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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization of two β-carotene ketolases, CrtO and CrtW, by complementation analysis in *Escherichia coli*

Seon-Kang Choi · Hisashi Harada · Satoru Matsuda · Norihiko Misawa

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Abstract The pathways from β-carotene to astaxanthin are crucial key steps for producing astaxanthin, one of industrially useful carotenoids, in heterologous hosts. Two β-carotene ketolases (β-carotene 4,4′-oxygenase), CrtO and CrtW, with different structure are known up to the present. In this paper, we compared the catalytic functions of a CrtO ketolase that was obtained from a marine bacterium *Rhodococcus erythropolis* strain PR4, CrtO derived from cyanobacterium *Synechosistis* sp. PCC6803, and CrtW derived from a marine bacterium *Brevundimonas* sp. SD212, by complementation analysis in *Escherichia coli* expressing the known *crt* genes. Results strongly suggested that a CrtO-type ketolase was unable to synthesize astaxanthin from zeaxanthin, i.e., only a CrtW-type ketolase could accept 3-hydroxy-β-ionone ring as the substrate. Their catalytic efficiency for synthesizing canthaxanthin from β-carotene was also examined. The results obtained up to the present clearly suggest that the bacterial *crtW* and *crtZ* genes are a combination of the most promising gene candidates for developing recombinant hosts that produce astaxanthin as the predominant carotenoid.

Keywords Astaxanthin · Carotenoid · CrtW · CrtO

S.-K. Choi · H. Harada · S. Matsuda · N. Misawa (✉)
Marine Biotechnology Institute,
Heita,
Kamaishi-shi, Iwate 026-0001, Japan
e-mail: norihiko.misawa@mbio.jp

S.-K. Choi
Gangneung Marine Bio Foundation,
Gangneung, Gangwon 210-340, South Korea

Introduction

Carotenoid pigments, which possess colors ranging from light yellow through orange to deep red, are biosynthesized in all of the photosynthetic bacteria, cyanobacteria, algae and higher plants, and also in some of nonphotosynthetic bacteria, yeasts, and fungi. More than 750 different carotenoids have been isolated from natural sources (Britton et al. 2001). Several carotenoids including astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) have attracted greater attention due to their beneficial effects on human health as well as their utility in food colorants and/or animal feeds. Astaxanthin has been shown to have inhibitory effect toward oxidation of low-density lipoprotein in human (Iwamoto et al. 2000), preventive effect of diabetic nephropathy in diabetic db/db mice (Naito et al. 2004), preventative effects against cancer or anticancer activities (Tanaka et al. 1994; Chen et al. 1999), and enhancement of immune responses (Jyonouchi et al. 1995; Chen and Park 2004).

The biosynthetic pathway of astaxanthin in bacteria was elucidated at enzyme and gene levels by in vitro (Fraser et al. 1997, 1998) and in vivo (Misawa et al. 1995) studies. Astaxanthin is synthesized from β-carotene (β,β-carotene) with the introduction of keto and hydroxyl moieties at the 4,4′ and 3,3′ positions of the β-ionone rings by a typical β-carotene ketolase (carotenoid 4,4′-oxygenase), CrtW, and a β-carotene hydroxylase (carotenoid 3,3′-hydroxylase), CrtZ, respectively, by way of the eight intermediates of hydroxylated and/or ketolated carotenoids, as shown in Fig. 1. We have recently characterized a variety of bacterial CrtW and CrtZ (and CYP175A1) enzymes by complementation analysis in *Escherichia coli*, and shown that their astaxanthin-productive efficiencies are different and that the CrtW and CrtZ derived from a marine bacterium *Brevundimonas* sp.

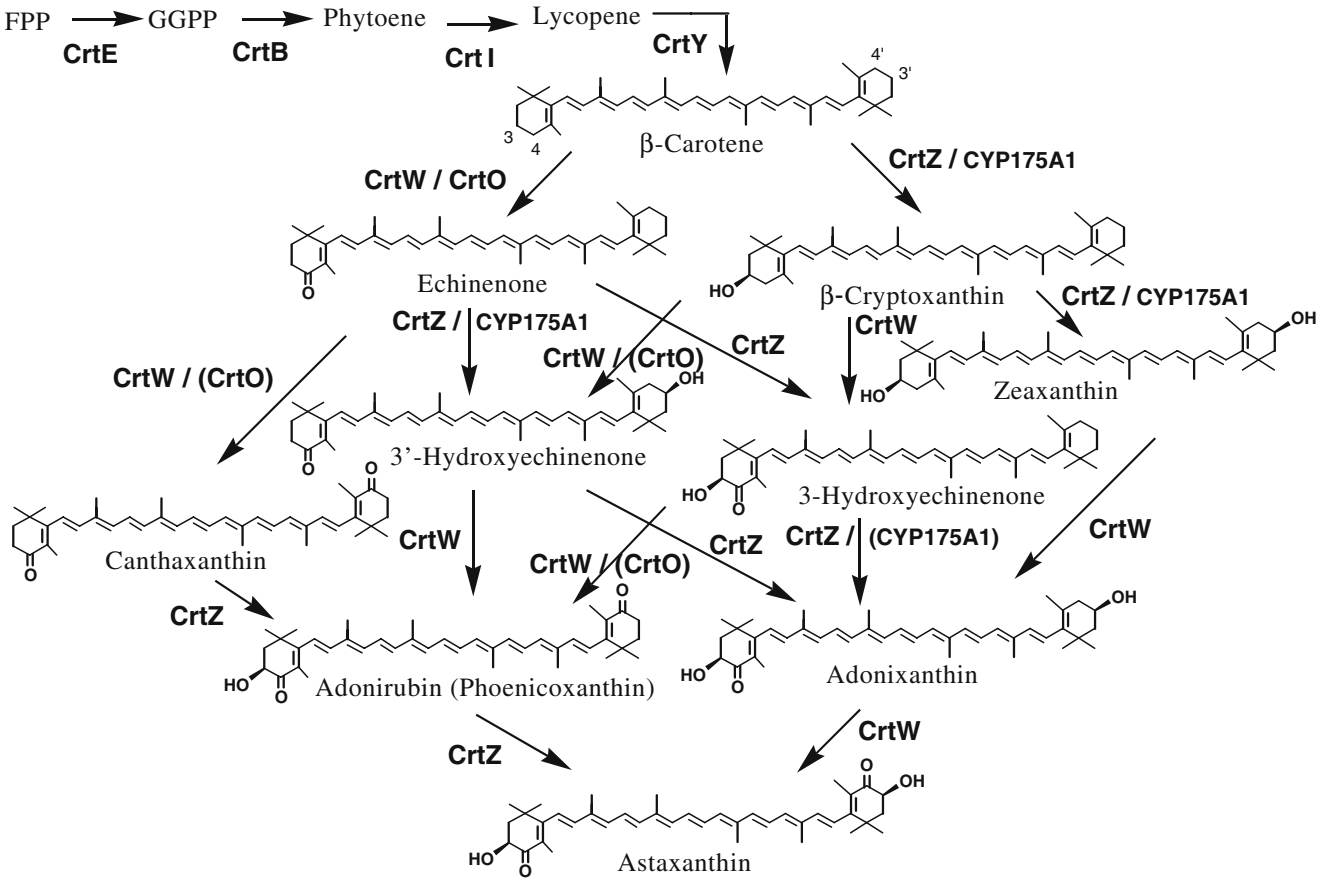

Fig. 1 Astaxanthin biosynthetic pathway in astaxanthin-producing bacteria and the catalytic functions of CrtZ, CrtW, and so forth. CYP175A1 was derived from *Thermus thermophilus* HB27 (Blasco et al. 2004), and its catalytic function was elucidated (Choi et al. 2006). The function of CrtO was elucidated in the present study. *Parentheses* represent weak or possible catalytic functions

strain SD212 belonging to α-*Proteobacteria* possess the most efficient catalytic functions for astaxanthin synthesis (Choi et al. 2005, 2006). Recently, mutational analysis of *crtW* has been carried out to obtain an artificial *crtW* gene favorable to the production of astaxanthin (Ye et al. 2006; Tao et al. 2006).

A new type of β-carotene ketolase that was structurally different from CrtW was first identified in cyanobacterium *Synechosystis* sp. PCC6803, and the corresponding gene was designated *crtO* (Fernandez-Gonzalez et al. 1997). This gene product CrtO (slr0088) was found to catalyze the reaction from β-carotene to echinenone (β,β-caroten-4-one; Fig. 1; Fernandez-Gonzalez et al. 1997). Mochimaru et al. (2005) reported that cyanobacterium *Anabaena* sp. PCC7120 possessed both CrtO and CrtW ketolases. In this cyanobacterium, CrtO and CrtW were suggested to mediate the synthesis of echinenone and ketomyxol, respectively (Mochimaru et al. 2005). Tao and Cheng (2004) isolated *crtO* genes from high GC gram-positive bacteria *Rhodococcus erythropolis* strain AN12 (ATCC47072) and *Deinococcus radiodurans* R1. These CrtO ketolases were shown to catalyze the reaction from β-carotene to canthaxanthin (β,β-carotene-4,4′-dione) via echinenone (Fig. 1; Tao and Cheng 2004).

In this study, we compare the catalytic functions of a CrtO ketolase that was obtained from a marine bacterium *R. erythropolis* strain PR4, CrtO derived from *Synechosistis* sp. PCC6803, and CrtW derived from *Brevundimonas* sp. SD212 by complementation analysis in *E. coli*, and show that only a CrtW-type ketolase is capable of synthesizing astaxanthin from zeaxanthin (3,3′-dihydroxy-β,β-carotene; Fig. 1).

Materials and methods

Recombinant DNA techniques

The restriction enzymes and DNA ligation kit were, respectively, purchased from New England BioLabs (Beverly, CA, USA) or Takara (Shiga, Japan) and Toyobo (Osaka, Japan). Plasmid DNA was prepared with the Miniprep DNA Purification Kit (Takara). The polymerase chain reaction (PCR) was carried out by an automated thermal

cycler (Takara) with KOD plus DNA polymerase. All recombinant DNA techniques were conducted by the standard molecular biology technique (Sambrook et al. 1989) or as instructed by the suppliers. Total genomic DNA was extracted from *R. erythropolis* PR4 (deposited as NBRC 100887 and MBIC 01337) as previously described (Teramoto et al. 2003).

Construction of expression plasmids

A *crtO* gene of *R. erythropolis* PR4 was amplified from genomic DNA by PCR using primers 5′-TACGAATTC GATGAGCGCATTTCTCGACGC-3′ and 5′-TAGAG GATCCTCACGACCTGCTCGAACGAC-3′ based on the genomic sequence (Sekine et al. 2006). The *crtO* gene of *Synechosistis* sp. PCC6803 was amplified from plasmid pslr0088 (Fernandez-Gonzalez et al. 1997) using primers 5′-TACGAATTCGATGATCACCACCGATG-3′ and 5′-TAGAGGATCCTTACCAAAAACGACGA-3′. The PCR products were digested with *Eco*RI and *Bam*HI, and then inserted into the corresponding sites of pUC18 vector (Toyobo) to, respectively, construct pUCPR4-O (for *R. erythropolis* PR4 *crtO*) and pUC6803-O (for *Synechosistis* sp. PCC6803 *crtO*), where ATG for the original start of each *crtO* gene was placed next to the *Eco*RI site to form the CrtO proteins which fused with the additional 7-amino-acid terminus of β-glucosidase (LacZ).

Nucleotide sequencing and computer analysis

The nucleotide sequences of the inserted fragments of the plasmids were confirmed using a DNA sequencing kit (Bigdye terminator cycle sequencing ready reaction kit version 3.1; Applied Biosystems) and a model 3730 DNA analyzer (Applied Biosystems) according to the manufacturer's instructions. Homologous protein sequences in the protein sequence database were retrieved from the GenBank database with the basic local alignment search tool (BLAST) program (Altschul et al. 1997) and aligned by Clustal W program (DDBJ version; http://www.ddbj.nig.ac.jp/search/ex_clustalw-j.html; Thompson et al. 1997).

Cultures of recombinant bacteria

E. coli JM109 (Takara) carrying plasmid pACCAR25ΔcrtX that contained the five carotenoid biosysthesis genes, *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ*, from *Pantoea ananatis* (formerly known as *Erwinia uredovora* 20D3) and plasmid pACCAR16ΔcrtX with *crtE*, *crtB*, *crtI*, and *crtY* from *P. ananatis* (Misawa et al. 1995), were, respectively, used as the host for synthesizing zeaxanthin and β-carotene. A Luria-Bertani medium (6 ml) containing appropriate antibiotics was inoculated with 60 μl of a fully grown culture of *E. coli* transformants, and incubated at 30°C while shaking. *E. coli* containing pACCAR25ΔcrtX or pACCAR16ΔcrtX required chloramphenicol (Cm) at a final concentration of 30 μg/ml, and *E. coli* carrying plasmid pACCAR25ΔcrtX or pACCAR16ΔcrtX, and pUCPR4-O, pUC6803-O or pUCBre-W (for *Brevundimonas* sp. SD212 *crtW*; Nishida et al. 2005) each required Cm and ampicillin (100 μg/ml). When OD_{600} of the culture had reached about 0.5, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After cultivating for 12, 24, and 48 h, the cells were harvested by centrifugation at 4°C and stored at −70°C.

Analysis of carotenoids accumulated in the recombinant bacteria

HPLC-PDA analysis was carried out as described previously (Choi et al. 2005). HPLC-PDA-APCI-MS analysis was performed by a modification of a previously described method (Choi et al. 2005) using a reverse-phase HPLC column Deverosil C30-UG5 (1.0×250 mm, Nomura chemical) with a Deverosil C30-UG-S precolumn. Column temperature was maintained 40°C. Mobile phases consisted of water-methanol, 5:95, v/v (A) and methanol-*tert*-butyl methyl ether, 30:70, v/v (B), and the elute was monitored continuously at 210–600 nm. The crude extracts from *E. coli* transformants were eluted at a rate of 0.1 ml/min with solvent A for 15 min, followed by a linear gradient from solvent A to B for 50 min, and hold for 20 min. Mass spectra were monitored continuously m/z 200–1,200. The capillary temperature was set to 150°C, the APCI vaporizer temperature was held at 400°C, the capillary voltage was optimized to 23 V, and the sheath nitrogen gas flow was set to 28 (arbitrary units).

Authentic samples of carotenoids were purchased from Sigma-Aldrich (St. Louis, MO, USA) or purified from the appropriate *E. coli* transformants expressing the *crt* genes (Nishida et al. 2005).

Spectral data for the individual carotenoids

Astaxanthin (1) HPLC-PDA, retention time (RT) 6.3 min, λmax 480 nm; HPLC-PDA-APCI-MS, RT 18.2 min, λmax 474 nm, m/z 597 $[M+H]^+$.

Adonixanthin (2) HPLC-PDA, retention time (RT) 8.2 min, λmax 478 nm; HPLC-PDA-APCI-MS, RT 23.1 min, λmax 461 nm, m/z 583 $[M+H]^+$.

Astaxanthin-cis-isomer (1') HPLC-PDA, retention time (RT) 8.5 min, λmax 371, 471 nm; HPLC-PDA-APCI-MS, RT 25.8 min, m/z 597 $[M+H]^+$.

Zeaxanthin (3) HPLC-PDA, RT 9.5 min, λmax 452, 479 nm; HPLC-PDA-APCI-MS, RT 27.9 min, λmax 450, 476 nm, m/z 569 $[M+H]^+$.

Canthaxanthin (4) HPLC-PDA, RT 10.3 min, λmax 478 nm.

Zeaxanthin-cis-isomer (3′) HPLC-PDA, RT 10.6 min, λmax 339, 445, 471 nm; HPLC-PDA-APCI-MS, RT 35.2 min, λmax 447, 473 nm, m/z 569 $[M+H]^+$.

3-Hydroxyechinenone (5) HPLC-PDA, RT 11.8 min, λmax 471 nm; HPLC-PDA-APCI-MS, RT 45.2 min, λmax 465 nm, m/z 567 $[M+H]^+$.

Rubixanthin (6) HPLC-PDA, RT 12.1 min, λmax 463, 492 nm; HPLC-PDA-APCI-MS, RT 46.8 min, λmax 438, 461, 487 nm, m/z 553 $[M+H]^+$.

Echinenone (7) HPLC-PDA, RT 12.4 min, λmax 451, 478 nm; HPLC-PDA-APCI-MS, RT 48.8 min, λmax 463 nm, m/z 551 $[M+H]^+$.

Lycopene (8) HPLC-PDA, RT 13.8 min, λmax 473, 504 nm; HPLC-PDA-APCI-MS, RT 58.5 min, λmax 445, 470, 500 nm, m/z 537 $[M+H]^+$.

β-Carotene (9) HPLC-PDA, RT 14.3 min, λmax 452, 481 nm.

Results

Functional expression of *crtO* and *crtW* in β-carotene-accumulating *E. coli*

From the sequence database of *R. erythropolis* PR4 (Sekine et al. 2006), we found one open reading frame (ORF) with high homology (99% identity) to the CrtO-type ketolase from *R. erythropolis* ATCC47072 (Tao and Cheng 2004). The PR4 *crtO* gene was amplified by PCR, and cloned in the expression vector pUC18 for *E. coli* to generate plasmid pUCPR4-O as shown in Materials and methods. The *Synechosystis* sp. PCC6803 *crtO* gene was also amplified and cloned into pUC18 to generate pUC6803-O. Plasmids pUCPR4-O and pUC6803-O, and pUCBre-W, in which the *crtW* gene from *Brevundimonas* sp. SD212 was similarly inserted into pUC18, were introduced into β-carotene- or zeaxanthin-accumulating *E. coli* due to the presence of plasmid pACCAR16ΔcrtX or pACCAR25 crtX. The cells of the respective *E. coli* transformants were cultivated and found to reach stationary phase in 12 h, as shown in Fig. 2.

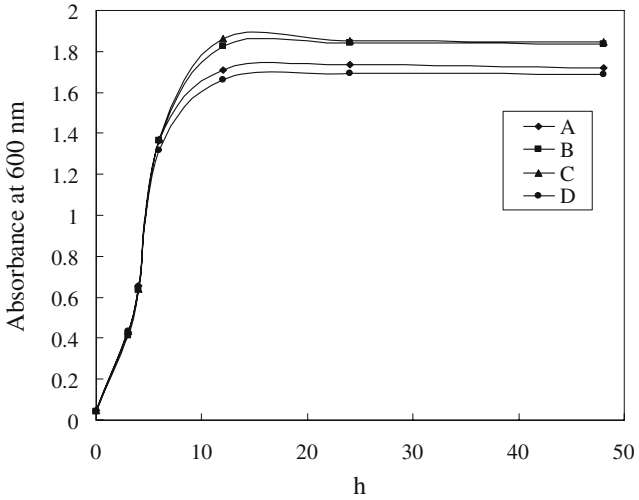

Fig. 2 Growth curve of *E. coli* transformants carrying (an) appropriate plasmid(s). **a** pACCAR25ΔcrtX; **b** pUC6803-O and pACCAR25ΔcrtX; **c** pUCPR4-O and pACCAR25ΔcrtX; **d** pUCBre-W and pACCAR25ΔcrtX

The cells were harvested after 12, 24, and 48 h of culture. Carotenoids were extracted from the cells, and analyzed by HPLC-PDA and HPLC-PDA-APCI-MS.

The *E. coli* control strain carrying pACCAR16ΔcrtX synthesized β-carotene (9) (Fig. 3a). When plasmids pUCPR4-O and pUCBre-W were introduced into *E. coli* (pACCAR16ΔcrtX), these recombinant strains produced canthaxanthin (4) by way of echinenone (7; Fig. 3c and d). Whereas, *E. coli* carrying plasmids pUC6803-O and pACCAR16ΔcrtX produced echinenone as the main product even after 48-h culture (Fig 3b). The catalytic efficiency between *E. coli* (pUCPR4-O and pACCAR16ΔcrtX) and *E. coli* (pUCBre-W and pACCAR16ΔcrtX) for synthesizing canthaxanthin was different, e.g., CrtW biotransformed β-carotene into canthaxanthin almost completely even after 24-h culture (Fig. 3d).

Functional expression of *crtO* and *crtW* in zeaxanthin-accumulating *E. coli*

The *E. coli* control strain carrying pACCAR25ΔcrtX synthesized zeaxanthin (3) in addition to small amounts of carotenoids (3′ and 6), which were considered to be a *cis* isomer of zeaxanthin and rubixanthin (3-hydroxy-γ-carotene), respectively, by their spectral data (Fig. 4a). When plasmids pUC6803-O and pUCPR4-O were introduced into *E. coli* (pACCAR25ΔcrtX), these recombinant strains produced large amounts of zeaxanthin in addition to 3-hydroxyechinenone (5), rubixanthin and lycopene (8) (Fig. 4b and c). Small amounts of astaxanthin (1), adonixanthin (2) and echinenone (7) were also observed in the 24-h culture of the *E. coli* cells carrying pUCPR4-O

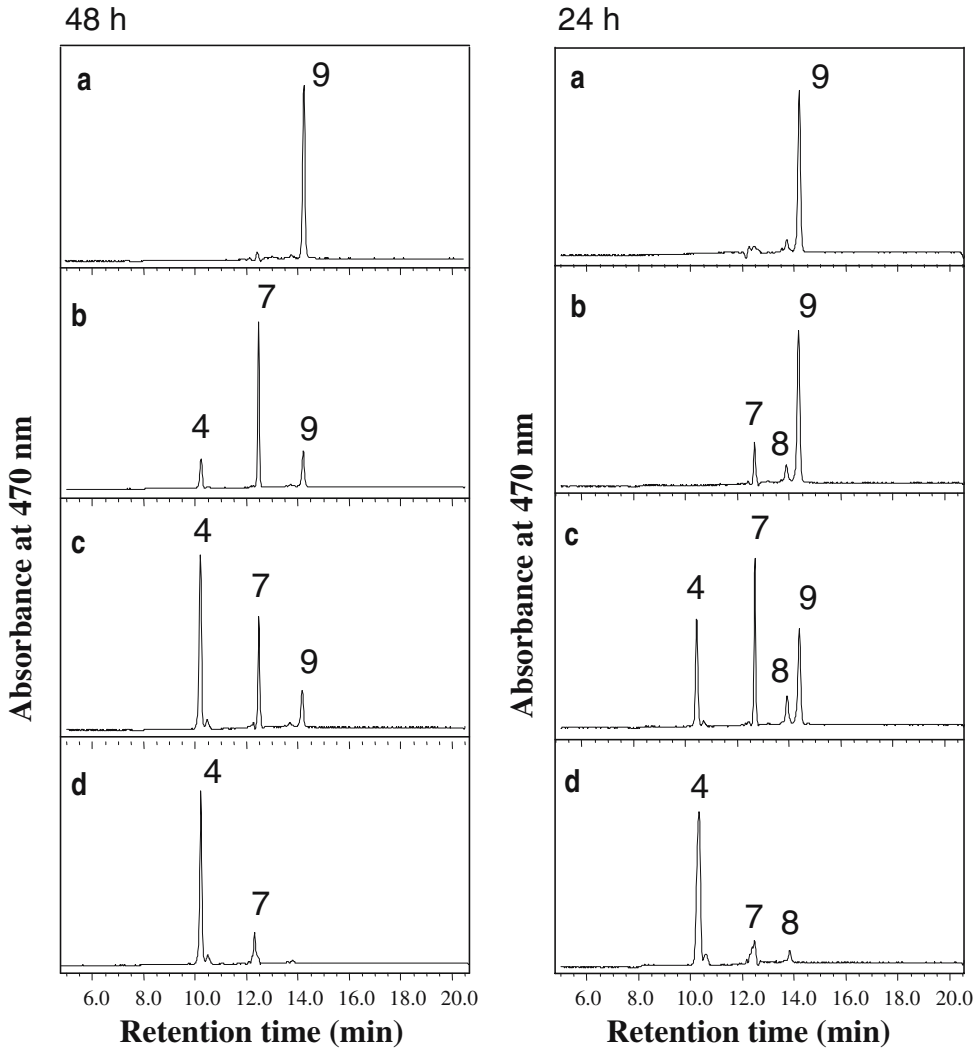

Fig. 3 HPLC-PDA traces of the carotenoids biosynthesized in 48-h and 24-h cultures of β-carotene-accumulating *E. coli* transformants carrying another plasmid. **a** pACCAR16ΔcrtX (control); **b** pUC6803-O and pACCAR16ΔcrtX; **c** pUCPR4-O and pACCAR16ΔcrtX; **d** pUCBre-W and pACCAR16ΔcrtX. *4*, canthaxanthin; *7*, echinenone; *8*, lycopene; *9*, β-carotene

and pACCAR25ΔcrtX (Fig. 4c). On the other hand, *E. coli* (pUCBre-W and pACCAR25ΔcrtX) accumulated astaxanthin (1) predominantly (Fig 4d).

Discussion

Plasmids pUC6803-O (which contained *crtO* from *Synechosystis* sp. PCC6803), pUCPR4-O (which contained *crtO* from *R. erythropolis* PR4), and pUCBre-W [which contained *crtW* from *Brevundimonas* sp. SD212 (AB181388)] were constructed as shown in Materials and methods. The expression levels of the individual *crtO* and *crtW* genes in these plasmids were expected to be similar because they utilized the same transcription and translation signals derived from vector pUC18.

The results obtained using *E coli* accumulating β-carotene (Fig. 3) indicated that CrtO from *Synechosystis* sp. PCC6803 catalyzed monoketolation reaction of β-carotene to synthesize echinenone, as described by Fernandez-Gonzalez et al. (1997), and that CrtO from *R. erythropolis* PR4 catalyzed diketolation reactions of β-carotene to synthesize canthaxanthin via echinenone, as suggested by Tao and Cheng (2004); whereas, CrtW from *Brevundimonas* sp. SD212 possessed much higher catalytic efficiency for canthaxanthin synthesis than that of the *R. erythropolis* PR4 CrtO enzyme.

On the other hand, the results obtained using *E coli* accumulating zeaxanthin (Fig. 4) indicated that the characteristics of CrtO-type and CrtW-type enzymes were largely different. The *E. coli* cells expressing the *Brevundimonas* SD212 *crtW* gene were able to produce astaxanthin as the predominant carotenoid. Whereas, the *E. coli* cells expressing the *crtO* gene from *Synechosystis* sp. PCC6803 or *R. erythropolis* PR4 accumulated large amounts of zeaxanthin. The generated zeaxanthin did not diminish according to cultivation time (data not shown). These results strongly suggest that the encoded CrtO enzymes are unable to utilize zeaxanthin to produce astaxanthin, which is a new finding

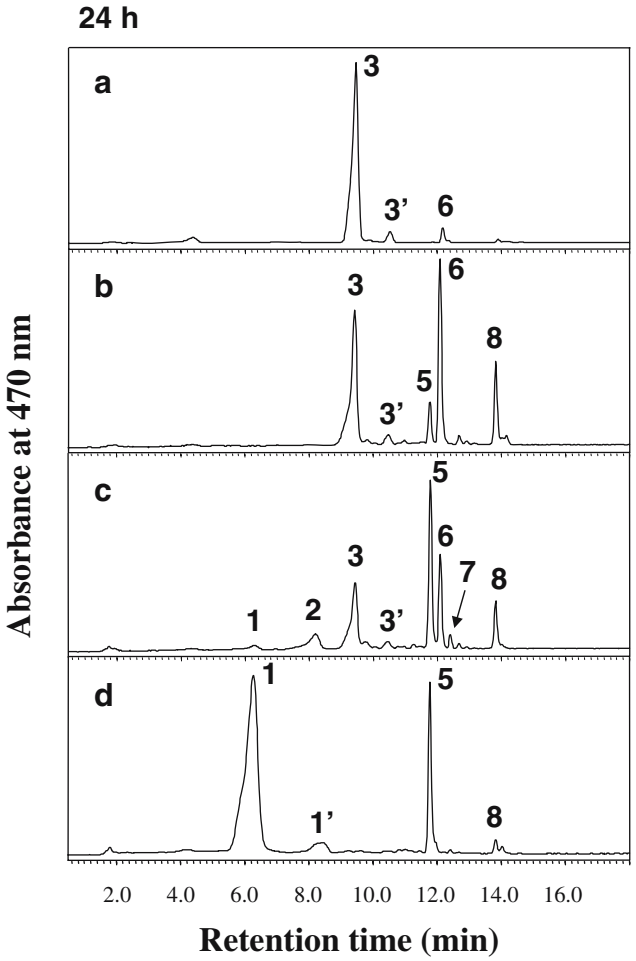

Fig. 4 HPLC-PDA traces of the carotenoids biosynthesized in a 24-h culture of zeaxanthin-accumulating *E. coli* transformants carrying another plasmid. **a** pACCAR25ΔcrtX (control); **b** pUC6803-O and pACCAR25ΔcrtX; **c** pUCPR4-O and pACCAR25ΔcrtX; **d** pUCBrevW and CAR25ΔcrtX. *1*, astaxanthin; *1′*, *cis*-astaxanthin; *2*, adonixanthin; *3*, zeaxanthin, *3′*, *cis*-zeaxanthin; *5*, 3-hydroxyechinenone; *6*, rubixanthin; *7*, echinenone; *8*, lycopene

on the characteristics of CrtO-type ketolases. It is very likely that CrtO cannot accept 3-hydroxylated β-ionone ring as the substrate. In *E. coli* (pUCPR4-O and pACCAR25ΔcrtX), very small amounts of astaxanthin as well as adonixanthin were observed in 24-h culture (Fig. 4c). Also in *E. coli* (pUC6803-O and pACCAR25ΔcrtX), very small amounts of adonixanthin was observed in 48-h culture (data not shown). Gerjets and Sandmann (2006) reported that zeaxanthin and adonixanthin accumulated in addition to very small amounts of astaxanthin in an *E. coli* transformant expressing the *Synechosystis* sp. PCC6803 *crtO* gene and the *P. ananatis crtEBIYZ* genes. In these *E. coli* transformants expressing *crtO*, astaxanthin is likely to be synthesized from adonirubin with the *P. ananatis* CrtZ enzyme (Fig. 1). Adonirubin should generate from canthaxanthin with CrtZ and probably from 3-hydroxyechinenone with CrtO (Fig. 1). Adonixanthin is likely to be synthesized from 3′-hydroxyechinenone and 3-hydroxyechnenone with CrtZ. 3-Hydroxyechnenone accumulated in all the *E. coli* (pACCAR25ΔcrtX) expressing the *crtO* or *crtW* gene (Fig 4b–d). This carotenoid decreased according to cultivation time (data not shown). Similar phenomenon was observed in *E. coli* carrying pACCAR25ΔcrtX and a plasmid for expressing each *crtW* (Choi et al. 2005). 3-Hydroxyechinenone may act like a detergent because of its highly monopolar structure, which may reduce an opportunity that the substrate and the enzymes meet. Lycopene also tended to accumulate in many *E. coli* transformants (Figs. 3 and 4), suggesting that CrtY activity is relatively weak in *E. coli* carrying plasmid pACCAR25ΔcrtX or pACCAR16ΔcrtX. Rubixanthin was observed in the *E. coli* transformants carrying pACCAR25ΔcrtX (Fig. 4a–c). This carotenoid decreased according to cultivation time (data not shown). It can be speculated that rubixanthin generated with CrtZ by the hydroxylation reaction of γ-carotene, which is the intermediate from lycopene to β-carotene with CrtY.

CrtW-type ketolases included four conserved regions containing three iron-binding motifs. On the other hand, CrtO-type ketolases included different six conserved regions (Tao and Cheng 2004), and had no significant sequence homology to CrtW, whereas they showed significant homology to bacterial phytoene dehydrogenases, CrtI. Therefore, it may not be surprising that CrtO-type and CrtW-type ketolases showed different catalytic functions.

The pathways from β-carotene to astaxanthin are crucial key steps for producing astaxanthin in heterologous hosts such as edible plants as well as bacteria including *E. coli*. We have previously elucidated that cytochrome P450 (CYP175A1) from *Thermus thermophilus* HB27, which had been shown to be β-carotene hydroxylase (Blasco et al. 2004), is unable to utilize canthaxanthin to produce astaxanthin, i.e., it cannot accept 4-ketolated β-ionone ring as the substrate. CYP175A1 is very likely to accept monoketolated carotenoids with the naked β-ionone ring (echinenone) as well as β-carotene and β-cryptoxanthin as the substrates (Fig. 1; Choi et al. 2006). In this case, an *E. coli* transformant expressing the *Paracoccus* sp. N81106 *crtW* gene, the CYP175A1 genes and the *P. ananatis crtEBIY* genes produced large amounts of adonirubin in addition to 3′-hydroxyechinenone and canthaxanthin.

The results obtained up to the present clearly suggest that the bacterial *crtW* and *crtZ* genes are a combination of the most promising gene candidates for developing recombinant hosts that produce astaxanthin as the predominant carotenoid.

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