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Characterization of Geranylgeranyl Pyrophosphate Synthase from the Marine Bacterium, *Paracoccus haeundaensis*

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Carotenoids such as β-carotene and astaxanthin are used as food colorants, animal feed supplements and for nutritional and cosmetic purposes. In a previous study, an astaxanthin biosynthesis gene cluster was isolated from the marine bacterium, *Paracoccus haeundaensis*. Geranylgeranyl pyrophosphate (GGPP) synthase (CrtE), encoded by the *ortE* gene, catalyzes the formation of GGPP from farnesyl pyrophosphate (FPP), which is an essential enzyme for the biosynthesis of carotenoids in early steps. In order to study the biochemical and enzymatic characteristics of this important enzyme, a large quantity of purified GGPP synthase is required. To overproduce GGPP synthase, the *crtE* gene was subcloned into a pET-44a(+) expression vector and transformed into the *Escherichia coli* BL21(DE3) codon plus cell. Transformants harboring the *crtE* gene were cultured and the *crtE* gene was over-expressed. The expressed protein was purified to homogeneity by affinity chromatography and applied to study its biochemical properties and molecular characteristics.

Key words: *Paracoccus haeundaensis*, Carotenoid, Farnesyl pyrophosphate (FPP), Geranylgeranyl pyrophosphate (GGPP), Geranylgeranyl pyrophosphate synthase (CrtE)

Introduction

Carotenoids are natural lipid-soluble pigments produced primarily in bacteria, algae, fungi, and plants. These pigments are responsible for the wide variety of colors seen in nature (Lee and Kim, 2006a; Lee and Kim, 2006b). Carotenoids have numerous biological functions including being a vitamin A precursor, enhancing gap junction communications, modulating the immune system, and possessing antitumor activities (Akyon, 2002; Amar et al., 2004; Bertra, 1999; Jyonouchi et al., 1994). Recently, carotenoids have received great attention for their significant antioxidant activities and for playing an important role in inhibiting the onset of chronic diseases (Amar et al., 2004; Edge et al., 1997). Carotenoids such as β-carotene and astaxanthin are commercially used as food colorants, animal feed supplements and for nutritional and cosmetic purposes (Chemler et al., 2006). All carotenoids are derived from the isoprenoid (or terpenoid) pathway as shown in Fig. 1. Two pathways, the mevalonate and the non-mevalonate (or pyruvate/glyceraldehyde-3-phosphate) pathways, lead to the formation of the first isoprene unit, an isopentenyl pyrophosphate (IPP). After IPP is synthesized, the various terpenoids are synthesized with the reaction of chain elongation by successive head-to-tail condensation of dimethylallyl pyrophosphate (DMAPP), which is a reactive IPP isomer. The short-chain length prenyl transferases synthesize geranyl PP (GPP; C_{10}), farnesyl PP (FPP; C_{15}) and geranylgeranyl pyrophosphate (GGPP; C_{20}). Most carotenoids, C_{40} derivatives, originate from phytoene formed by the condensation reaction of two molecules of GGPP. GGPP synthase is an essential enzyme in the carotenoid biosynthesis pathway and catalyses the consecutive condensation of an allylic diphosphate with three molecules of IPP to produce GGPP, an essential linear precursor for carotenoid biosynthesis (Math et al., 1992). GGPP synthase is generally thought to be an important branch point enzyme for isoprenoid biosynthesis, since it catalyzes an FPP branching reaction with the condensation of FPP and IPP to produce C_{20} GGPP, which contains 4 isoprene units (Wang and Ohunma, 1999).

In a previous study, we isolated and characterized a marine bacterium, *Paracoccus haeundaensis*, which

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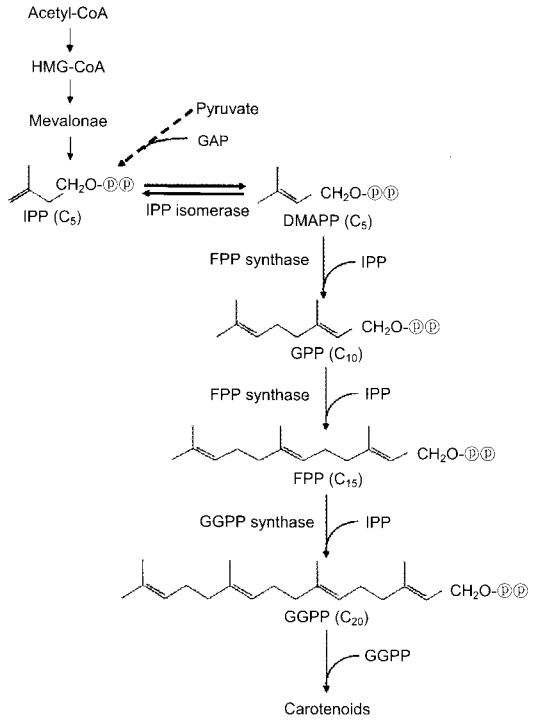

Fig. 1. Summary of isoprenoid biosynthetic pathway. Mevalonate pathway from acetyl-CoA to IPP via HMG-CoA is well known to exist in the eukaryotes from yeasts to animals. Non-mevalonate pathway, which is shown by the dotted arrow is well known in bacteria and plant chlorloplasts. Abbreviations used: CoA, coenzyme A; HMG-CoA, 3-hydrosy-3-methyl-glutaryl-coenzyme A; GAP, glyceraldehyde-3-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylally pyrophosphate; GPP, dimethyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

produces astaxanthin (Lee et al., 2004). In addition, we reported the structural and functional analysis of genes encoding the astaxanthin biosynthetic enzymes: GGPP synthase (CrtE), phytoene synthase (CrtB), phytoene desaturase (CrtI), lycopene cyclase (CrtY), β-carotene hydroxylase (CrtZ), and β-carotene ketolase (CrtW) derived from *P. haeundaensis* (Lee and Kim, 2006a). In the present study, we described the overexpression, purification and characterization of GGPP synthase originated from the marine bacterium, *P. haeundaensis*. Overexpression of GGPP synthase from *P. haeundaensis* was achieved by introducing the *crtE* gene into prokaryotic expression vector. A recombinant protein was produced in the cytoplasm of the transformed *Escherichia coli* cells. Also, we reported the purification procedures of expressed proteins from the transformed cells and the characterization of biochemical and enzymatic properties of the purified GGPP synthase. These results might provide a wider knowledge of the primary structures of GGPP synthase at the molecular level and facilitate the biotechnological applications of the carotenoid biosynthesis enzymes.

Materials and Methods

Strains and growth conditions

The *E. coli* strain BL21(DE3) codon plus harboring the pET-44a(+)-CrtE plasmid was cultured at 37°C in LB medium containing ampicillin (50 μg/mL).

Cloning of *crtE* gene onto expression vector

In order to overexpress the *crtE* gene originated from the carotenoid biosynthesis gene cluster of *P. haeundaensis*, the *crtE* expression plasmid was constructed as the following. The coding region of the *crtE* gene was amplified by PCR using a pair of corresponding oligonucleotides (Table 1) specifically designed for *crtE* gene cloning and a plasmid pCR-XL-TOPO-CrtE containing the carotenoid biosynthesis gene cluster as a template (Lee and Kim, 2007). The amplified fragment was subcloned into the vector pGEM-T (Promega, USA) and its nucleotide sequence was confirmed by DNA sequencing. The subcloned plasmid was digested with restriction enzymes, *Nde*I and *Xho*I. The digested fragment including the *crtE* gene was ligated into the expression plasmid pET44-a(+) vector. The resulting plasmid was designnated into pET-44a(+)-CrtE (Fig. 2). The pET-44a(+)-CrtE plasmid was then transformed into the *E. coli* BL21(DE3) codon plus cell.

Overexpression of *crtE* gene in *E. coli*

The cell harboring plasmid pET-44a(+)-CrtE was cultured at 37°C in 2 X Yeast extract tryptone (DYT) medium containing ampicillin (50 μg/mL) and induced by the adding of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM at a cell density corresponding to OD_{600}=0.5. Then,

Table 1. Oligonucleotide primers used for this study

Primers	Nucleotide sequences	Remarks
CrtE- 1	5' CCATATGAGACGAGACGTCAA 3'	For *crtE* cloning: forward (*Nde*I site)
CrtE-2	5' GCCGCCTCGCTAGGCGC 3'	For *crtE* cloning: reverse (*Xho*I site)

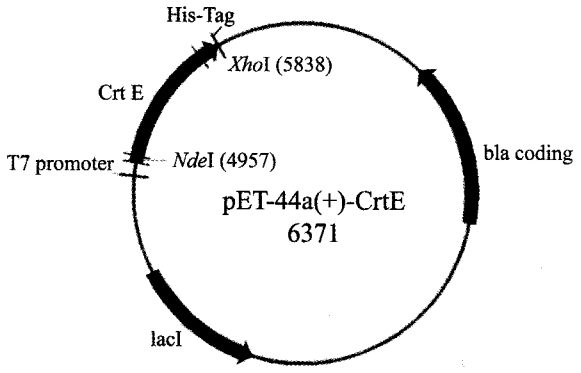

Fig. 2. Genetic map of plasmid pET-44a(+)-CrtE.

the cells were harvested by centrifugation after additional incubation times: 1, 2, 3 and 4 hours, respectively.

Purification procedures of CrtE protein

The cell pellets (1 g/wet weight) containing the expressed CrtE protein were resuspended with 20 mL of resuspension buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9), sonicated with a 50 W ultrasonic sonicator (VC130, Sonics and Material Inc., USA) for 15 sec, and then harvested by centrifugation at 13,000 rpm for 10 min. The supernatant which contains expressed CrtE protein was saved for the next step of purification (soluble fraction). The pellets were resuspended by adding isolation buffer (2 M Urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100, pH 7.9), sonicated with Ultra sonicatior, and again harvested by centrifugation. Finally, the pellets were resuspended with binding buffer (6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 7.9; solubilization of the pellet fraction). The saved soluble fraction and solubilization of the pellet fraction were each filtrated through 0.45 μm filter. Prepared samples were loaded onto an affinity column using a His-Trap Chelating HP column Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacture's instructions.

Analysis of expressed protein on SDS-PAGE

The cultures induced by the addition of IPTG at the designated induction times were centrifuged and the cell pellet was mixed with 2 X SDS loading buffer and heated at 100°C for 3 min. The treated samples were centrifuged at 13,000 rpm for 2 min and then stored on ice. Twenty microliters of each sample were loaded on a 12% SDS-polyacrylamide gel and electrophoresed under the constant current of 200 mA for 2 hours. The gel was stained with Comassie brilliant blue and then destained with destaining buffer (7% acetic acid, 15% methanol).

Western blot analysis

After proteins were separated on a 12% SDS-polyacrylamide gel, the gel was transferred to nitrocellulose membrane at 100 mA for 16 hours in transfer buffer (25 mM Tris, 120 mM glycine, 20% methanol). The nitrocellulose membrane was then washed with PBS buffer (20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 2.5 mM KCl and 0.1% tween-20) and blocked with PBS buffer containing 5% skim milk for 1 hour. Then, the membrane was washed out three times for 10 min each time with PBS buffer and treated for 1 hour with His-tag primary antibody (1:10,000 dilutions). After washing three times for 10 min each time, the membrane was treated for 1 hour with alkaline phosphatase conjugated anti-goat IgG in PBS containing 5% skim milk. The membrane was washed out three times for 10 min each time in PBS buffer and developed with nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, containing 0.1 M NaCl and 0.1 M $MgCl_2$).

Enzyme assay

The enzymatic activity of the CrtE protein was measured with a slight modification of the method as described in Lee and Kim (2006). Four hundred microliters of substrate mixture was made with 0.1 M Tris-HCl buffer (pH 8.0) containing 50 μM IPP, 100 μM FPP, 10 mM ATP, 1 mM dithiothreitol, 6 mM $MnCl_2$, 4 mM $MgCl_2$. Purified CrtE protein (0.1 μg) was added. The incubation was carried out at 37°C for 15 hours with constant shaking in the dark. After termination of the enzymatic reaction by adding 2.5 mL of methanol, the synthesized carotenoids were partitioned into 10% diethylether in petrol, evaporated, redissolved in acetone, and then separated and quantified by HPLC analysis. HPLC was performed using a Waters HPLC system (Inertsil ODS-3 column; 150 mm long x 4.6 mm). The reverse phase was a methanol/water (85/15, v/v). The flow rate was 0.5 mL/min. The injection volume and column temperature were 20 μL and room temperature, respectively. The products of farnesyl pyrophosphate (FPP), isopentenyl pyrophosphate (IPP), and Geranylgeranyl pyrophosphate (GGPP) were detected by absorbance at 214 nm. The FPP, IPP, and GGPP standards were purchased from Sigma (U.S.A.) as an authentic sample.

Results and Discussion

Overexpression of the *crtE* gene in *E. coli*

The *crtE* gene isolated from *P. haeundaensis* was composed of 879 bp encoding a polypeptide of 293 amino acid residues. In order to express the *crtE* gene in a prokaryotic system, the *crtE* gene was subcloned into the pET44-a(+) expression vector, which allows the expression of a recombinant protein with C-terminal fusion His-tag and designated pET-44a(+)-CrtE plasmid (Fig. 2). This plasmid was transformed into *E. coli* strain BL21(DE3) Codon plus cells and the expression of the recombinant *crtE* gene was induced by the addition of IPTG at a final concentration of 0.1 mM. Recombinant CrtE protein was properly expressed after addition of IPTG. The expression pattern of induced proteins from the transformed cells was analyzed by 12% SDS-PAGE (Fig. 3). The optimal induction time of recombinant CrtE protein was achieved at 3 hours after IPTG induction. The predicted size of recombinant CrtE protein is approximately 33 kDa, including a C-terminal His-Tag fusion.

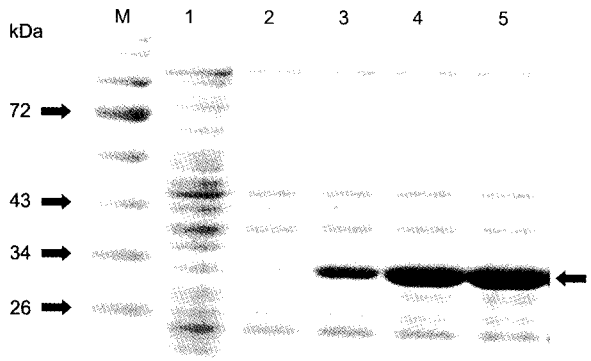

Fig. 3. The expression patterns of recombinant CrtE gene by SDS-PAGE. The expressed proteins were separated by 12% SDS-PAGE. Lane M, standard protein molecular weight markers; lane 1, proteins from uninduced cell extracts (control); lanes 2-5, proteins from induced cell extracts 1, 2, 3 and 4 hours after IPTG induction, respectively.

Purification of recombinant CrtE protein and Western blot analysis

The cell harboring the pET-44a(+)-CrtE plasmid was cultured at 37°C in DYT medium containing ampicillin (50 μg/mL) and induced by adding IPTG. The overexpressed CrtE protein was purified by the methods described in "Materials and Methods". A typical elution profile from affinity column was shown in Fig. 4. The fractions of eluted CrtE protein was confirmed by Western blot analysis. The purified CrtE protein is approximately 33 kDa at a single band, corresponding to its estimated molecular weight (Fig. 5).

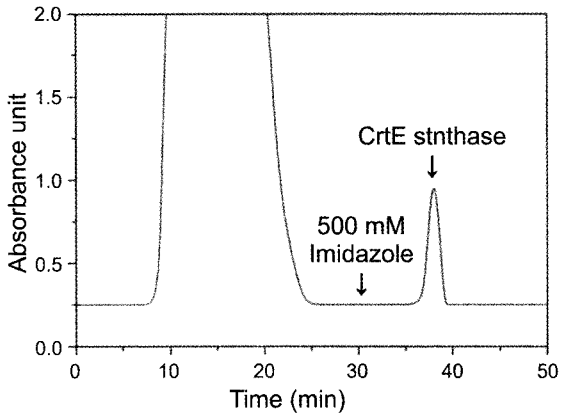

Fig. 4. Chromatogram of His-trap affinity chromatography. Purification of GGPP synthase by chromatography on His-tag column. The column was washed with buffer A containing 40 mM imidazole and bound CrtE protein was eluted with buffer A containing 500 mM Imidazole. Each fraction (1 mL) was collected.

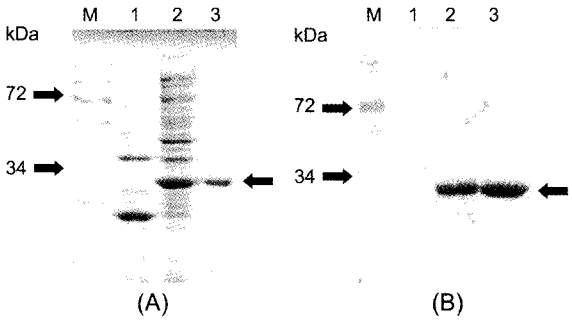

Fig. 5. SDS-PAGE (A) and Western blot (B) analysis of the purified CrtE protein. Lane M indicates molecular weight markers; lane 1, proteins from uninduced cell extracts (control); lane 2, proteins from induced cell extracts; lane 3, purified CrtE protein by affinity chromatography.

GGPP synthase activity

In order to determine the enzymatic activity of purified GGPP synthase, the purified GGPP synthase was incubated with its substrates, and the products of the reaction mixture were analyzed by HPLC. As shown in Fig. 6, the elution peaks of the HPLC analysis from the reaction mixture incubated with purified GGPP synthase (CrtE) were found to be three peaks. Peaks 1, 2, and 3 were found to be three pigments corresponding to IPP, FPP and GGPP, respectively, when these peaks were compared with standards (Fig. 6A-C). These peaks were eluted at retention times of 5.8, 4.0, and 2.9 min, respectively.

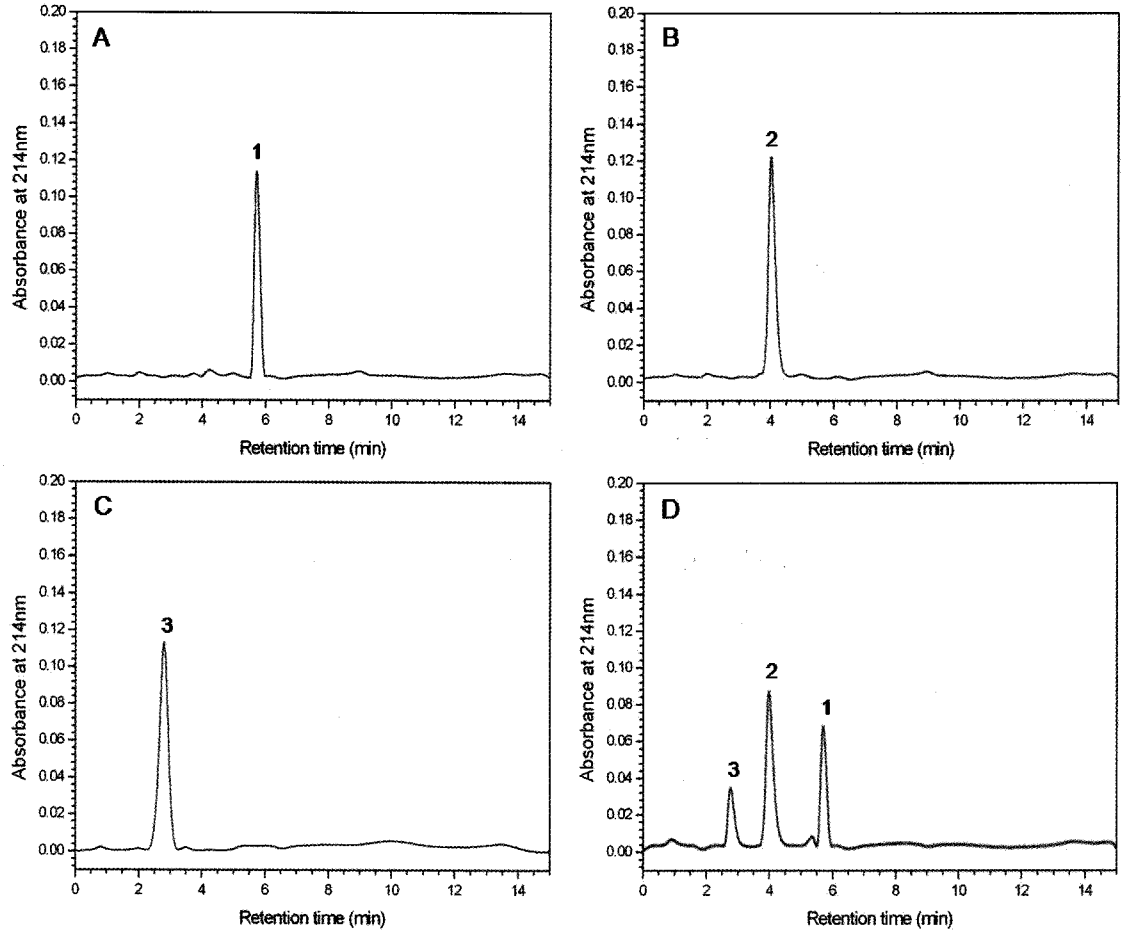

Fig. 6. HPLC patterns of the products from enzymatic assays of GGPP synthase on Inertsil ODS-3 column. Standard samples of (A) IPP (peak 1), (B) FPP (peak 2), and (C) GGPP (peak 3) for the control. (D) Samples isolated from incubation mixtures were injected and turned out to be of IPP (peak 1), FPP (peak 2) and GGPP (peak 3), respectively.

The main peak (peak 3) of the HPLC analysis was found to be GGPP by comparing it to the standard GGPP. The enzymatic properties of the purified GGPP synthase (CrtE) exhibited catalytic activity to be able to convert its substrate FPP to the product, GGPP. The maximum specific activity of purified enzyme was 1.3 μmoL/min/mg with FPP and IPP as the allelic substrate.

In the present study, we have reported the over-expression of *crtE* gene from *P. haeundaensis*, purification of the expressed protein and characterization of the purified GGPP synthase using chromatographic and spectroscopic analyses. This observation may provide a very useful model in which to study the mechanism of carotenoid biosynthesis. In addition, the results of this study can be used to enhance the production of useful carotenoids of high economic value through the manipulation of carotenoid bio-synthesis genes in *P. haeundaensis*. These data will provide a wider base of knowledge on the primary functions of the *crtE* gene at the molecular level as well as further the biotechnological applications of carotenoids.

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