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Overexpression, purification and characterization of geranylgeranyl pyrophosphate synthase from marine bacterium, Paracoccus haeundaensis

by

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Pukyong National University

August 2007

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해양 미생물 *Paracoccus haeundaensis*서 유래된
geranylgeranyl pyrophosphate synthase의 대량 발현,
순수 분리 및 특성 연구

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by

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ABSTRACT

Carotenoids such as β-carotene and astaxanthin are used as food colorants, animal feed supplements and for nutritional and cosmetic purposes. Recently, carotenoids have received a great attention for their significant antioxidant activities and for playing an important role in inhibiting the onset of chronic diseases. In addition to its antioxidative activity, astaxanthin has been shown to gave biological functions as diverse as serving as a vitamin A precursor, enhancing gap junction communications, and modulating the immune system.

A carotenoid biosynthesis gene cluster for the production of astaxanthin was isolated from the marine bacterium *Paracoccus haeundaensis*. Early steps in the biosynthesis of carotenoid include the

formation of the C_{20} compound geranylgeranyl pyrophosphate (GGPP) from farnesyl pyrophosphate (FPP) by GGPP synthase, an enzyme encoded by the *crtE* gene. Geranylgeranyl (GGPP) synthase is thought to be a key enzyme in the carotenoid biosynthesis pathway. In order to obtain a large quantity of the purified GGPP synthase, the *crtE* gene was subcloned into pET-44a(+) expression vector, transformed into the *E. coli* BL21(DE) codon plus cell, and the expressed protein was purified to homogeneity by affinity chromatographic method. Finally, the biochemical properties of the purified GGPP synthase have been characterized

INTRODUCTION

Carotenoids are natural lipid-soluble pigments produced primarily within bacteria, algae, fungi, and plants. These pigments are responsible for the wide variety of colors seen in nature (Lee and Kim, 2006a). The majority of the 600 known structures of carotenoids are C_{40}-carotenoids, while a few bacterial carotenoids exist with 30, 45 or 50 carbon atoms (Chemler J. A., 2006). Carotenoids such as β-carotene and astaxanthin are commercially used as food colorants, animal feed supplements and for nutritional and cosmetic purposes (Chemler J. A., 2006). 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; Bertram, 1999; Jyonouche *et al.*, 1994). More recently, carotenoids have received a 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 R. *et al.*, 1998).

All carotenoids are derived from the isoprenoid (or terpenoid) pathway as shown in Figure 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). In archaea, fungi, animals, and plants, IPP is synthesized from mevalonic acid with acetyl-CoA as a starting precursor. In eubacteria, IPP synthesis proceeds via an initial condensation reaction between pyruvate and glyceraldehyde-3-phosphate

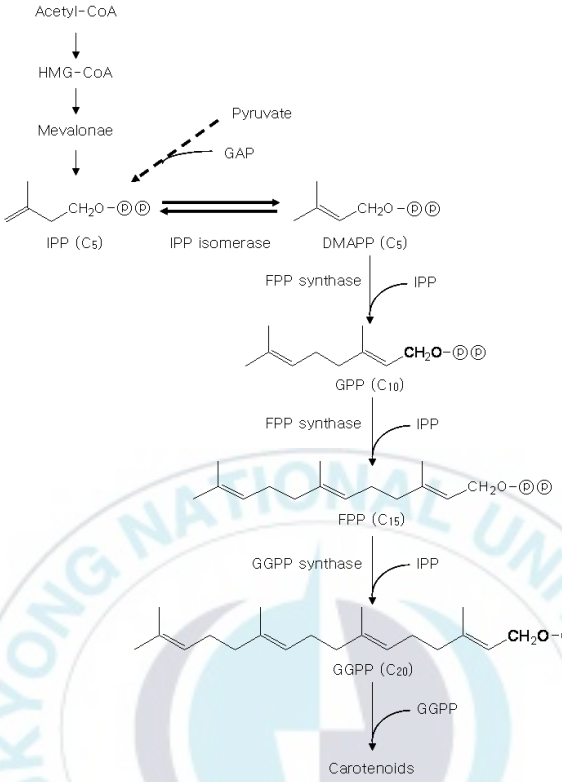

Figure 1. Summary of isoprenoid biosynthetic pathway.

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 dotted arrow, is suggested to be present in bacteria and plant chlorlplasts (Lichtenthaler *et al.*, 1997; Panidan *et al.*, 1981). Abbreviations used: CoA, coenzyme A; HMG-CoA, 3-hydrosy-3-methylglutaryl-coenzyme A; GAP, glyceraldehyde-3-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylally pyrophosphate; GPP, dimethyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

followed by several additional reaction steps, some of which are yet to be discovered (Eisenreich *et al.*, 2001). After IPP synthesized the various terpenoids are synthesized with the reaction of chain elongation by successive head-to-tail condensation of dimethylallyl pyrophosphate (DMAPP), the reactive IPP isomer. The reaction of IPP to DMAPP is catalyzed by isopentenyl 이phosphate isomerase (*idi*). Chain elongation is catalyzed by prenyl transferases with different chain length specificities (Ogura and Koyama, 1998). The short-chain length prenyl transferases synthesize geranyl PP (GPP, C_{10}), farnesyl PP (FPP, C_{15}) or geranylgeranyl PP (GGPP, C_{20}), which are the precursors of mono-, di-, and tri-terpenes and carotenoids. Medium and long-chain prenyl transferases synthesize the tails of quinones. FPP (C_{15}) and GGPP(C_{20}) are the immediate precursors of C_{30} and C_{40} carotenoids. FPP is synthesized by an FPP synthase (ispA in *Escherichia coli*), whereas GGPP formation is catalyzed by a GGPP synthase (Lee and Kim, 2004).

Most carotenoids are C_{40} derivatives originating from phytoene which is formed by condensation of two molecules of geranylgeranyl pyrophosphate (GGPP). This part of the carotenoid biosynthesis pathway proceeds in two steps. Although phytoene synthase is the first specific enzyme in carotenogenesis, most organisms synthesize the C_{20} substrate for the enzyme in two distinct stages. The first is the synthesis of FPP from the fundamental C_5 building blocks isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) by FPP synthase (Poulter *et al.*, 1978). GGPP synthase catalyzes an additional FPP branching reaction with the condensation of FPP and IPP to

provide the C_{20} precursor needed for biosynthesis of carotenoids. Early steps in the biosynthesis of carotenoids include the formation of geranylgeranyl pyrophosphate (GGPP) from farnesyl pyrophosphate (FPP) by GGPP synthase, an enzyme encoded by the *crtE* gene (Lee and Kim, 2006b). The enzymes and reactions catalyzed in the isoprenoid biosynthetic pathways are summarized in Table 1.

Astaxanthin (3,3-dihydroxy-β-carotene-4,4-dione) is an unique carotenoid widely distributed in nature providing coloration characteristics to some birds, crustacean, and salmons (Johnson, 1991). Organisms that produce astaxanthin include the basidiomycetous yeast *Phaffia rhodozyma* (Miller *et al.*, 1976), the green alga *Haematococcus pluvialis* (Bubrick, 1991), the Gram-negative bacteria *Agrobacterium aurantiacum* (Yokoyama *et al.*, 1994), *Paracoccus marcusii* (Harker *et al.*, 1998), and *Paracoccus haeundaensis* (Lee *et al.*, 2004). Since animals cannot synthesize astaxanthin, it must be included in their feed for them to obtain their appealing color.

Significant advances have been made in our understanding of the genes coding for the enzymes involved in carotenoid biosynthesis (Misawa *et al.*, 1995). Many carotenoid biosynthesis genes have been cloned from various organisms, and their functions have been determined (Armstron, 1994; Sandmann, 1994; Wieland *et al.*, 1994).

GGPP synthase is one of the important enzymes in the carotenoid biosynthesis pathway. GGPP synthase catalyses the consecutive condensation of an allylic diphosphate with three molecules of IPP to produce GGPP, an essential linear precursor for biosynthesis of

Table 1. Isoprenoid biosynthetic enzymes and reaction catalysed.

Enzyme	Reaction catalysed
Acetly-CoA acetyltransferase (atoB, phoA)	Acetoacetyl-CoA biosynthesis
HMG-CoA synthase (hcs)	Hydroxymethyl-glutanyl-CoA (HMG-CoA) biosynthesis
HMG-CoA reductase (mvaA)	L-mevalonic acid biosynthesis
Mevalonate kinase (muk)	Mevalonic-5-phosphate biosynthesis
Phosphomevalonate kinase (pmk)	Mevalonic-5-diphosphate biosynthesis
Diphosphomevalonate decarboxylase (mvd)	Isopentenyl diphosphate (IPP) biosynthesis
Isopentenyl disphosphate isomerase (idi)	Dimethylallyl diphosphate (DMAPP) biosynthesis
Farnesyl diphosphate synthase (ispA)	Geranyl diphosphate (GPP) biosynthesis

carotenoid. GGPP synthase is generally thought to be an important branch point enzyme for isoprenoid biosynthesis, since it catalyzes a FPP branching reaction with the condensation of FPP and IPP to produce C_{20} GGPP, which contains 4 isoprene units (Wang and Ohnuma, 1999).

In this thesis, we have described the overexpression, purification and characterization of GGPP synthase from marine bacterium, *Paracoccus haeundaensis*. Overexpression of GGPP synthase from *P. haeundaensis* was achieved by introducing *crtE* gene into the prokaryotic expression vector. The recombinant protein was produced in the cytoplasm of the transformed *E. coli* cells. Also, we have described the purification procedures of expressed proteins from the transformed cells. And then, we have characterized the biochemical and enzymatic properties of the purified GGPP synthase. These results will provide a wider knowledge of the primary structures of CrtE enzyme at the molecular level and facilitate the biotechnological applications of the carotenoid biosynthesis enzymes.

MATERIALS AND METHODS

Construction of crtE expression vector

In order to express *crtE* gene from the carotenoid biosynthesis gene cluster of *Paracoccus haeundaensis*, the *crtE* expression plasmid was constructed as follows. The coding region of the *crtE* gene was amplified by PCR using a pair of corresponding oligonucleotides specifically designed for cloning *crtE* gene and a template, which was originally loaded onto the plasmid pCR-XL-TOPO-CrtE (Table 2). Contained the carotenoid biosynthesis gene cluster (Lee and Kim, 2006a). 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 *NdeI* and *XhoI* restriction enzymes. The excised fragment was then ligated into the expression plasmid pET44-a(+) vector, and the resulting plasmid carrying an *crtE* gene of the carotenoid biosynthesis enzymes was designated into pET-44a(+)-CrtE (Figure 2). The pET-44a(+)-CrtE plasmid was then transformed into the *E. coli* BL21(DE3) codon plus cell.

Table 2. Oligonucleotide primers used for this study.

Primers	Sequences	Remarks
CrtE- 1	5' CCATATGAGACGAGACGTCAA 3'	for crtE forward (*NdeI* site)
CrtE-2	5' GCCGCCTCGCTAGGCGC 3'	for crtE reverse (*XhoI* site)

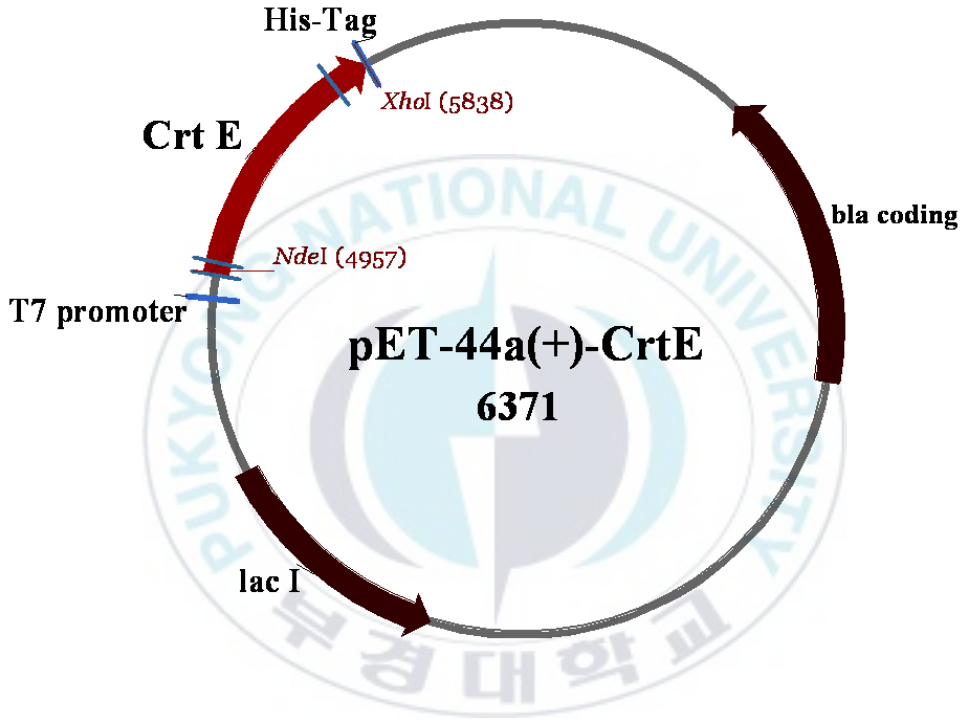

Figure. 2 The pET44a(+)-CrtE expression vector.

Overexpression of crtE gene in E. coli

The cell harboring the pET-44a(+)-CrtE plasmid that contains the *crtE* gene was cultured at 37℃ in DYT medium containing 50㎍/ml ampicillin. The cells were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM at a cell density corresponding to OD_{600} = 0.5～1.0. Then the cells were harvested by centrifugation after designated induction times; 1, 2, 3 and 4 hours, respectively. The conditions for the overexpression of the CrtE protein were optimized.

Purification procedures of CrtE protein

Purification scheme of the expressed CrtE protein is described in Figure 3. The cell pellets (1 g/wet weight) were resuspended with 20 ml of resuspension buffer (20 mM Tris-HCl, pH 7.9) and sonicated three times for 10 sec with Ultra sonicator, for 30 sec to 90-100 % power and harvested again by centrifugation at 13,000 rpm for 10 min. The supernatant was saved for the next purification. The cell pellets were resuspended by adding isolation buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM NaN, pH 7.9). The resuspended cell debris was discarded and the supernatant (20 vol.) was obtained. The sample was filtrated with 0.45 ㎛ filter. The saved sample was dialyzed against the buffer A (20 mM Tris-HCl, 0.5 M NaCl, 1 mM NaN_3, pH 8.0) for affinity chromatography.

Cell culture

Medium : DYT broth, Culture temperature : 37℃

Induction agent : IPTG 0.1 mM (final concentration)

Induction time : 3 hours

Cell lysis & purification

Centrifugation (3000 rpm, 20 min. 4℃)

⇩

Pellets resuspended (cell of 1 g addition 20 ml resuspension buffer)

⇩

Sonication (for 30 sec at 90-100% power, three times for 10 sec)

⇩

Centrifuge (13,000 rpm, 20 min. 4℃)

⇩

Supernatant obtained

⇩

Affinity chromatography using a His-Trap Chelating HP column

⇩

Analysis of the purified protein
(SDS-PAGE, Western Blot)

Figure 3. Purification Scheme of CrtE protein.

The sample was loaded onto the His-Trap chelating HP-column (GE healthcare, USA). The column was washed with buffer A containing 40 mM Imidazole and the bound proteins were eluted by the elution buffer (Buffer A containing 500 mM Imidazole).

Analysis of expressed protein on SDS-PAGE

The cells induced proteins by the addition of IPTG were centrifuged and the pellet was mixed with 2× sample loading buffer and heated at 100℃ for 3 min. The sample was centrifuged 13,000 rpm for 2 min and stored on ice. Twenty microliters of each sample was loaded on a 12% SDS-polyacrylamide gel, and electrophoresed under the constant current of 200 mA for 2 hours. The gel was stained with coomassie brilliant blue and then destained with destaining buffer (7% acetic acid, 15% methanol).

Western blot analysis

After total proteins containing CrtE-His protein 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, 0.5 M NaCl, 2.5 mM KCl, 0.1% Tween-20) and blocked with PBS buffer containing 5% skim milk for 1 hour. The membrane was washed out three times for

10 min at each time with PBS buffer and treated for 1 hour with His-tag primary antibody which was diluted to 1:10,000 in PBS containing 5% skim milk. After washing three times for 10 min at 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 at each time in PBS buffer and developed with NBT and BCIP solution in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 1 M $MgCl_2$).

Enzyme assay

The enzymatic activity of the CrtE protein was mea녁ed with a slight modification of the method as described in (Math *et al.*, 1992). Four hundred microliters of incubation mixture was made with 0.1 M Tris-HCl buffer (pH 8.0), containing the substrate in a concentration 50 μM IPP and 100 μM FPP, 10 mM ATP, 1 mM dithiothreitol, 6 mM $MnCl_2$, 4 mM $MgCl_2$ and 1 μg protein of purified CrtE protein. The assays were carried out at 37℃ for 15 hour at constant shaking in dark. After termination of the enzymatic reaction by addition of 2.5 ml of methanol, the synthesized carotenoid was partitioned into 10% diethylether in petrol, evaporated, redissolved in acetone, and then separated and quatified by HPLC analysis. For HPLC separation, chromatography was performed using an Waters HPLC system. The column used was Inertsil ODS-3 column (5 micron, steel, 150 mm long x 4.6 mm I.d., GL Sciences). The reverse phase was a methanol/water

(85/15, v/v) with the following parameters. The flow rate was 0.5 ml/min. The injection volume and column temperature were 20 ㎕ and room temperature, respectively. The products of farnesyl pyrophosphate (FPP) and isopentenyl pyrophosphate (IPP) were detected by absorbance at 214 nm. The FPP and IPP standards were purchased from Sigma (Germany) as an authentic samples.

RESULTS

Overexpression of crtE gene in E. coli

In order to express the GGPP synthase gene (*crtE*) in a prokaryotic system, the *crtE* gene was subcloned into the pET44-a(+) expression vector which allows the expression of the recombinant protein with C-terminal fusion His-tag and designated pET-44a(+)-CrtE plasmid (Figure 2). The plasmid was transformed into *E. coli* strain BL21(DE3) Codon plus cells and the expression of the recombinant CrtE protein was induced by the addition of IPTG at a final concentration of 0.1 mM.

The expression of the recombinant CrtE protein was analyzed by 12% SDS-PAGE (Figure 4). The conditions for the expression of the recombinant CrtE protein was measured after designated induction times; 1, 2, 3 and 4 hours after addition of IPTG, respectively. As shown in Figure 4, 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 30 kDa, including a C-terminal His-Tag fusion.

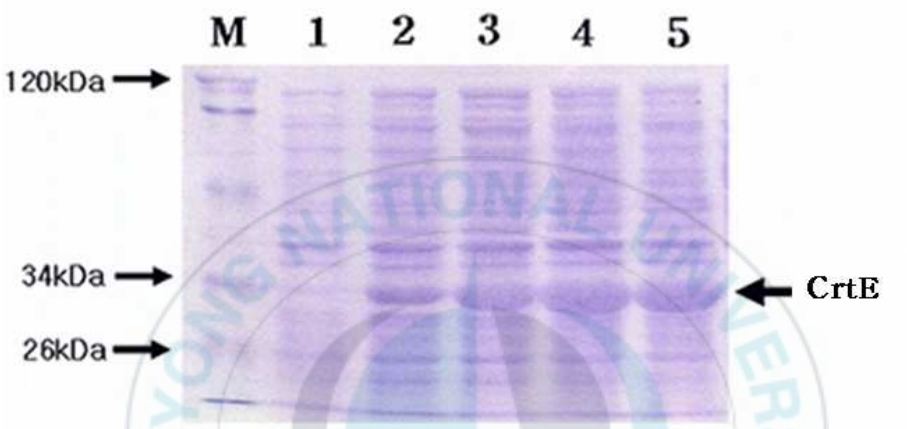

Figure. 4 The expression patterns of recombinant CrtE protein by SDS-PAGE.

The expressed proteins were analyzed 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

The *crtE* gene was subcloned into the pET-44a(+) expression vector which produces a recombinant CrtE protein tagged with histidine residues in the carboxyl terminus. The plamid pET-44a(+)-CrtE was cultured upto cell density reached to $O.D_{600}$ = 0.5 and the CrtE protein was overexpressed by the addition of IPTG. After 3 hours induction, the cells were harvested by centrifugation, and sonicated three times for 10 sec with Ultra sonicator, and precipitated by centrifugation at 13,000 rpm for 10 min. The supernatant was discarded. The cell pellets were resuspended with isolation buffer and then centrifuged again. The precipitated cell debris was discarded and the supernatant was saved. The saved sample was dialyzed against the buffer A (20 mM Tris-HCl, 0.5 M NaCl, 1 mM NaN_3, pH 8.0) for affinity chromatography. The dialyzed sample was loaded onto the His-Trap chelating HP-column. The column was washed with buffer A containing 40 mM Imidazole and the bound proteins were eluted by the elution buffer (Buffer A containing 500 mM Imidazole).

A typical elution profile from affinity column was shown in Figure 5. The majority of the total proteins was washed out with buffer A containing 40 mM Imidazole and the bound CrtE protein was eluted at buffer A containing 500 mM Imidazole. The fractions collected from the affinity column were analyzed on 12% SDS-PAGE as shown in Figure 6. The purified CrtE protein appears to be pure and its estimated a molecular weight was approximately 30 kDa at a single band.

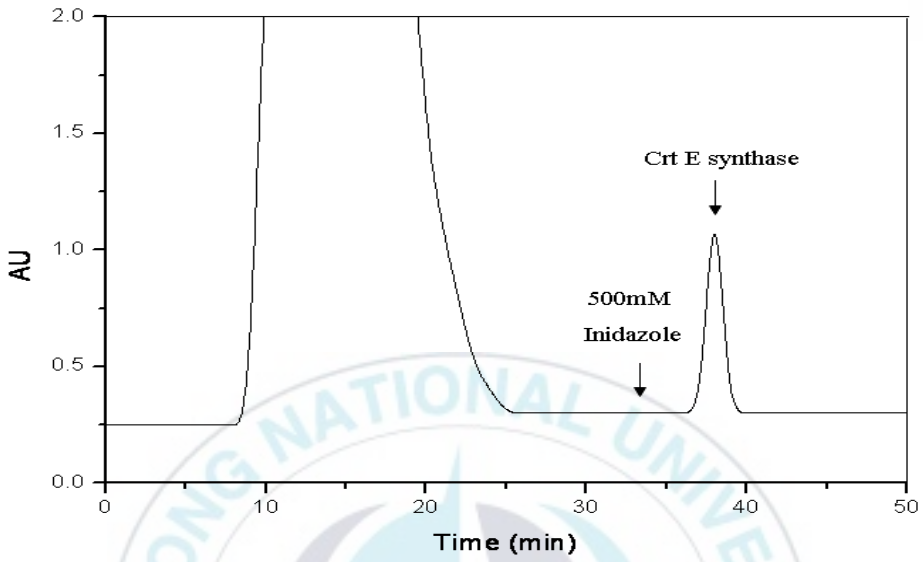

Figure. 5 Chromatogram of His-trap affinity chromatogrphy.

Purification of GGPP synthase by chromatography on His-tag column. The column was washed with buffer A containing 40 mM Imidazole. And the bound CrtE protein was eluted with 500 mM elution buffer (buffer A containing 500 mM Imidazole). Each fraction (1 ml) was collected.

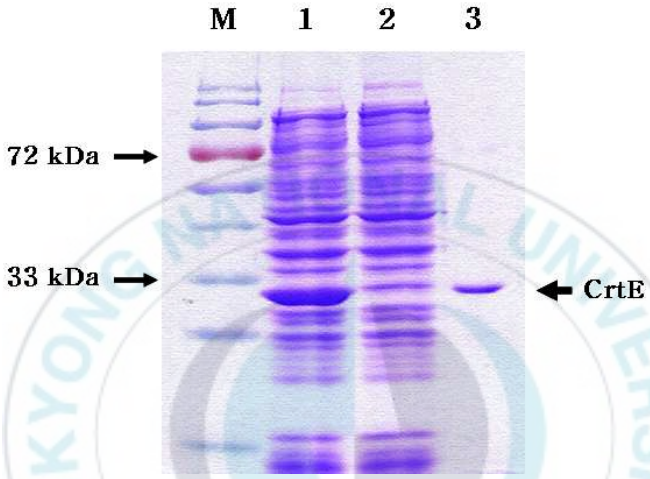

Figure 6. SDS-PAGE Patterns of affinity chromatogram

The lane M indicates molecular weight marker; lane 1, proteins from induced cell extracts; lane 2, proteins by affinity chromatography; lane 3, purified CrtE protein by affinity chromatography.

Conformation of purified GGPP synthase by Western blot analysis

The purified recombinant CrtE protein was confirmed by Western blot analysis and the result was shown in Figure 7. In Western blot analysis, proteins were electrophoretically transferred from a SDS-PAGE gel to a nitrocellulose membrane, probed with mouse antiserum against 6-His tag, and incubated with alkaline phosphatase coupled to mouse antibody against mouse IgG. The nitrocellulose membrane was developed using NBT/BCIP. As shown in Figure 7, the recombinant CrtE protein was recognized as a His-tag protein by Western blot.

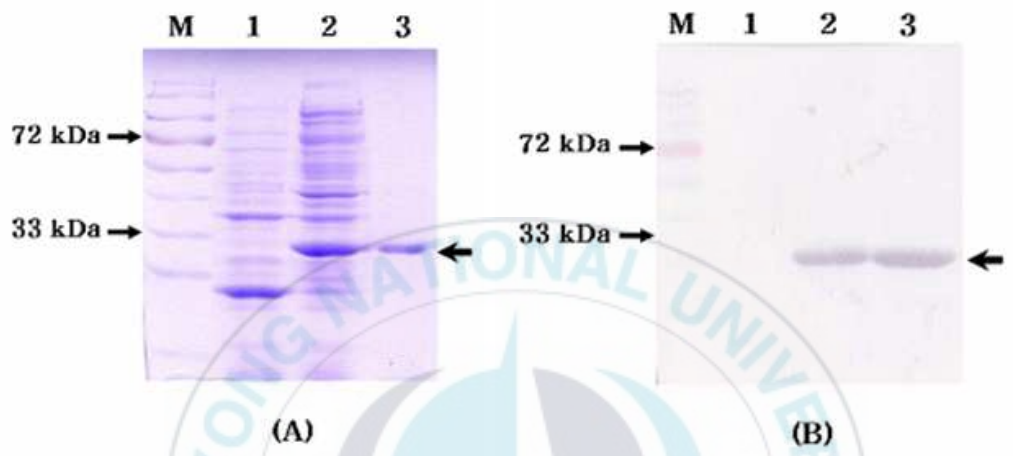

Figure. 7 SDS-PAGE (A) and Western blot (B) analysis of the purified CrtE proteins. The lane M indicates molecular weight marker; lane 1, proteins from uninduced cell extracts (control); lane 2, proteins from induced cell extracts; lane 3, purified CrtE protein by affinity chromatography.

GGPP synthase activity assay

In order to determine the enzymatic activity of purified GGPP synthase, the product of the reaction mixture was incubated with GGPP synthase and its substrates, and analyzed by HPLC. The enzyme assay was performed the reaction mixture in a total volume of 400 ㎕ in 0.1 M Tris-HCl buffer, pH 8.0, containing the substrates in a concentration of 50 ㎛M IPP and 100 ㎛M FPP, 10 mM ATP, 1 mM dithiothreitol, 6 mM $MnCl_2$, 4 mM $MgCl_2$, and 1 ㎛g protein of purified CrtE protein. The assays were carried out at 37℃ for 15 hours incubation with constant shaking in the dark.

The products from the reaction mixture were analyzed by the HPLC analysis monitored at 214 nm. The result of HPLC analysis was shown in Figure 8. The standard samples of IPP, FPP and GGPP were injected onto the Inertsil ODS-3 column (Figure 8A). GGPP synthesized from incubation mixture was analyzed onto the same column (Figure 8B). As shown in Figure 8, The activity could be demonstrated with the expressed enzyme. After incubation, three peaks appeared having retention times of 4.0, 4.6 and 5.5 min (Figure 8A). The elution peaks (peak 1 and 2) of the HPLC analysis from the incubation mixtures were found to be two pigments corresponding to IPP and FPP, respectively. The main peak (peak 3) of the HPLC analysis was found to be GGPP by comparing it to the standard GGPP. This peak was eluted at retention time at 5.5 min and absorbed at maximal wavelength at 214 nm.

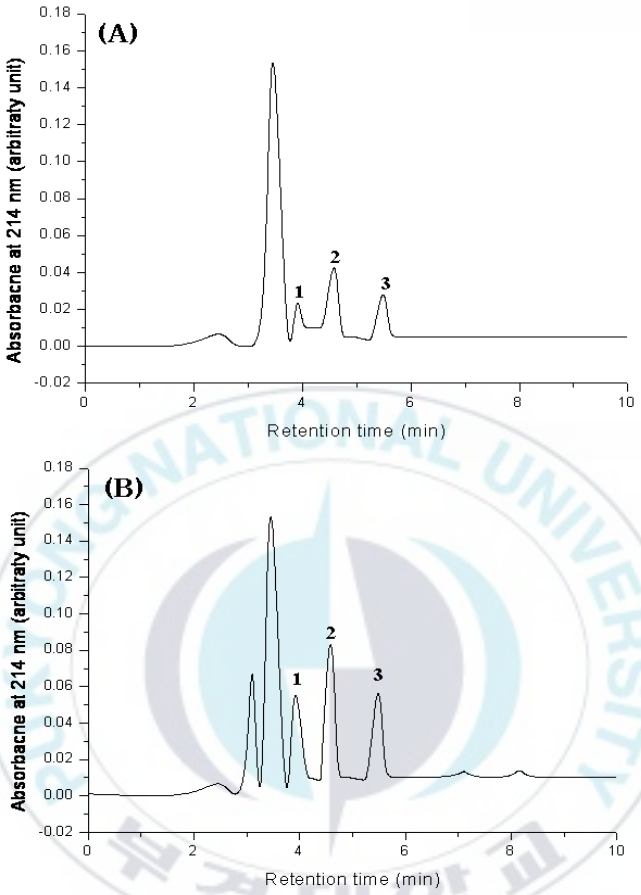

Figure 8. HPLC patterns of the products from the GGPP synthase assays. (A) Standard samples of IPP (peak 1), FPP (peak 2) and GGPP (peak 3) were need for the control. (B) Samples isolated from incubation mixtures were coinjected with authentic samples of IPP (peak 1), FPP (peak 2) and GGPP (peak 3) on Inertsil ODS-3 column (GL Science, USA) and eluted with H_2O/methanol, 15:85 (vol/vol). The products from the enzymatic assay mixtures were monitered at 214 mM.

DISCUSSION

GGPP synthesis is the early steps of the biosynthesis of the carotenoids, which mediate the formation of GGPP from FPP and IPP. This pathway is catalyzed by a specific enzyme, GGPP synthase, encoded by the *crtE* gene. We previously reported that the isolation of a new marine bacterium, *Paracoccus haeundaensis*, which produces carotenoids, mainly in the form of astaxanthin (Lee *et al.*, 2004) and described the cloning and sequence analysis of genes encoding the astaxanthin biosynthetic enzymes from this organism (Lee and Kim, 2006a). All six genes of the astaxanthin biosynthesis gene cluster of *P. haeundaensis* (*crtW, crtZ, crtY, crtI, crtB*, and *crtE*, which contain 726, 486, 1158, 1503, 912, and 879 base pairs, respectively) are necessary for astaxanthin biosynthesis, and those genes were cloned and characterized (Lee and Kim, 2006a; Lee and Kim, 2006b). Detailed studies of the involvement of the astaxanthin biosynthetic enzymes on carotenoid biosynthesis, however, have not yet been conducted. In order to elucidate the mechanism responsible for controlling the astaxanthin biosynthetic pathway and the intracellular carotenoid concentration, it would therefore be necessary to conduct a comparative analysis of the structure, expression, and function of the carotenoid biosynthesis genes.

The *crtE* gene isolated from *P. haeundaensis* was composed of 879 bp encoding a polypeptide of 293 amino acid residues. To create the expression plasmid for the *crtE* gene, *crtE* gene was amplified by PCR using a pair of gene-specific oligonucleotides (Table 2) with the

plasmid pCR-XL-TOPO-Crt-full as a template, which carries the full-length astaxanthin biosynthesis gene cluster (Lee and Kim, 2006a). The amplified fragment was subcloned into the vector pET-44a(+) for overexpression of the CrtE protein The resulting plasmid carrying an *crtE* gene of the carotenoid biosynthesis enzymes was designated pET-44a(+)-CrtE (Figure 2). The plasmid, pET-44(a)-CrtEB, was transformed into *E. coli* BL21(DE3) Codon Plus. The cells harboring the *crtE* gene were cultured in LB medium (containing 50 g/ml ampicillin) and induced by adding final concentration of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at a cell density corresponding to OD_{600} = 0.5. Induced cells were incubated in a rotary shaking incubator at 37°C and 150 rpm for 3 hours.

The expression patterns of the *crtE* gene were analyzed on the SDS-PAGE as shown in Figure 4 and the molecular weight of the expressed CrtE protein turned out to be approximately 30 kDa. The optimal induction time of a recombinant CrtE protein was achieved at 3 hours after induction. The recombinant CrtE protein was purified using affinity chromatography and confirmed by Western blot analysis (Figure 7). The goal of affinity chromatography is to separate all the molecules of a particular specificity from total protein. Affinity chromatography is designed to purify a particular protein from a mixed sample and can be performed using a number of different protein tags. Proteins sieve through matrix of affinity beads. Proteins interact with affinity ligand with some binding loosely and others tightly. Wash off proteins that do not bind and bind loosely. Elute proteins that bind tightly to ligand and

collect purified protein of interest. The prepared sample was loaded onto the His-Trap affinity column and eluted by the addition of eluting buffer. The CrtE protein was purified to homogeneity and the purified protein shows enzymatically active. The biological activity of purified GGPP synthase was tested by incubation with the substrates and appears to be strong enzymatic activity after analysis of the incubation products.

The products from the enzymatic assay mixture incubated with the purified CrtE enzyme were dissolved in 2-propanol and subjected to high-performance liquid chromatography (HPLC). Chromatography was performed using an Inertsil ODS-3 column equipped with a temperature-controlled autosampler and a diode array detector.

The result of the HPLC analysis from incubation mixture assayed with purified CrtE protein was shown in Figure 8. The main peak of the HPLC analysis from the products of the assay mixture with purified CrtE protein was found to be GGPP by comparing it to the standard. This peak was eluted at a retention time of 5.5 min and absorbed at maximal wavelength of 214 nm [Figure 8(B)]. The elution peaks of the HPLC analysis from the incubation mixture were found to be three pigments corresponding to IPP, FPP and GGPP when these peaks were compared with standards. These peaks were eluted at a retention time of 4.0, 4.6, and 5.5 min, respectively [Figure 8(B)].

In this study, we have conducted a comparative study of the overexpression, purification and characterization of the GGPP synthase of *crtE* gene from *P. haeundaensis* using chromatographic and

spectroscopic analyses. The observations and results of this study 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 astaxanthin through the manipulation of carotenoid biosynthesis genes in *P. haeundaensis*, an important application since astaxanthin is a pigment of high economic value. These data will provide a wider base of knowledge on the primary structure of the astaxanthin biosynthesis gene cluster at the molecular level as well as further the biotechnological applications of carotenoids.

국문초록

GGPP synthase (CrtE)는 아스타잔틴을 비롯한 카로티노이드계 생리활성물질을 생합성 하는 과정에 관여하는 중요한 핵심 효소이다. carotenoid는 식품색소, 동물사료 첨가제, 건강보조식품과 화장용등의 산업적 응용을 위한 위하여 현재 활발히 연구되고 있다. 또한 carotenoid는 비타민 A의 전구물질로 작용하기 때문에 시력에 직접 관여하고 또한 세포 성장 및 분화 그리고 면역체계에도 관여하고 있다. 최근에는 carotenoid의 중요한 항산화 작용으로 인하여 암 예방과 치료 및 만성질환 치료제로도 중요한 역할을 하는 것으로서 밝혀져 더욱 주목받고 있다. 이러한 carotenoid계 물질의 생합성은 FPP로부터 GGPP를 생합성하는 것으로부터 시작되며 이 과정은 GGPP synthase (CrtE protein)에 의한 효소반응을 통하여 이루어진다.

해양 미생물 *Paracoccus haeundaensis*로부터 유래된 *crtE* gene의 생화학적, 효소학적 특징을 구명하기 위해서는 대량의 순수 분리된 GGPP synthase가 필요하다. 따라서 GGPP syunthase의 대량 발현을 위하여 *P. haeundaensis*로부터 분리된 *crtE* gene을 pET-44a(+) 발현 벡터에 subcloning 시킨 후, 이를 대장균 BL21(DE3) codon plus에 형질전환 시켰다. 0.1 mM IPTG를 사용하여 3시간 동안 발현 유도를 시켰을 때 CrtE protein의 발현이 최적으로 나타났다. 대량 발현으로 시킨 GGPP synthase를 순수 분리 정제하기 위하여 affinity chromatography를 실시하여 GGPP synthase 만을 순수 분리할 수 있었다. 실험에 His-tag column을 사용하였다. 분리해 낸 GGPP synthase의 효소학적인 특징을 알아보기 위해서 기질물질인 IPP와 FPP를 정제한 효소와 반응시켜 반응 산물을 HPLC를 이용하여 분석하여 GGPP synthase의 효소활성을 동정하였다.

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