Journal of Oleo Science

doi : 10.5650/jos.ess26022
J. Oleo Sci. **75**, *(6) 703-711 (2026)*

Domain analysis and engineering of multifunctional β-carotene synthase, CrtIBY, for lycopene and phytoene production

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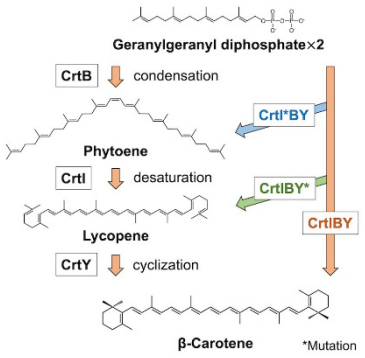
Abstract: The multifunctional β-carotene synthase, CrtIBY, derived from *Aurantiochytrium* sp. KH105 catalyzes to produce β carotene from geranylgeranyl diphosphate through three serial enzymatic reactions with a trace amount of intermediates such as phytoene and lycopene. In this research, the functions of domains of CrtIBY were studied and the product specificity was modified by protein engineering techniques including the removal of functional motif and site-directed mutagenesis. *Saccharomyces cerevisiae* transformants expressing the functionally modified CrtIBY mutants accumulated phytoene and lycopene. These mutants are expected to be useful to supply raw materials of pharmaceuticals, cosmetics, and health foods.



Keywords: carotenoid production, *Aurantiochytrium* sp, protein engineering, heterologous expression

1 Introduction

Carotenoids are polyene pigments generally present red or yellow and coloration. Among these, β-carotene and astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) are well-studied and have various physiological activities including improvement of lipid metabolism[1)], antitumor[2)], and anti-inflammatory activities[3)]. Due to such beneficial properties, these carotenoids are highly demanded in the fields of pharmaceuticals, cosmetics, and health foods. While the major carotenoids have been commercialized, useful properties have also been reported for their biosynthetic intermediates such as phytoene (15-*cis*-7,7′,8,8′,11,11′,12,12′-octahydro-ψ,ψ-carotene) and lycopene (ψ,ψ-carotene)[4)] but their industrial applications remain limited.

Phytoene is a colorless carotenoid that has been reported to exhibit physiological activities such as lipoprotein protection[5)]. Phytoene offers advantages including light stability and extended shelf life compared to other colored carotenoids[6)]. On the other hand, lycopene is a reddish carotenoid found in some plants[7)]. Notably, Mascio *et al.* measured the singlet oxygen scavenging activity of 16 carotenoids including β-carotene and astaxanthin, and concluded that lycopene exhibited the highest activity[8)]. Therefore, healthcare products containing phytoene and lycopene can be expected to demonstrate new and higher functionality than existing carotenoid products.

Although some plants are reported to produce phytoene and lycopene[7, 9)], their yield is typically low since these compounds serve as biosynthetic intermediates. Metabolic modification using genetic engineering techniques is an effective strategy to produce these carotenoids in plants and microorganisms. However, the introduction of multiple genes involved in carotenoid synthesis and precise control of each gene's expression remain significant challenge.

The authors previously identified the trifunctional β-carotene synthase, CrtIBY, which catalyzes condensation of

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Accepted April 2, 2026 (received for review February 6, 2026)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
https://www.jstage.jst.go.jp/browse/jos/ https://mc.manuscriptcentral.com/jjocs

geranylgeranyl diphosphate (GGPP), desaturation of phytoene, and cyclization of lycopene[10], in a carotenoid-producing microorganism, *Aurantiochytrium* sp. KH105[11]. Since CrtIBY efficiently converts GGPP to β-carotene and accumulates little amount of the intermediates, this enzyme is considered more advantageous than three independent enzymes in terms of reaction rate and reaction efficiency. In this study, we investigated the domain functions of CrtIBY and the production of intermediates, phytoene and lycopene, using mutant enzymes generated by removal of functional motif and site-directed mutagenesis.

2 Material and Methods

2.1 Microorganisms, culture media, and regents

Transformants of *Escherichia coli* DH5α were cultured in LB medium (10 g/L Bacto tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, 20 g/L agar for plates) containing 50 mg/L ampicillin. *Saccharomyces cerevisiae* INVSc1 (Invitrogen, Carlsbad, CA, USA) were cultured in YPD medium (20 g/L glucose, 20 g/L hipolypepton, 10 g/L yeast extract, and 20 g/L agar for plates), and transformants of *S. cerevisiae* were selected on SD medium (20 g/L glucose, 6.7 g/L yeast nitrogen base, 1.9 g/L yeast synthetic dropout medium w/o uracil, 18 g/L agar for plates) and cultured in SCT medium (6.7 g/L yeast nitrogen base, 1.9 g/L yeast synthetic dropout medium w/o uracil, 20 g/L raffinose). Reference standards of β-carotene, (*E*/*Z*)-phytoene (mixture of isomers), and lycopene were purchased from Merck (Darmstadt, Germany).

2.2 Amino acid sequence comparison of CrtIBY and related enzymes

Alignment was performed by CLUSTAL W computer program[12]. Reference sequences including phytoene desaturase (P21685.1, P54979.1, and P54978.2), phytoene synthase (P21683.2, P37294.1, and P54975.2), and lycopene cyclase (Q9P854.1, B0R753.1, and E6ZZ11.1) were obtained from Swiss-Prot database[13].

2.3 Structure prediction and molecular docking

Three-dimensional structure of the CrtIBY was predicted using AlphaFold3[14], and the top-ranked model was used for docking analysis. Ligands (geranylgeranyl pyrophosphate （GGPP), phytoene, and lycopene) were prepared using RDKit[15] and converted to PDBQT format using Meeko[16]. The protein structure was also prepared using Meeko and ChimeraX (v1. 11. 1)[17]. Docking simulations were performed using AutoDock Vina (v1. 2. 7)[18] with domain-specific grid boxes centered on each catalytic domain. Docking results were visualized using ChimeraX.

2.4 Construction of plasmid carrying the genes of CrtIBY and its mutants

The CrtIBY gene was amplified from genomic DNA of *Aurantiochytrium* sp. KH105 using KOD Neo DNA polymerase (Toyobo, Osaka, Japan) and the oligonucleotide primers shown in **Table 1**. The PCR conditions were 30 cycles of 98 ºC for 10 seconds and 68 ºC for 4 minutes. The obtained fragment was digested by *Bam*HI and *Eco*RI and ligated into pYES2 vector (Invitrogen) digested by same restriction enzymes, and the constructed plasmid was transformed into *E. coli* DH5a.

Table 1 Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5' to 3')	Purpose
CrtIBY-F	TACGGATCCATGCATCACCATCATCAC-GCGCGCAGGGCG	Amplification of the CrtIBY gene
CrtIBY-R	CTTGAATTCTCAGGCATTCTT-GTACAGCG	Amplification of the CrtIBY gene
CrtY-Mut	GGGCAAATATGCACTTGGTCTCGCGG-CAGCTCTCTTCTTCTTGG	Site-specific mutation in the lycopene cyclase domain
CrtI-Δ35	TACGGATCCATGGGCGCGA-GAGGCCCGGGGCGC	Removal of 35 amino acids from the N-terminal end of CrtIBY
CrtI-Δ40	TACGGATCCATGGGGCGCGA-GAGCGACGGCGACCG	Removal of 40 amino acids from the N-terminal end of CrtIBY
CrtI-Δ50	TACGGATCCATGAAGCG-CATCGCCGTGCTCGGGGCC	Removal of 50 amino acids from the N-terminal end of CrtIBY
CrtI-Δ75	TACGGATCCATGGTCGTGGTTCTTGA-GAAGAACG	Removal of 75 amino acids from the N-terminal end of CrtIBY

The oligonucleotide primers shown in **Table 1** were designed to introduce nucleotide mutations for substitution of amino acids of the lycopene cyclase motif (1222~1227 amino acids) in CrtIBY. The plasmid carrying the *CrtIBY* gene was used as a template, and the site-directed mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Mutant CrtIBY genes with deletions of 35, 40, 50, and 75 amino acids from the N-terminus of the enzyme were created by PCR.

The nucleotide sequence of the plasmid was determined using BigDye Terminator v3.1 cycle sequencing kit and ABI 3130xl genetic analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5 Expression of CrtIBY genes in yeast

pYES2 plasmids carrying wild type and mutant CrtIBY genes were introduced into *S. cerevisiae* INVSc1 using the lithium acetate method[19]. Transformants were selected on SD plate medium and cultured in SCT medium for at 28 ºC for 6 hours with rotary shaking at 180 rpm. Galactose was added to a final concentration of 20 g/L, and transformants were cultured for an additional 17 hours to induce expression of the CrtIBY gene.

2.6 Carotenoid extraction

Carotenoid extraction was performed following the method of Watanabe *et al.*[20] with some modifications. Briefly, yeast cells collected from 50 mL of culture broth were washed with distilled water and lyophilized. An equivalent volume of glass beads (0.5 mm in diameter) and 1 mL of *tert*-butyl methyl ether/methanol (2:1, v/v) were added to lyophilized cells, and vigorously mixed using beads crusher μT-12 (Taitec, Aichi, Japan). The supernatant was recovered by centrifugation at 3,500 rpm for 10 min, and 1 mL of distilled water was added and mixed vigorously using a vortex mixer. The *tert*-butyl methyl ether layer was recovered by centrifugation and transferred to a new test tube. After evaporation under N_2 stream, carotenoids were dissolved in acetone/methanol (7:3, v/v).

2.7 Carotenoid analysis by high-performance liquid chromatography

Analysis of carotenoids was performed using high-performance liquid chromatography (HPLC) equipped with a diode array detector (DAD). Carotenoids were separated by the COSMOSIL 5C_{18}-MS-II column (4.6 mm × 150 mm, Nacalai Tesque) using acetonitrile/dichloromethane/methanol (7:2:1, v/v) as the mobile phase. Identification of carotenoids was performed by comparison of retention times and absorption spectra with those of authentic standards, and quantification of carotenoids was performed using DAD mainly at 470 nm.

3 Results

3.1 Prediction of functional regions and membrane topology of CrtIBY

Amino acid sequence comparison with homologous enzymes from other organisms revealed that CrtIBY from *Auranti-ochytrium* sp. KH105 contains conserved motifs including the FAD binding motif (57-GAGYAGLSAACELSRLGH-74) in the phytoene desaturase (CrtI) domain, two aspartate-rich motifs in the phytoene synthase (CrtB) domain, and two lycopene cyclase motifs (989-PVEEYMF-995 and 1123-PLEEALF-1129) in the lycopene cyclase (CrtY) domain (**Fig. 1A**). These motifs have been reported as important for the activities of related enzymes from bacteria, fungi, and plants[21-23]. The AlphaFold3-predicted structure of CrtIBY revealed a multi-domain architecture composed of CrtI, CrtB, and CrtY domains connected by linker regions (**Fig. 1B**). Docking simulations suggested that phytoene, GGPP, and lycopene may associate with the CrtI, CrtB, and CrtY domains, respectively. The docked phytoene was located near the FAD-binding motif near the CrtI domain (**Fig. 1C**). In the CrtY domain, lycopene was positioned in the vicinity of the two conserved cyclase motifs (residues 989–995 and 1123–1129), suggesting a possible role of these regions in substrate interaction (**Fig. 1D**).

3.2 Removal of FAD binding motif in CrtIBY caused phytoene accumulation

The wild type CrtIBY and a series of mutants from which the N-terminus 35, 40, 50, and 75 amino acids were removed (CrtIBY-Δ35, -Δ40, -Δ50, and -Δ75) were expressed in *S. cerevisiae* that has no capacity to convert GGPP to β-carotene. Consequently, β-carotene was accumulated at 3.1-4.0 mg/L-culture in the yeast strains expressing wild-type CrtIBY, CrtIBY-Δ35, -Δ40, and -Δ50, but not in the strain expressing CrtIBY-Δ75 whose FAD binding motif was eliminated (**Fig. 2A**). HPLC analysis on the strain expressing CrtIBY-Δ75 using a diode array detector revealed the accumulation of the substance whose retention time is similar to that of phytoene with absorbance at 280 nm (**Fig. 2B**). The absorption spectrum of this peak exhibits a maximum absorption near 280 nm, showing good agreement with that of the authentic phytoene standard (**Fig. 2C**). The accumulated phytoene was quantified at 0.5 mg/L-culture. Taken together, although

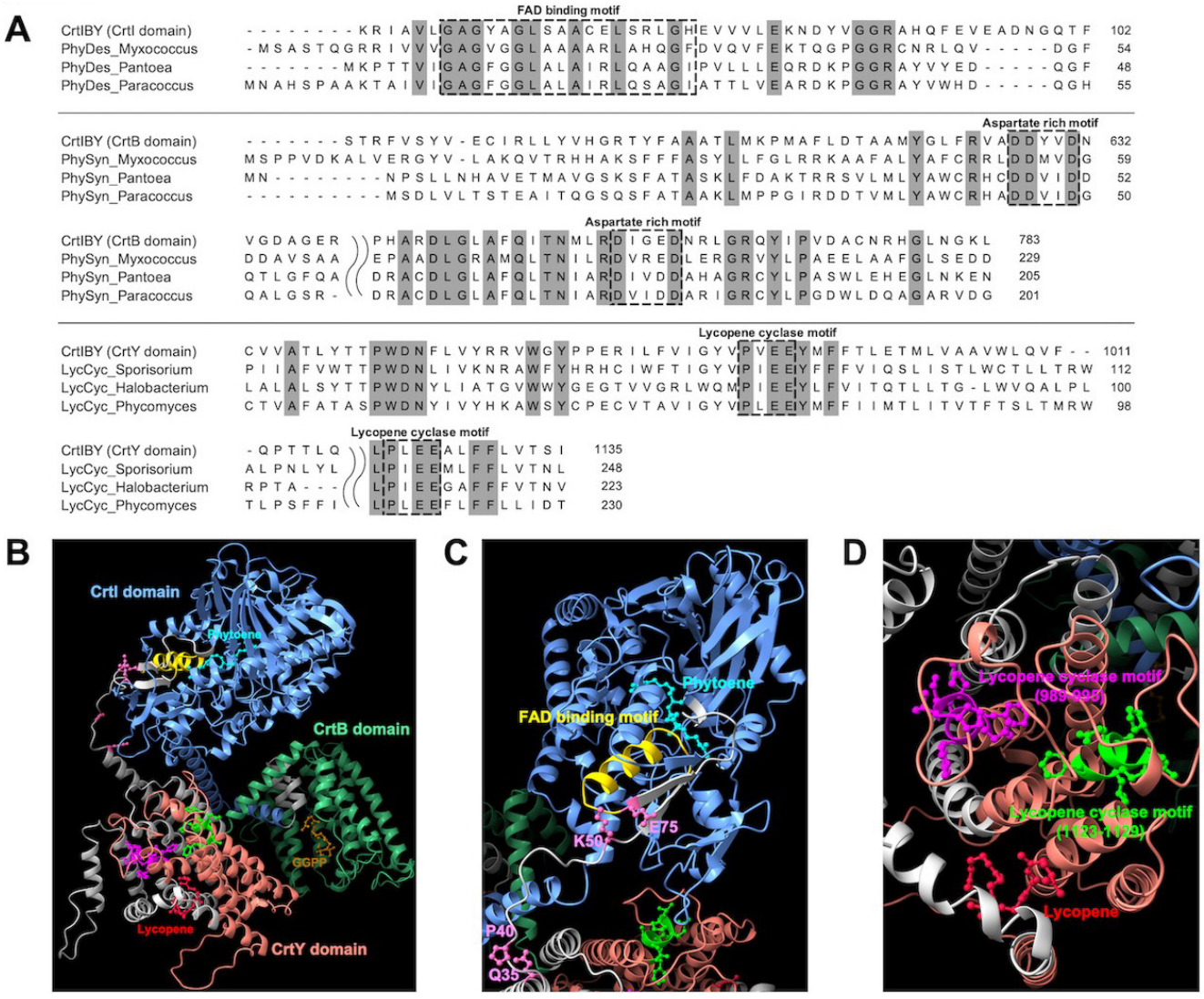

Fig. 1 Partial amino acid sequences of domains within CrtIBY were compared with those of homologous enzymes, and identical amino acids among all sequences were shade in gray (A). Overall structure of CrtIBY predicted by AlphaFold3, showing the CrtI (blue), CrtB (green), and CrtY (salmon) domains connected by linker regions, with docked phytoene (cyan), GGPP (orange), and lycopene (red) (B). Close-up of the CrtI domain showing the FAD-binding motif (yellow), residues of interest in this study (Q35, P40, K50, and E75; magenta), and docked phytoene (cyan) (C). Close-up of the CrtY domain showing docked lycopene (red) and two conserved lycopene cyclase motifs (989–995, magenta; 1123–1129, green) (D).

the FAD-binding motif removed in CrtIBY-Δ75 was essential for phytoene desaturation, the CrtB domain in CrtIBY-Δ75 was presumed to retain the phytoene synthase activity, leading to phytoene accumulation.

3.3 Lycopene production by site-specific mutagenesis in CrtY

There are two lycopene cyclase motifs in the CrtY domain according to a previous report[24]. Based on the docking model, the C-terminal motif was selected for mutational analysis because it was located slightly closer to the docked lycopene than the N-terminal motif, as indicated by a shorter distance between the center of the two glutamate residues, which are considered to be critical for cyclization activity, and lycopene (19.51 Å vs 20.69 Å) (**Fig. 1D**). Site-directed mutations were introduced into the C-terminus one (P1123G, E1125A, E1126A) in CrtIBY, and the generated mutant (CrtIBY-mY) was expressed in S. cerevisiae. As a result, production of β-carotene was not observed in the yeast strain expressing

CrtIBY-mY, while a peak whose retention time was similar to that of lycopene was detected (**Fig. 3A**).
By extracting the absorption spectrum of this peak, the shape of spectrum showed good agreement with that of the authentic lycopene standard (**Fig. 3B**). The accumulated lycopene was quantified at 2.4 mg/L-culture. Therefore, the introduced mutation in the C-terminus lycopene cyclase motif specifically abolished the lycopene cyclase activity of CrtIBY and induced high accumulation of lycopene.

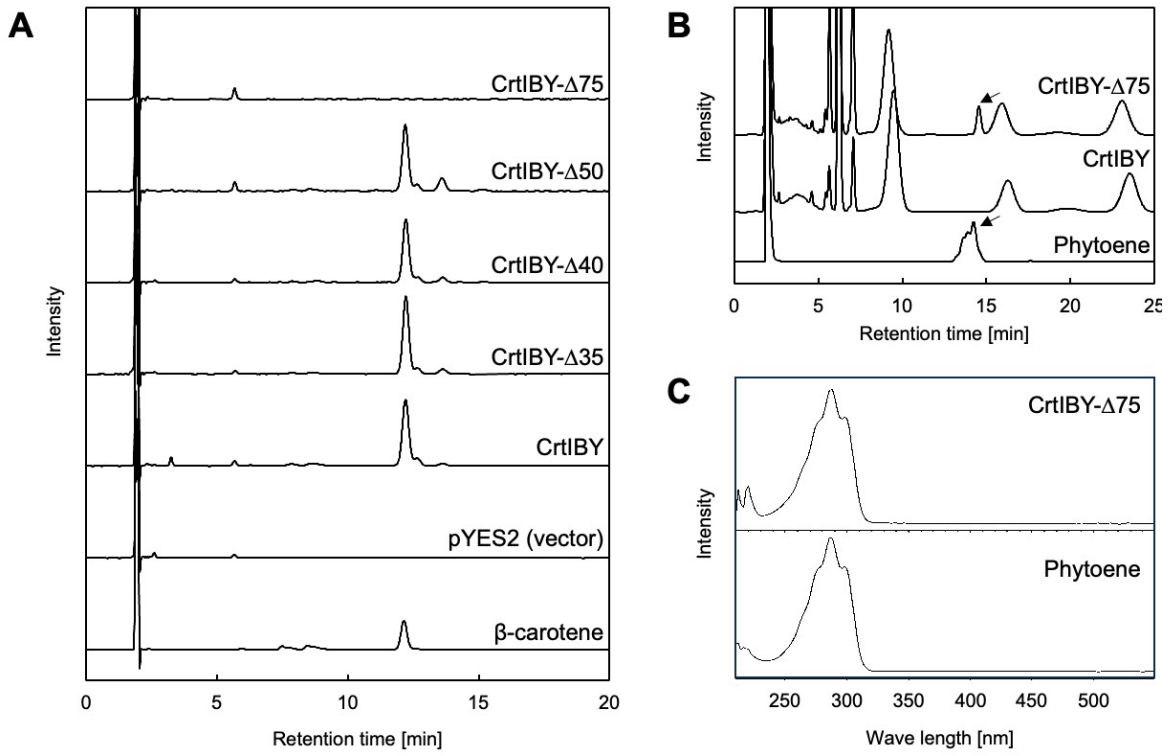

Fig. 2 Carotenoids extracted from *S. cerevisiae* were separated with a reverse phase column and detected by a diode array detector at 470 nm (A) and 280 nm (B). The absorption spectrum of a peak exhibiting a retention time close to that of standard phytoene was extracted and compared with that of the standard (C).

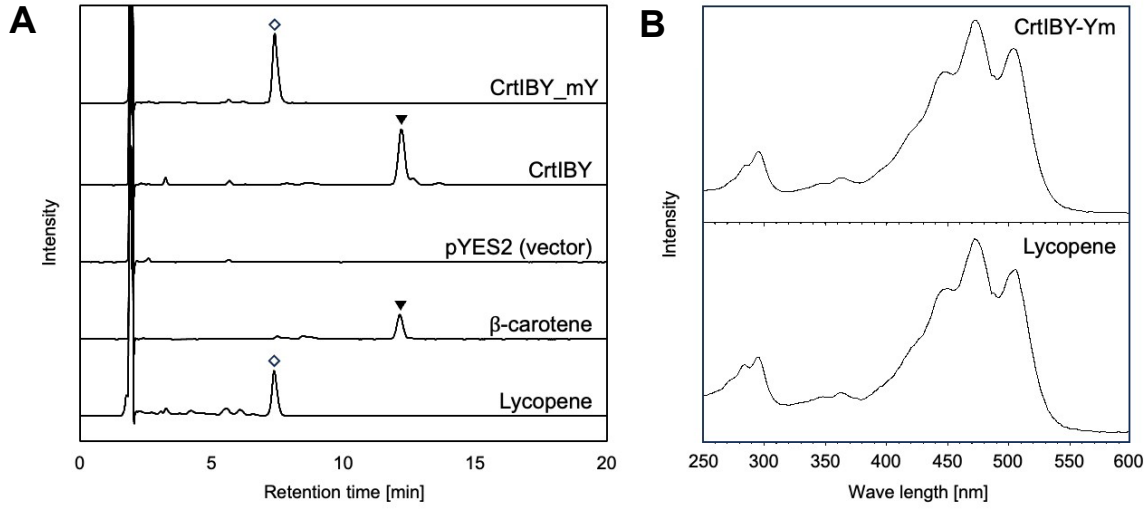

Fig. 3 Carotenoids extracted from *S. cerevisiae* were separated with a reverse phase column and detected by a diode array detector at 470 nm (A). The absorption spectrum of a peak exhibiting a retention time close to that of standard lycopene was extracted and compared with that of the standard.

4 Discussion

CrtIBY derived from *Aurantiochytrium* sp. KH105 is a multifunctional enzyme catalyzing a three-step biosynthetic reaction from GGPP to β-carotene. Since the amino acid sequence of each domain of CrtIBY shows high similarity with the sequences of the related bacterial Crt enzymes[10], it is speculated that these genes were acquired by horizontal gene transfer or phagocytosis and fused into a single gene during evolution. Since KH105 produces only small amounts of phytoene and lycopene, which are β-carotene biosynthetic intermediates, CrtIBY is considered to be an efficient biocatalyst for ß-carotene production compared to using a group of monofunctional enzymes. Indeed, individual expression of CrtI and CrtYB in *S. cerevisiae* has been reported to result in substantial accumulation of biosynthetic intermediates, particularly phytoene[25], and artificial fusion of these enzymes via peptide linkers reduces the relative levels of intermediates[26]. This is likely because the spatial proximity of catalytic domains in a multifunctional enzyme prevents the diffusion of intermediates and enhances the efficiency of substrate–enzyme association at each successive catalytic step. In the present study, we aimed to expand the utility of CrtIBY as an efficient biocatalyst for the production of phytoene and lycopene by modifying the product specificity of CrtIBY.

The phytoene desaturase (CrtI) domain of CrtIBY contains a FAD-binding motif similar to that of CrtI derived from bacteria, fungi, and plants (**Fig. 1A**). FAD bound to this motif is presumed to be involved in the removal of hydrogen from the substrate, phytoene[27]. The analysis of enzymatic activity of CrtIBY mutants lacking the partial N-terminal regions revealed that the yeast strain expressing CrtIBY-Δ75, which lacks the FAD-binding motif, accumulated a substance presumed to be phytoene without the ß-carotene production (**Fig. 2B** and **2C**). This result demonstrates that this motif is essential for CrtI activity. To gain structural insight into the substrate binding of the CrtI domain, molecular docking simulation of phytoene into the AlphaFold3-predicted CrtIBY structure was performed (**Fig. 1A**). The docking analysis revealed that phytoene binds within a hydrophobic channel of the CrtI domain, in proximity to the FAD-binding motif (residues 57–74) (**Fig. 1C**). This structural analysis is consistent with the established role of FAD in the oxidative dehydrogenation of phytoene[22, 27]. The peak derived from CrtIBY-Δ75 showed a retention time similar to that of the chemically synthesized *E*/*Z* phytoene mixture standard, but eluted slightly later (**Fig. 2B**). In reverse-phase chromatography, the *trans* form exhibits a shorter retention time than the *cis* form, suggesting that CrtIBY-Δ75 may generate phytoene with a different geometric isomer composition than chemically synthesized phytoene. In chemical synthesis, thermodynamically stable *trans*-phytoene is mainly produced, whereas in biosynthesis, *cis*-phytoene is mainly produced due to the stereochemical properties of enzymes. Therefore, this difference in retention time suggests that CrtIBY-Δ75 produces phytoene with a high ratio of *cis* isomers.

The loss of desaturase activity in CrtIBY-Δ75 can be explained by two possible mechanisms. First, since the FAD-binding motif in bacterial phytoene desaturase has been reported to play an important role in enzyme binding to biological membranes[22], the removal of this motif from CrtIBY may have disrupted proper membrane association, thereby impairing enzyme function. However, since CrtB activity was retained in the CrtIBY-Δ75-expressing yeast, the structural impact of removing the FAD-binding motif appears to be limited to the CrtI domain rather than affecting the overall enzyme architecture. This localized effect supports the modular nature of the CrtIBY enzyme, where individual domains can be selectively impaired without compromising the function of other domains. Alternatively, the removal of the FAD-binding motif may have directly eliminated the catalytic capability by removing the essential cofactor binding site. FAD serves as the electron acceptor in the desaturation reaction[22, 28], and without proper FAD binding, the enzyme would be unable to perform the oxidative dehydrogenation of phytoene to phytofluene and subsequent intermediates.

The AlphaFold3-predicted structure of CrtIBY revealed that the CrtY domain contains multiple transmembrane helices, which are likely to play an essential role in both the localization of CrtIBY to biological membranes and the maintenance of proper enzyme structure (**Fig. 1B**). Indeed, in the bifunctional enzyme CarRP from *Mucor circinelloides*, which contains both CrtB and CrtY domains, the proper conformation of the CrtY domain has been reported to be crucial for CrtB activity[29].

To investigate the functional importance of the lycopene cyclase motifs present in the CrtY domain (**Fig. 1A**), molecular docking of lycopene into the AlphaFold3-predicted CrtIBY structure was performed to select the primary mutagenesis target (**Fig. 1D**). Previous studies have demonstrated that the proline and glutamate residues within conserved lycopene cyclase motifs are essential for cyclase activity, with the glutamate residues serving as acid-base catalysts for ß-ionone ring closure[4] and proline substitutions abolishing activity[24, 30]. The catalytic EE residues of the C-terminal motif (E1125–E1126) were located 19.51 Å from the center of mass of the docked lycopene molecule, whereas those of the N-terminal motif (E991–E992) were located 20.69 Å away. Although the difference was modest, this structural analysis suggested that the C-terminal PLEEALF motif is positioned closer to the substrate lycopene, and therefore the C-terminal motif (PLEEALF, residues 1123–1129) was selected as the primary mutagenesis target. Accordingly, CrtIBY-mY, which contains P1123G, E1125A, and E1126A mutations in its C-terminal lycopene cyclase motif, was generated.

Analysis of this mutant revealed complete loss of lycopene cyclization activity, as evidenced by lycopene accumulation and absence of ß-carotene production (**Fig. 3**). Since the role of the N-terminal motif (PVEEYMF, residues 989–995) remains to be elucidated, future mutagenesis studies targeting this motif will be necessary to fully understand the individual contributions of each motif to the overall cyclase activity of CrtIBY. Nevertheless, our results clearly demonstrate that the C-terminal motif plays an essential for cyclase activity. These findings, together with the presence of the N-terminal motif, suggest that the two motifs may cooperatively contribute to the overall cyclase activity of CrtIBY.

In the metabolic engineering of plants and microorganisms aimed at enhancing the productivity of various carotenoids, intermediate accumulation at each enzymatic step often reduces the yield of the final product, representing a significant bottleneck in efficient carotenoid production. Multifunctional enzymes such as CrtIBY offer a promising solution to this challenge because they efficiently channel intermediates through the entire biosynthetic pathway without significant accumulation.

From a practical standpoint, introducing a single gene encoding a multifunctional enzyme also offers several technical advantages over introducing multiple genes for individual enzymes. These benefits include reducing the number of required selection markers, simplifying cloning procedures, and facilitating more straightforward gene expression control through the use of a single promoter system. Additionally, the use of multifunctional enzymes can reduce metabolic burden on the host organism by decreasing the total number of genes that need to be expressed and maintained.

Therefore, CrtIBY and its engineered mutants represent particularly valuable tools for carotenoid production. Intact CrtIBY serves as an efficient biocatalyst for ß-carotene production, while the characterized mutants offer new possibilities for producing specific carotenoid intermediates. The CrtIBY-Δ75 mutant, which lacks phytoene desaturase activity, enables efficient phytoene production without further conversion to downstream products. Similarly, the CrtIBY-mY mutant, in which the lycopene cyclase activity is abolished, allows for high-level lycopene accumulation. By overexpressing these CrtIBY mutants in organisms that naturally accumulate high levels of GGPP, the substrate for carotenoid biosynthesis, or by modifying the function of endogenous CrtIBY in high carotenoid-producing *Aurantiochytrium* strains[20] through genome editing approaches[31], it may be possible to achieve high-level production of phytoene and lycopene, respectively.

Acknowledgements

This study was supported in part by the Core Research of Evolutional Science and Technology program (CREST) of the Japan Science and Technology Agency (JST).

Author Contribution Statement

K.W. designed this research, analyzed the data, and drafted the original manuscript. A.O. designed this research, performed the experiments, and analyzed the data. A.N. designed this research, performed the experiments, and analyzed the data. R.S. designed this research, performed the experiments and analyzed the data. Y.H. provided invaluable advice on the three-dimensional structure prediction and docking simulations for CrtIBY. T.A. designed this research and revised the draft critically for important intellectual content. All authors reviewed and revised the manuscript draft and approved the final version for submission.

Conflict of interest

The authors declare no conflict of interest.

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