

# Production of Sepiapterin in *Escherichia coli* by Coexpression of Cyanobacterial GTP Cyclohydrolase I and Human 6-Pyruvoyltetrahydropterin Synthase

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***Synechocystis* sp. strain PCC 6803 GTP cyclohydrolase I and human 6-pyruvoyltetrahydropterin synthase were coexpressed in *Escherichia coli*. The *E. coli* transformant produced sepiapterin, which was identified by high-performance liquid chromatography and enzymatically converted to dihydrobiopterin by sepiapterin reductase. Aldose reductase, another indispensable enzyme for sepiapterin production, may be endogenous in *E. coli*.**

Sepiapterin is a precursor as well as a by-product of tetrahydrobiopterin (BH4) synthesis. BH4 is a well-known cofactor for aromatic amino acid hydroxylation and nitric oxide synthesis in higher animals (8, 12) and is used for treatment of hyperphenylalaninemia and endothelial dysfunction (2, 14). De novo synthesis of BH4 starts with GTP (7, 8, 10, 11). GTP is converted to dihydroneopterin triphosphate by GTP cyclohydrolase I (GTPCH) (EC 3.5.4.16), and the dihydroneopterin triphosphate is converted to 6-pyruvoyltetrahydropterin (PPH4) by 6-pyruvoyltetrahydropterin synthase (PTPS) (EC 4.6.1.10). Finally, sepiapterin reductase (SR) (EC 1.1.1.153) reduces both carbonyl groups of PPH4 to form BH4. In addition, aldose reductase (AR) (EC 1.1.1.21) catalyzes reduction of the C-2' oxo group in PPH4, yielding 6-lactoyltetrahydropterin (LPH4). LPH4 is further reduced to BH4 by SR or is oxidized nonenzymatically to sepiapterin, which is its dihydro form. In the salvage pathway of BH4, sepiapterin is converted to 7,8-dihydrobiopterin by SR and then to BH4 by dihydrofolate reductase (8). *Escherichia coli* is not known to produce BH4 or sepiapterin. However, in the study described here, we found that coexpression of cyanobacterial GTPCH and human PTPS (hPTPS) in *E. coli* resulted in sepiapterin production.

A coexpression vector harboring the sequences encoding *Synechocystis* sp. strain PCC 6803 GTPCH (cGTPCH) and hPTPS was constructed in T7-based pET-28a (Novagen). Both DNAs were available as the clones in *E. coli* expression vectors which have been reported to produce functional recombinant proteins (6, 9). The GTPCH gene of *Synechocystis* sp. strain PCC 6803 was previously cloned in pET-15b (pET-cGTPCH) (6). The hPTPS cDNA was amplified from the original vector in pMal-c2 (9), which was a gift from N. Blau and B. Thöny of Zürich University. The oligonucleotides used for the PCR were 5'-GGAATTCATATGAGCACGGAAGGTGGT-3' (forward primer) and 5'-CGGGATCCTATTCTCCTTTATAAACCA-3' (reverse primer). The DNA was cloned into the pGEM-T vector (Promega) and subsequently cloned as an *NdeI/BamHI* restric-

tion fragment into pET-28a (pET-hPTPS). Finally, a *BglII/HindIII* fragment of pET-cGTPCH was introduced into the *BamHI/HindIII* site of the pET-hPTPS plasmid, giving rise to the coexpression vector pET-cGTPCH/hPTPS. This construct was transformed into *E. coli* BL21(DE3) (Novagen). This strain has been deposited in the Korean Collection for Type Cultures as strain KCTC 1020BP. The N-terminal His-tagged recombinant proteins were purified with an Ni-nitrilotriacetic acid gel (QIAGEN) by following the instructions in the product manual. Pteridine compounds were oxidized for fluorescence detection by mixing them with an equal volume of an acidic iodine solution (2% KI and 1% I<sub>2</sub> in 1 N HCl) as previously described (6). The solution was centrifuged at 10,000 × g, and the supernatant was reduced with ascorbic acid and subjected to high-performance liquid chromatography (HPLC). HPLC was performed with a Kontron model 430 equipped with an Inertsil ODS-3 C<sub>18</sub> column (5 μm; 150 by 2.3 mm; GL Sciences, Inc., Tokyo, Japan) and an HPLC detector (model 430) or a fluorescence detector (model 1046A; Hewlett-Packard). Pteridines were eluted with 10 mM sodium phosphate (pH 6.0) at a flow rate of 1.2 ml min<sup>-1</sup> and were monitored at an excitation wavelength of 350 nm and an emission wavelength of 450 nm (6). Sepiapterin was eluted with 12% aqueous methanol at the same flow rate and was monitored at 420 nm. Pteridine compounds were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed under denaturing conditions.

The pET-cGTPCH/hPTPS transformant formed yellow colonies after overnight growth even without isopropyl-β-D-thiogalactopyranoside (IPTG) induction. After 1 day the whole medium became yellow, indicating that a remarkable amount of some compound(s) had been synthesized and excreted. The production was quantitatively assayed in a batch culture by monitoring the color absorbance at 420 nm for 23 h (Fig. 1A). The transformant constitutively produced the compound without IPTG induction, and the concentration approached saturation at the stationary phase. Although the reason remains unclear, induction with IPTG at various concentrations was rather inhibitory, as shown for a concentration of 50 μM in Fig. 1A. Yellow color formation was never observed with individual

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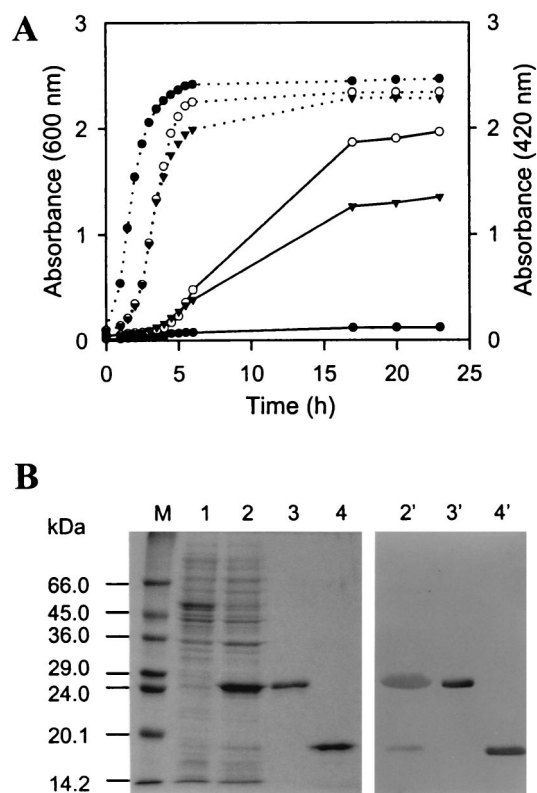


FIG. 1. (A) Time profiles of cell growth (dotted lines) and sepiapterin production in the medium (solid lines), measured at 600 and 420 nm, respectively, for nontransformed strain BL21 (●) and the coexpression transformant with (▼) and without (○) IPTG induction. (B) SDS-PAGE (left panel) and Western blot (right panel) analyses of the recombinant proteins in crude extract of the transformant. Lane 1, BL21; lanes 2 and 2', transformant; lanes 3 and 3', recombinant cGTPCH; lanes 4 and 4', recombinant hPTPS. The molecular masses of the marker proteins (lane M) are indicated on the left. His-Tag polyclonal antibody (Cell Signaling Technology) was used for the Western blot analysis.

pET-cGTPCH and pET-hPTPS transformants. Therefore, the yellow color of the transformant most likely resulted from the activities of both recombinant proteins in single cells. To identify expression of the proteins in the cells, the crude extract was analyzed by SDS-PAGE (Fig. 1B, left panel) and Western blotting (Fig. 1B, right panel). The recombinant cGTPCH in the transformant extract was distinguishable by its abundance relative to the abundance of the protein in the wild-type extract, although hPTPS was not apparent. The deduced molecular masses of the recombinant cGTPCH and hPTPS were 27.5 and 18.6 kDa, respectively. Western blot analysis with anti-His-tag antiserum clearly demonstrated the presence of both recombinant proteins migrating at the same rates as their purified counterparts.

Because the consecutive actions of cGTPCH and hPTPS should produce PPH4, a derivative was presumed to cause the yellow color. The supernatant of the medium showed maximal absorbance at 420 nm and no fluorescence under UV light (data not shown), strongly suggesting the presence of sepiapterin in the medium. To verify this, we analyzed the supernatant directly with HPLC monitored at 420 nm and found a

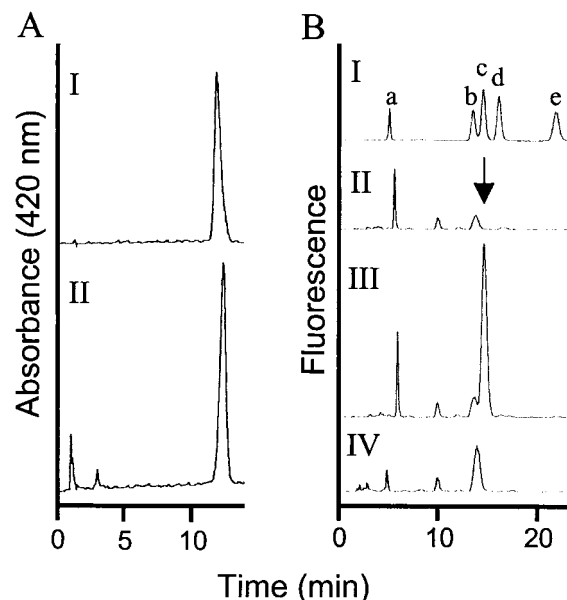


FIG. 2. Identification of sepiapterin in the medium of the coexpression transformant. (A) Direct HPLC analysis of sepiapterin in the medium. Trace I, standard sepiapterin; trace II, centrifugal supernatant of the medium. (B) Enzymatic analysis of sepiapterin with SR. Trace I, standard pteridines (a, neopterin; b, pterin; c, biopterin; d, 6-hydroxymethylpterin; e, dictyopterin); trace II, pteridines in the medium before enzymatic reaction; trace III, pteridines in the medium after incubation with SR and NADPH; trace IV, pteridines in the medium of pET-cGTPCH transformant before (and after) the SR reaction. The arrow in trace II corresponds to the peak in trace III.

large peak at the position corresponding to the position of the sepiapterin standard (Fig. 2A). Furthermore, a new fluorescent peak corresponding to the position of biopterin was seen when the supernatant was incubated with SR and NADPH and subsequently oxidized by iodine (Fig. 2B, trace III); this peak was not observed in the sample incubated without the enzyme (Fig. 2B, trace II), while the other minor peaks remained unchanged. It was therefore clear that the dominant compound produced by the *E. coli* transformant was sepiapterin. The medium of the pET-cGTPCH transformant contained a larger amount of pterin (Fig. 2B, trace IV) but no sepiapterin to generate the same chromatogram pattern after incubation with SR. No pteridine compound was detected in the medium of the pET-hPTPS transformant, as was the case for the wild type (data not shown). Therefore, we were quite sure that sepiapterin was a result of coexpression of cGTPCH and hPTPS. However, coexpression of these compounds in the cell is not enough to explain the synthesis of sepiapterin. Another required enzyme is AR, which converts PPH4 to LPH4, a non-enzymatic precursor of sepiapterin. A homology search of the finished *E. coli* genome sequence (1) revealed two open reading frames of the *yqhE* and *yafB* genes, whose products had significant protein sequence identities (39 and 34%, respectively) with human AR (GenBank accession number J04795). The *E. coli* genes were previously found to encode 2,5-diketo-D-gluconate reductases (16), which are members of the aldoketo reductase superfamily. The activities identified also are not endogenous in *E. coli* because they are important for ascorbate production in other bacteria. In higher animals AR be-

longs to an aldo-keto reductase superfamily of enzymes that reduce a wide variety of carbonyl compounds, including xenobiotic aldehydes and ketones (15). Therefore, it is likely that there is one or more aldo-keto reductases in *E. coli* that could be involved in the conversion of PPH4, although PPH4 is not an endogenous substrate. Finally, the reduced product LPH4 should be oxidized to sepiapterin and excreted into the medium. The estimated yield of sepiapterin was 32.7 mg per liter.

Our results provide the first demonstration of sepiapterin production in *E. coli* by coexpression of a cyanobacterial GTPCH and hPTPS. Sepiapterin is a useful substitute for BH4 (3, 5, 13), because it is converted to BH4 by the salvage pathway (2, 8) and is more stable than BH4, which is rapidly oxidized in solution (4). As the method currently used for organic synthesis of sepiapterin seems to be costly, our method may be valuable as an alternative for producing sepiapterin.

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