Cloning and expression in *Escherichia coli* of the gene encoding β-C-4-oxygenase, that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*

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Abstract In the green alga *Haematococcus pluvialis* the ketocarotenoid astaxanthin accumulates under stress conditions. Astaxanthin is a red carotenoid pigment which possess antioxidative activity. We have cloned the gene for β-C-4 oxygenase (β-carotene ketolase) from the green algae *H. pluvialis*. The cloning method took advantage of a strain of *E. coli* which was genetically engineered to produce β-carotene. An expression cDNA library of *H. pluvialis* was transfected to cells of this strain and visually screened for brown-red pigmented colonies. One colony out of 100,000 transformants showed color change due to accumulation of canthaxanthin. The cDNA clone in this transformant colony encodes the enzyme β-C-4 oxygenase that catalyzes the conversion of β carotene to canthaxanthin via echinenone. This enzyme does not convert zeaxanthin to astaxanthin. It is concluded that in *H. pluvialis* astaxanthin is synthesized via canthaxanthin and therefore an additional enzyme is predicted, which converts canthaxanthin to astaxanthin.

Key words: Carotenoid; Astaxanthin; Canthaxanthin; Biosynthesis; Cloning; *Haematococcus pluvialis*

1. Introduction

Carotenoids are widely distributed in nature. More than 630 different naturally-occurring carotenoids have been characterized. They are synthesized de novo from isoprenoid precursors only in photosynthetic organisms and some microorganisms [1]. The early steps in this pathway consist of phytoene synthesis by condensation of two molecules of geranylgeranyl pyrophosphate (GGPP), followed by desaturation of phytoene to phytofluene, ζ-carotene, neurosporene and lycopene. Two cyclization reactions convert lycopene to β-carotene. The addition of various oxygen-containing side groups to carotenes form the various xanthophyll species [2].

While this pathway has been known for several decades, only recently have some of the molecular mechanisms involved been elucidated. Genes for carotenoid biosynthesis enzymes have been cloned from several microorganisms (reviewed in [3]). In algae and plants genes for only the early steps up to β-carotene have been studied (reviewed in [4]) — these include: GGPP syntase, phytoene synthase, phytoene desaturase, ζ-carotene desaturase (from cyanobacteria only) [5] and lycopene cyclase [6]. Relatively little is known about the formation of xanthophylls [7].

The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a red pigment that is common in many marine animals where it contributes to the pinkish color of their flesh. Only few animals can synthesize it de novo from other carotenoids and most of them acquire it in their food. In the plant kingdom astaxanthin occurs mainly in some species of cyanobacteria, lichens and algae. However, it is found rarely also in petals of higher plants [1].

The unicellular fresh-water green alga *Haematococcus pluvialis* accumulates large amounts of astaxanthin when exposed to unfavorable growth conditions, or following different environmental stresses such as phosphate or nitrogen starvation, high concentration of salt in the growth medium or high light intensity [8–10]. During this process the vegetative cells of the alga form cysts and change their color from green to red.

We report here the cloning of a cDNA from *H. pluvialis*, which encodes β-C-4-oxygenase (ketolase), the enzyme that converts β-carotene to canthaxanthin, an intermediate step of astaxanthin synthesis.

2. Material and methods

2.1. Algae and growth conditions

Haematococcus pluvialis (strain 34/7 from the Culture Collection of Algae and Protozoa, Windermere, UK) was kindly provided by Dr. Andrew Young from the University of Liverpool. Suspension cultures of the alga were grown in a liquid medium as described [8]. For induction of astaxanthin biosynthesis cells were harvested, washed in water and resuspended in a nitrogen-depleted medium. The cultures were maintained under continuous light (photon flux of 75 μE/m²/s) at 25°C.

2.2. Construction of cDNA library

Total RNA was extracted from alga cells grown for 5 days under nitrogen starvation (cell color brown-red). Cells from 50 ml culture were harvested and RNA was extracted using Tri reagent (Molecular Research Center Inc.). Poly(A) RNA was isolated by two cycles of fractionation on oligo dT-cellulose (Boehringer). The final yield was 1.5% of the total RNA. cDNA library was constructed in the Uni-ZAP-XR vector, using ZAP-cDNA synthesis kit (Stratagene).

XL1-Blue MRF′ (Stratagene) cells were used for the amplification of the cDNA library.

2.3. Plasmids and E. coli strains

Plasmid pPL376, which contains all of the genes for the carotenoid biosynthesis pathway in the bacterium *Erwinia herbicola* was obtained from Tuveson [11]. Cells of *E. coli* strain JM109 that carry this plasmid accumulate the bright yellow zeaxanthin glycoside. A 1.1 kb *Sal*I-*Sal*I fragment was deleted from this plasmid to inactivate the gene *crtX* (for zeaxanthin glucosyl transferase). In a second step, partial *Bam*HI cleavage and self ligation, followed by screening for orange-yellow colonies, deleted a 0.8 kb fragment which inactivated *crtZ*, encoding β-carotene hydroxylase. A partial *Bgl*II cleavage generated a fragment of 7.4 kb which was cloned in the *Bam*HI site of the plasmid vector pACYC184. The resulting recombinant plasmid carried the genes *crtE*, *crtB*,*crtI* and *crtY* and was designated pBCAR (Fig. 1).

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Plasmid pBCAR was transfected into Solar strain cells of *E. coli* (Stratagene). Colonies of Solar strain that carry the this plasmid develop a deep yellow-orange color due to accumulation of β-carotene (Fig. 2A).

An additional plasmid, pZEAX, which confers zeaxanthin accumulation in *E. coli* was similarly constructed. Solar strain was used as a host for the pZEAX plasmid.

E. coli cells were grown on Luria Broth (LB) medium. Ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml) were added to the medium for selection of appropriate transformed cells.

2.4. Excision of the phage library and the screening for the β-carotene oxygenase gene

Mass excision of the cDNA library was carried out using the ExAssist helper phage (Stratagene) in Solar strain that carried the plasmid pBCAR. The excised library was plated on LB plates containing 1 mM isopropylthio-β-D-galactosidase (IPTG), 50 mg/ml ampicillin and 30 mg/ml chloramphenicol in a concentration that yielded about 100–150 colonies on each plate. The plates were incubated at 37°C overnight and further incubated for two more days at room temperature. From then on the plates were kept at 4°C until screened for changes in colony colors.

2.5. Carotenoid analysis

Carotenoids were extracted with acetone from *E. coli* cells which were grown in suspension in LB medium and washed once in water. The carotenoids were separated by high-performance liquid chromatography (HPLC) on a reverse-phase C18 column, Spherisorb ODS2 (5 mm particle size). The mobile phase was pumped by Merck-Hitachi L-6200A high pressure pump, at a flow rate of 1 ml/min. Peaks were detected at 450 nm by Merck-Hitachi diode-array detector L-4500. The solvent system included two solutions: acetonitrile/H_2O (9:1) and ethylacetate. The pigments were separated by linear gradient between the two solvents for 25 min. Individual carotenoids were identified by absorption spectra and their typical retention times, as compared to standard samples of chemically pure β-carotene, canthaxanthin and echinenone (the latter two were kindly provided by Dr. Andrew Young from the University of Liverpool). In addition, the HPLC peaks were separated, dried and further analyzed by a spectrophotometry (Hewlett Packard 8452A Diode Array Spectrophotometer), using petroleum ether as a solvent, and by mass spectrometry. Mass spectrometry analysis was carried out in the analytical service unit of the Technion, Israel Institute of Technalogy, Haifa. The mesurments were done on a Finnigen mass spectrometer model TSQ-70 by direct injection using CI for both positive and negative ions, and isobutanol as gas.

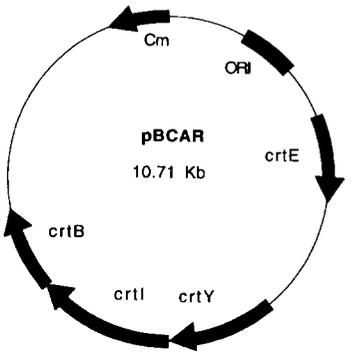

Fig. 1. Structure of the plasmid pBCAR.

3. Results

3.1. Cloning the β-carotene oxygenase gene

A cDNA library was constructed in lambda ZAP II vector from RNA of *Haematococcus pluvialis* cells that had been induced to synthesize astaxanthin by nitrogen deprivation. The entire library was excised into β-carotene-accumulating cells of *E. coli* strain Solar, which carried plasmid pBCAR (Fig. 1). The screening for a β-carotene oxygenase gene was based on color visualization of colonies of a size of 3 mm in diameter. Astaxanthin and other oxygenated forms of β-carotene have distinct

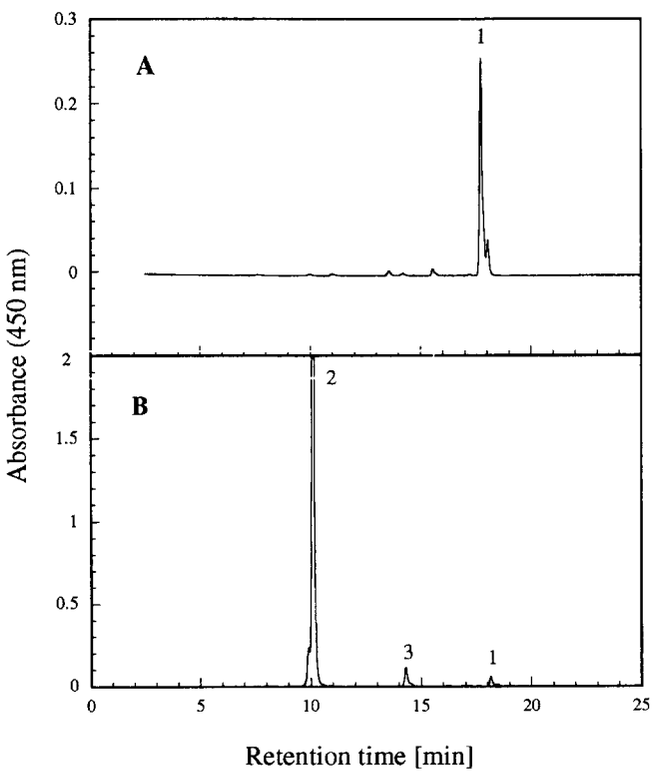

Fig. 2. HPLC analysis of carotenoid pigments extracted from cells of *E. coli* carrying plasmids pBCAR (panel A) or pBCAR and pHPK (panel B). 1-β-Carotene; 2-canthaxanthin; 3-echinenone.

darker colors and thus can be detected on the yellow β-carotene background. The screening included approximately 100,000 colonies which were grown on LB medium plates containing ampicillin and chloramphenicol that select for both plasmids. Several colonies showed different color tones but only one exhibited a conspicuous brown-red pigment.

3.2. Analysis of the products of β-carotene oxygenase enzyme

The colony presumed to contain a xanthophyll biosynthesis gene was streaked and further analyzed. First, the plasmid carrying the cDNA clone, designated pHPK, was isolated and used to transform a β-carotene-producing *E. coli* cells. Carotenoids from the transformant as well as from the host cells were extracted by acetone and analyzed by HPLC. It was determined that the brown-red colony contained the ketocarotenoid canthaxanthin (β,β-carotene-4,4-dione).

HPLC analysis of carotenoids of the host bacteria which synthesized β-carotene, and of the brown-red colony producing canthaxanthin, are shown in Fig. 2A and B, respectively. The absorbance spectra of the HPLC peaks are shown in Fig. 3. The retention times of the echinenone and canthaxanthin in the HPLC, and their absorbance spectra were identical to those of the standard compounds. Only traces of β-carotene were observed in the transformant cells (Fig. 2B) while a new major peak of canthaxanthin and another minor peak of echinenone appeared. Further verification of the carotenoids identity was provided by mass spectrometry analysis which showed a molecular weight of M^- 564.3 for canthaxanthin and M^- 550.3 for echinenone. These results indicate that the cDNA in plasmid pHPK encodes an enzyme with β-C-4-oxygenase activity, which converts β-carotene to canthaxanthin via echinenone (β,β-carotene-4-one). It is concluded that a single enzyme catalyzes this two-step conversion by acting symmetrically on the β-carotene molecule.

Zeaxanthin has been postulated in the past as a possible intermediate in astaxanthin biosynthesis from β-carotene [2] (Fig. 4). To find out whether β-C-4-oxygenase can convert zeaxanthin to astaxanthin, plasmid pHPK, containing the cDNA of β-C-4-oxygenase, was transfected into *E. coli* cells that carried the plasmid pZEAX and accumulated zeaxanthin.

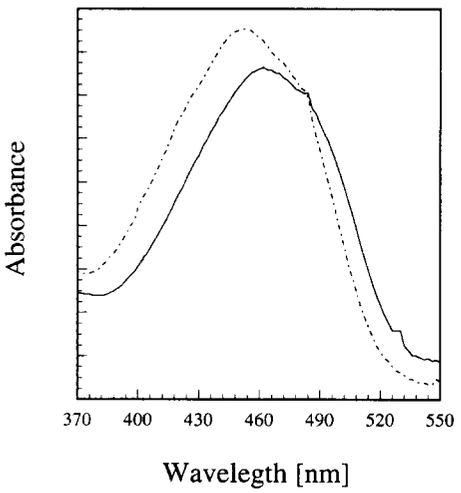

Fig. 3. The absorbance spectra in petroleum ether of peaks 2 and 3 from the HPLC analysis. The full-line represents peak 2 (canthaxanthin) and the dashed-line represents peak 3 (echinenone).

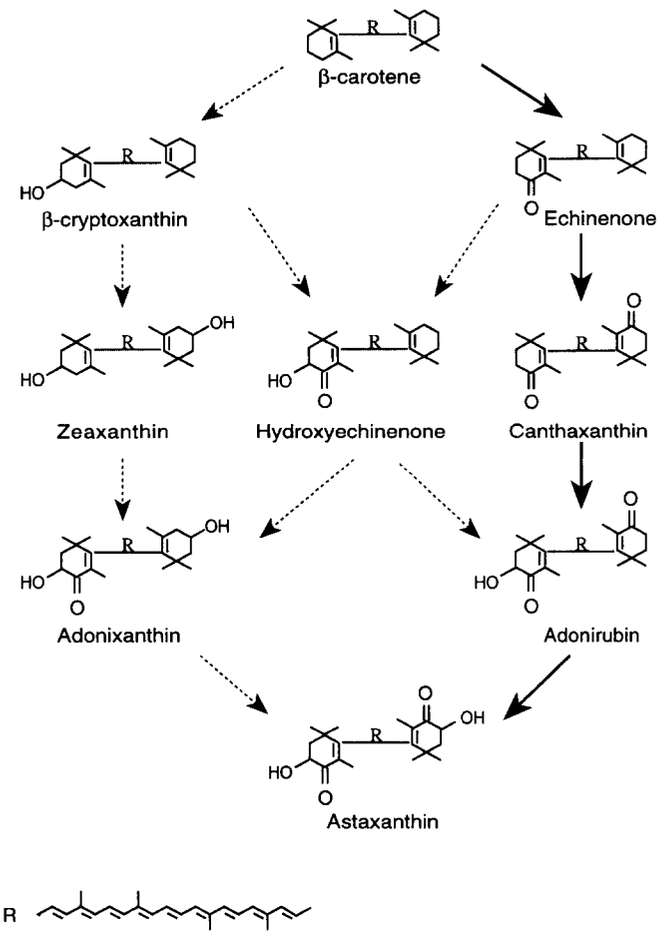

Fig. 4. Proposed pathway (solid arrows) for astaxanthin biosynthesis in *H. pluvialis*. Alternative pathways are indicated by broken arrows.

Carotenoids in the resulting transformed cells were extracted and analyzed by HPLC. The major carotenoid in these cells was zeaxanthin and no astaxanthin was found (data not shown). This result suggests that zeaxanthin is not a substrate for the β-C-4-oxygenase.

4. Discussion

The results presented in this work illustrate a unique strategy for cloning novel genes in the carotenoid biosynthesis pathway. This approach takes advantage of the ability of *E. coli* to synthesize carotenoids by expressing foreign gene and thus displaying a variety of colors. Using a complementation assay in *E. coli* cells between carotenogenic genes that produce a specific precursor and a foreign cDNA, it is possible to identify a new gene by screening for color changes in the host cells. A similar approach has been previously described for cloning of a prokaryotic carotenogenic gene [5].

The precise pathway by which astaxanthin is synthesized in *H. pluvialis* has not been elucidated, even though several hypotheses have been proposed [10,12,13]. It is believed that astaxanthin is formed from β-carotene. In algae it is synthesized as a (*3S,3'S*)-stereoisomer [14], whereas the yeast *Phaffia rhodozyma* produces the (*3R,3'*R)-stereoisomer [15]. It was therefore postulated that in the algae, the C-3 hydroxy groups are

introduced first, to give (*3R,3'R*)-zeaxanthin, into which the C-4-keto groups are then introduced [2]. We have previously observed (Fan et al., manuscript submitted for publication), that treatment with diphenylalamine (DPA) of *H. pluvialis* inhibits the synthesis of canthaxanthin and astaxanthin and leads to accumulation of β-carotene. Consequently, we have suggested that echinenone and canthaxanthin are bona fide intermediates in the conversion of β-carotene and that these two ketocarotenoids are likely to be synthesized by the same enzyme in *H. pluvialis*. Cloning the gene for β-C-4 oxygenase confirms this hypothesis. This is in contrast to the case of *P. rhodozyma*, where a keto group is introduced first to give echinenone, which is converted to 3-hydroxy-echinenone and adonirubin [15]. The presence of echinenone and canthaxanthin in two species of *Haematococcus* has been previously reported [16,17].

The enzyme β-C-4 oxygenase (β-carotene ketolase) catalyzes the conversion of β-carotene to canthaxanthin. This is a two-step reaction in which echinenone is an intermediate, as is indicated by the small amounts of this ketocarotenoid in the *E. coli* cells. We therefore conclude that the same enzyme carries out this reaction symmetrically on each half of the β-carotene molecule. It is predicted that an additional enzyme converts canthaxanthin to astaxanthin by hydroxylation reaction in the C-3 position. The finding that β-C-4 oxygenase does not convert zeaxanthin to astaxanthin by inserting keto groups in the C-4 position, support the hypothesis that astaxanthin is synthesized through canthaxanthin. The proposed pathway of astaxanthin biosynthesis is depicted in Fig. 4.

Astaxanthin is a carotenoid pigment of immense economical value. It is commercially used as food supplement in aquaculture of fish and other marine animals. It is also a desirable and effective non-toxic coloring for the food industry and is valuable in cosmetics. Natural (*3S,3'S*) astaxanthin is limited in availability and it is commercially extracted from some crustacea species. Cloning one of the two genes in the biosynthetic pathway of astaxanthin and expressing it in *E. coli*, paves the way for various biotechnological applications for the production of this ketocarotenoid.

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References

[1] Goodwin, T.W. (1980) The Biochemistry of Carotenoids, Vol. 1, Chapman-Hill, New York.
[2] Britton, G. (1988) in: Plant Pigments (Goodwin, T.W. ed.) pp. 133–180, Academic Press, Ney York.
[3] Armstrong, G.A. (1984) J. Bacteriol. 176, 4795–4802.
[4] Bartley, G.E., Scolnik, P.A. and Giuliano, G. (1994) Annu. Rev. Plant Physiol. 45, 287–301.
[5] Linden,H., Vioque, A. and Sandmann, G. (1993) FEMS Microbiol. Lett. 106, 99–104.
[6] Cunningham Jr., F.X., Chamovitz, D., Misawa, N., Gratt, E. and Hirschberg, J. (1993) FEBS Lett. 328, 130–138.
[7] Bouvier, F., Hugueney, P., Dharlingue, A., Kuntz, M. and Camara, B. (1994) Plant J. 6, 45–54.
[8] Boussiba, S. and Vonshak, A. (1991) Plant Cell Physiol. 32, 1077–1082.
[9] Kobayashi, M., Kakizono, T., Yamaguchi, K., Nishio, N. and Nagai, S. (1992) J. Ferment. Bioeng. 74, 17–20.
[10] Yong, Y.Y.R. and Lee, Y.K. (1991) Phycologia 30, 257–261.
[11] Tuveson, RW., Larson, R.A. and Kagan, J. (1988) J. Bacteriol. 170, 4675–4680.
[12] Droop, M.R. (1954) Arch. Mikrobiol. 20, 391–397.
[13] Johnson, E.A. and An, G.-H. (1991) Crit. Rev. Biotechnol. 11, 297–326.
[14] Andrewes, A.G., Borch, G., Liaaen-Jensen, S. and Snatzke, G. (1974) Acta Chem. Scand. B28, 730–736.
[15] Andrewes, A.G., Phaff, H.J. and Starr, M.P. (1976) Phytochemistry 15, 1003–1007.
[16] Donkin, P. (1976) Ketocarotenoid Biosynthesis by *Haematococcus pluvialis*, Phytochemistry 15, 711–718.
[17] Grung, M., D'Souza F.M.-L., Borowitzka, M. and Liaaen-Jensen, S. (1992) J. Appl. Phycol. 4, 165–171.