30	0.37	100.0	1.0
Sephadex G-100 chromatography	162.29	156.57	0.96	113.2	2.6
Phenyl-Sepharose chromatography	11.66	232.22	19.92	167.9	53.3
DEAE-Sepharose chromatography	1.41	51.21	36.32	37.0	97.2
Methotrexate-agarose chromatography	0.14	16.64	118.83	12.0	317.9
Mono-Q chromatography	0.0084	2.66	316.21	1.9	846.0

One unit of activity corresponds to 1 μmol of biopterin-glucoside formed per minute.

duced form of BH4-glycoside in the cell as demonstrated in other pteridine glycosides [6,11,12]. Not only to prove the suggestion but also to identify the sugar moiety in the compound a putative pteridine glycosyltransferase activity was examined in the crude extract of *Synechococcus* sp. PCC 7942 using BH4 and various kinds of UDP-sugars. The enzyme activity was observed only with UDP-glucose, but not with other sugar donors (Fig. 1C). The oxidized enzymatic product, which eluted after a peak of biopterin, coincides well in its retention time with that of the intracellular pteridine glycoside shown in Fig. 1B. Therefore, it is evident that the pteridine glycoside synthesized in *Synechococcus* sp. PCC 7942 is a BH4-glucoside. Its exact chemical structure remains to be determined by chemical analyses but is expected to be 1-*O*-(L-*erythro*-tetrahydrobiopterin-2′-yl)-β-glucose, considering the determined chemical structures of biopterin glycosides [4,11,12], in which the sugars are linked in β-configurations at C2′-OH of the biopterin moiety.

3.2. Purification and molecular properties of BGluT

The above result of the identification of a pteridine glycosyltransferase activity in *Synechococcus* sp. PCC 7942 paved the way to purify the enzyme. The enzyme was named UDP-glucose:BH4 glucosyltransferase (BGluT) according to its catalytic property and substrate specificity. BGluT was purified 846-fold from the cytosolic fraction of *Synechococcus* sp. PCC 7942, with a recovery of 1.9% relative to the preparation of ammonium sulfate fraction, as summarized in Table 1. The final purified fraction gave a single band on SDS-PAGE (Fig. 2). The apparent molecular mass of the purified enzyme was determined to be 39.2 kDa by its mobility on 12.5% SDS-PAGE. The native enzyme was eluted as a single peak corresponding to a 40 kDa protein by gel permeation chromatography on Superose 6 HR (data not shown). These results indicate that the enzyme migrates as a monomer in gel filtration under the mild conditions used and thus may also be present and active as a monomer in solution.

3.3. Effects of pH, temperature, and metal ions

In a preliminary assay of BGluT activity with a desalted ammonium sulfate fraction of *Synechococcus* cells, the enzyme was active at pH 6.5–7.0 and stimulated more by Mg^{2+} than by Mn^{2+} or Ca^{2+}. However, the purified BGluT was active over a wider range of pH 6.0–10.5 (Fig. 3); the highest activity was observed with 50 mM glycine-NaOH buffer, pH 10.0 with a plateau of lower

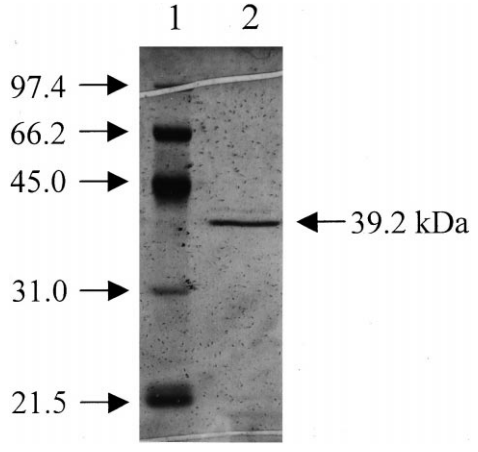

Fig. 2. SDS-PAGE of the purified BGluT from *Synechococcus* sp. PCC 7942. The final purified fraction was analyzed by a SDS-PAGE of 12.5% polyacrylamide under reducing condition (lane 2). The molecular masses of the marker proteins (lane 1, Bio-Rad low range) are depicted on the left. Protein was visualized by silver staining.

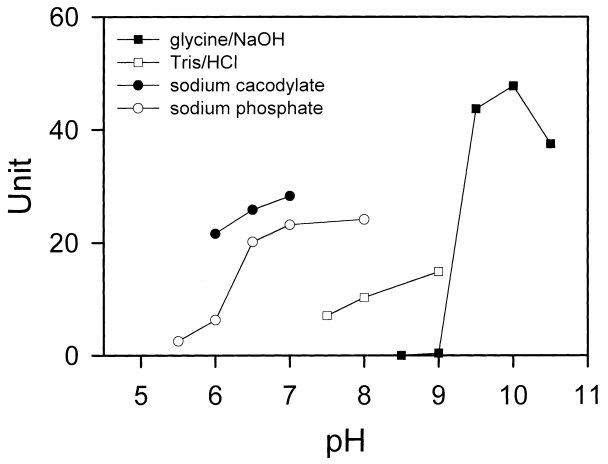

Fig. 3. Effect of pH on the activity of BGluT. The enzyme activity was assayed at the pH values indicated. Sodium phosphate (○; pH 5.5–8.0), sodium cacodylate (●; pH 6.0–7.0), Tris-HCl (□; pH 7.5–9.0), and glycine/NaOH (■; pH 8.5–10.5). Buffers were 50 mM final concentrations.

Table 2
Effect of metal ions on BGluT activity

Metal ion	Relative activity (%)
None	100.0
EDTA (1 mM)	38.9
Ni^{2+}	7.8
Co^{2+}	15.5
Zn^{2+}	81.2
Cu^{2+}	91.1
Ca^{2+}	154.7
Mg^{2+}	195.9
Mn^{2+}	253.2

The purified enzyme was assayed in the standard assay condition with 10 mM metal ions in chloride forms.

activity around pH 7.5. Probably the nature of the buffers might be important on BGluT activity. The enzyme required divalent metal ions for activity (Table 2). In contrast to the partially purified enzyme, which was stimulated more by Mg^{2+} than by Mn^{2+}, the purified BGluT activity was enhanced most effectively with 10 mM Mn^{2+} but less with Mg^{2+} or Ca^{2+}. The enzyme showed its highest activity at about 42°C (Fig. 4).

3.4. Substrate specificity and kinetic parameters

BGluT was highly specific to substrates, accepting only UDP-glucose and BH4. None of UDP-galactose, UDP-glucuronic acid, UDP-xylose, and UDP-*N*-acetylglucosamine was used as a substrate by BGluT. The tetrahydro or dihydro form of 6-hydroxymethylpterin and dihydrobiopterin was not utilized as acceptor.

Kinetic parameters of the enzyme were determined from Lineweaver-Burk plots of the two substrates, BH4 and UDP-glucose (Table 3). The apparent K_m and V_{max} values for BH4 were determined at a fixed concentration of 500 μM UDP-glucose, and were 4.3 μM and 16.6 pmol min^{-1}, respectively. The values for UDP-glucose determined with 50 μM BH4 were 188 μM and 15.1 pmol min^{-1}, respectively.

3.5. N-Terminal amino acid sequence

The N-terminal sequence analysis of the purified enzyme revealed 13 residues of Thr-Ala-His-Arg-Phe-Lys-Phe-Val-Ser-Thr-Pro-Val-Gly-. Homology search of the sequence against the Cyanobase [14] presented a highly homologous open reading frame (ORF) but no more in the NCBI database. As shown in Fig. 5, seven residues match perfectly with the deduced amino acid sequence at the N-terminal end of the unidentified ORF slr1166. The ORF encodes a polypeptide of 354 amino acids with a deduced molecular mass of 39 289 Da, quite similar to the determined mass of BGluT by SDS-PAGE or gel permeation chromatography. Although the predicted sequence of slr1166 showed no overall similarity to any sequence in databases, it exhibited a region of highly conserved sequence at the C-terminal half for glycosyltransferases (data not shown), indicating that the putative protein is a glycosyltransferase. Furthermore, the slr1166 was classified in a family of glycosyltransferases based on its amino acid sequence similarity [18]. These results strongly support that the protein encoded by slr1166 is the corresponding homologue of BGluT in the organism and further imply that BGluT as well as the protein of slr1166 consists of two structural domains: one for sugar binding in the conserved C-terminal sequence and the other for pterin binding in the variable N-terminal half. This kind of two-domain structure seems common in a variety of glycosyltransferases having different acceptor specificities, such as baculovirus ecdysteroid UDP-glucosyltransferase, *Erwinia herbicola* zeaxanthin glucosyltransferase, flavonol 3-*O*-galactosyltransferase from *Petunia hybrida*, and ceramide UDP-galactosyltransferase from rat brain [19–22]. Therefore, we propose that BGluT and the putative protein of slr1166 belong to a novel type of glycosyltransferases, which we named pteridine glycosyltransferase.

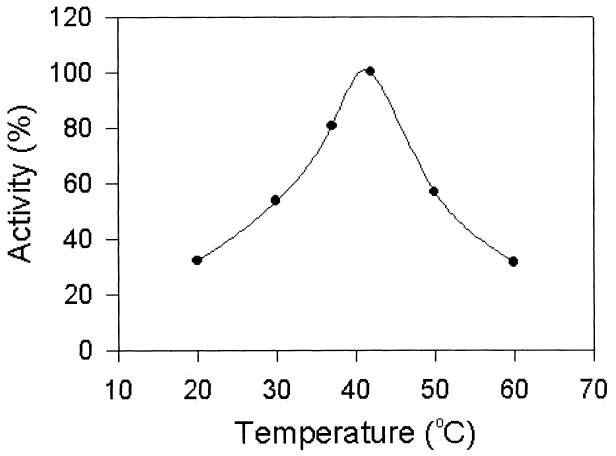

Fig. 4. Effect of temperature on the activity of BGluT. The enzyme activity was measured in the standard assay condition at the indicated temperatures.

Table 3
Kinetic parameters of BGluT

Substrate	K_m (μM)	V_{max} (pmol min^{-1} mg^{-1})
BH4	4.3	16.6
UDP-glucose	188.0	15.1

The kinetic parameters were determined with 500 μM UDP-glucose and 50 μM BH4, respectively.

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PCC 7942: TAHRFLFVSTPVG-
PCC 6803:M---RLLFVSSPVSSLNSGRLGGVAL-
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Fig. 5. N-Terminal amino acid sequence of BGluT purified from *Synechococcus* sp. PCC 7942. The sequence is compared with the corresponding region of the deduced polypeptide from the ORF slr1166 in *Synechocystis* sp. PCC 6803. Identical residues are boxed.

4. Conclusions

In this study BGluT catalyzing the synthesis of BH4-glucoside was purified from *Synechococcus* sp. PCC 7942 and characterized, following the structural identification of the compound produced in the organism. BGluT belongs to a novel type of glycosyltransferases, named pteridine glycosyltransferase, as supported by the results of homology analyses using the determined N-terminal amino acid sequence. Taken together, these results provide valuable information for further research. First, our results establish a new model system in *Synechococcus* sp. PCC 7942 for the research of pteridine glycosides. Second, the determined N-terminal sequence of BGluT will allow cloning of the gene from *Synechococcus* sp. PCC 7942, enabling more thorough investigation of BGluT and molecular research in the organism. Third, the occurrence of a highly homologous ORF slr1166 in the genome sequence of *Synechocystis* sp. PCC 6803 will make it possible to resume the unfinished work related to the biosynthesis and function of cyanopterin [15]. The BGluT gene and the ORF slr1166 are useful targets for gene disruption study in the respective organisms, which is believed helpful for elucidating the physiological function of pteridine glycosides abundant in cyanobacteria.

Acknowledgements

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