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ORIGINAL PAPER

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Cloning of the astaxanthin synthase gene from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) and its assignment as a β-carotene 3-hydroxylase/4-ketolase

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Abstract A gene has been cloned from *Xanthophyllomyces dendrorhous* by complementation of astaxanthin formation in a β-carotene accumulating mutant. It consists of 3,166 bp and contains 17 introns. For the β-carotene mutant ATCC 96815, a single point mutation in the splicing sequence of intron 8 was found. The resulting improper splicing of the mRNA results in an inactive protein. The cDNA of this β-carotene oxygenase encodes a cytochrome P450 monooxygenase belonging to the 3A subfamily. P450-specific domains were identified including a cytochrome P450 and an oxygen binding motif. Electrons are provided by a cytochrome P450 reductase. Functional characterization of the enzyme by genetic modification of *X. dendrorhous* demonstrated that this P450 monooxygenase is multifunctional catalyzing all steps from β-carotene to astaxanthin formation by oxygenation of carbon 3 and 4. The reaction sequence is first 4-ketolation of β-carotene followed by 3-hydroxylation. A hydroxylation mechanism at allylic carbon atoms has been proposed for the generation of 4-keto and 3-hydroxy groups at both β-ionone ends.

Keywords Astaxanthin synthase · Cytochrome P450 · Genetic complementation · Monooxygenase

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Introduction

The basidiomycetous yeast *Xanthophyllomyces dendrorhous*, the sexual state of *Phaffia rhodozyma* (Golubev 1995), accumulates substantial amounts of astaxanthin (Andrewes et al. 1976) which due to its antioxidative function protects this fungus against singlet oxygen. Mainly the all-*trans* isomer is found together with small amounts of 9-*cis* and 13-*cis* (Visser et al. 2005). In contrast to astaxanthin from all other natural sources, the compound from *X. dendrorhous* has the 3R,3′R-configuration (Andrewes and Starr 1976). This ketocarotenoid is of commercial interest especially as feed additive in salmon and trout farming (Johnson and An 1991). *X. dendrorhous* has a high potential as a natural production system for astaxanthin (Sandmann and Misawa 2002). Certain physiological conditions regulate the biosynthesis of astaxanthin. They include singlet oxygen (Schroeder and Johnson 1995) and light (An and Johnson 1990). Due to the development of appropriate molecular genetic techniques, carotenogenesis can be improved in *X. dendrorhous* by metabolic engineering of the pathway (Verdoes et al. 2003). This approach may be useful to complement classical strain optimization by mutagenesis and positive selection against oxidative agents and other compounds (Johnson and An 1991). So far, it is possible to engineer the early steps of the carotenoid biosynthetic pathway in *X. dendrorhous* for increased metabolic channeling of metabolites into carotenogenesis and decreased formation of 3-hydroxy-4-keto-torulene, a byproduct formed alternatively to astaxanthin (Verdoes et al. 2003).

The carotenoid biosynthetic pathway of *X. dendrorhous* is well understood to the stage of β-carotene formation. For this section of the pathway, only two genes are responsible (Sandmann and Misawa 2002). Both genes have been cloned and their functions were established. One is a fusion gene encoding a phytoene synthase and a lycopene cyclase (Verdoes et al. 1999a). The other is the gene for a phytoene desaturase (Verdoes et al. 1999b). Its product catalyzes the introduction of four double bonds into phytoene yielding all-*trans* lycopene as the reaction product. However, very little is known about the final steps involving the introduction of 3-hydroxy and 4-keto groups at both ionone end groups. But first, attempts have been made to clone a gene involved in the late reactions of astaxanthin biosynthesis (Hoshino et al. 2000). Identification of pathway intermediates between β-carotene and astaxanthin gave no definite information on the establishment of the reaction sequence (Sandmann and Misawa 2002). In the green algae *Haematococcus pluvialis*, the most likely succession is ketolation first followed by the hydroxylation reaction (Breitenbach et al. 1996). The ketolase genes known to date are quite homologous regardless of their pro- or eukaryotic origin (Kajiwara et al. 1995). In contrast, β-carotene 3-hydroxylase genes are much more diverse. They include the cyanobacterial *crtR* closely related to the ketolase gene (Masamoto et al. 1998) and *crtZ* found in many other bacteria. The gene *crtZ* corresponds to the plant type β-carotene 3-hydroxylase gene *bhy*. It contains four conserved histidine motifes for iron binding which are typical for iron-containing monoxygenases (Bouvier et al. 1998). Very recently, a P450 monooxygenase from *Thermus thermophilus* was identified as a β-carotene 3-hydroxylase (Blasco et al. 2004).

In the present investigation, the cDNA and gene for an enzyme was cloned from *X. dendrorhous* which catalyzes the oxidation of β-carotene to astaxanthin. The gene function was elucidated and a mechanism for the synthesis of its product established.

Materials and methods

Strains and their cultivation

Xanthophyllomyces dendrorhous strain CBS6938 (=ATCC96594) and the related β-carotene producing mutants PR-1-104 (Girard et al. 1994) and ATCC96815 (described as Yan-1, Schroeder and Johnson 1995) were grown in 30 ml of YPD broth (20 g/l bacto-peptone, 10 g/l yeast extract, and 20 g/l glucose) in 250-ml Erlenmeyer flasks at 21°C on a rotary shaker for 96 h. To select for G418 resistance, 40 μg/ml of geneticin (G-418 sulfate, Invitrogen Life Technology) were added. *Escherichia coli* strains DH5α, JM101 and JM110 were grown on LB medium. Antibiotics were added in the following concentration when appropriate, ampicillin (100 μg/ml), kanamycin (25 μg/ml) and chloramphenicol (34 mg/l) (Sambrook et al. 1989). In the complementation experiment, *E. coli* cultures were supplemented with 10 μM hemine and 0.5 mM δ-aminolevulinic acid.

Construction of genomic DNA and cDNA libraries from *X. dendrorhous* ATCC96594

For the isolation of genomic DNA, cells from an overnight culture of *X. dendrorhous* ATCC96594 were harvested and disrupted by enzymatic lysis using lyticase (2 mg/ml, Sigma, St. Louis, USA). DNA extraction and purification were carried out with the Qiagen Genomic Kit. Prior to the construction of a genomic library, a plasmid harboring a drug resistant marker cassette was constructed by inserting a G418 resistance gene between the promoter and terminator region of the *X. dendrorhous* glyceraldehyde-3-phosphate dehydrogenase encoding gene and ligating this cassette into the *Kpn*I- and *Hin*dIII-digested pUC19. Then, a *Cla*I linker was ligated into the unique *Eco*RI site of this vector. The resulting plasmid was used as a vector for the construction of the *X. dendrorhous* genomic library. The chromosomal DNA was partially digested with *Hpa*II and separated by agarose gel electrophoresis. Partially digested DNA fragments from 4 to 10 kb were recovered and ligated into the *Cla*I-digested cloning vector mentioned above. The genomic DNA library of 20,000 individual clones was amplified in *E. coli* DH5α.

Total RNA was isolated from glass beads disrupted *X. dendrorhous* ATCC96594 cells by phenol extraction and mRNA was purified using an mRNA separation kit (Clontech, Palo Alto, USA). cDNA was synthesized with the Smart cDNA Construction Kit (Clontech). A cDNA library was constructed with the Copy Kit (Invitrogen, Carlsbad, USA). After ligation of an *Eco*RI adaptor (Stratagene), synthesized cDNA was fractionated by agarose gel electrophoresis. The collected cDNAs from 1.9 to 2.3 kb were purified by Qiaex II (Qiagen) and ligated into the *Eco*RI-digested and dephosphorylated lambda ZAPII vector (Stratagene). The overnight ligation mixture was in vitro packed with Gigapack III gold packaging extract (Stratagene) and used to infect an *E. coli* XL1-Blue MRF' strain. All molecular techniques for gene cloning and plasmid constructions were used, according to Sambrook et al. (1989).

Library screening for the astaxanthin synthase gene and cDNA

The genomic DNA library was screened for the astxanthin synthase gene by transformation into the yellow β-carotene producing *X. dendrorhous* mutant ATCC96815 and visual screening for red colonies. The integrated plasmids were rescued from several red

transformants by digestion of their chromosomal DNA with *Hin*dIII, self-ligation and transformation to *E.coli* DH5α. The 0.7 and 2.7 kb *Eco*RI fragments which were obtained from the insert of the rescued plasmid were used as probes to screen the cDNA library. One plaque which strongly hybridized to these probes was selected for plasmid excision.

The Universal Genome Walker Kit (Clontech) was used to determine the complete genomic nucleotide sequence of the gene. The complete astaxanthin synthase gene was amplified from ATCC96815 by PCR. The primers used in both cases are listed in Table 1.

Construction of plasmids for *X. dendrorhous* and *E. coli* transformation

The plasmid pPR2TN2BPAT for the transformation of *X. dendrorhous* was constructed by overlapping PCR of the promoter and terminator from the glyceraldehyde-3-phosphate dehydrogenase gene from pPR2TN (Verdoes et al. 2003) and the full length astaxanthin synthase cDNA (1,674 bp including the stop codon). For the transformation of mutant PR-1-104 with the *crtZ* and *bkt* gens, an expression vector pPRcDNA1 was constructed by creating an expression cassette consisting of a *Not*I site, the *X. dendrorhous gpd* promoter (P*gpd*), an *Eco*RI site, an 8 bp spacer, a *Xho*I site, the *X. dendrorhous gpd* terminator and a *Hind*III site by overlapping PCR. Primer sets PGPD1- PGPD2 and TGPD1-TGPD2 were applied in a PCR using pPR2TN as a template in order to amplify the P*gpd* and T*gpd* sequences respectively. Both fragments were fused and the product amplified. The resulting expression cassette was sub-cloned in pGEM-T-Easy. pPR2TN was digested with *Eco*RI and *Kpn*I and the resulting 4.5 kbp fragment ligated to the 1.4 kbp *Eco*RI-*Kpn*I rDNA fragment. Then the plasmid was digested with *Eco*RI, treated with Klenow polymerase and religated in order to remove the *Eco*RI site. Finally, the expression cassette was inserted in the *Not*I and *Hind*III sites of the rearranged pPR2TN vector.

For the construction of pPRcDNA1bkt830, a shortened version of the *Haematococcus pluvialis* β-carotene ketolase (*bkt*) cDNA was amplified using plasmid pUC19BKT as the template (Breitenbach et al. 1996). Subsequently, the truncated *bkt* cDNA was ligated in the *Eco*RI and *Xho*I sites of vector pPRcDNA1.

Due to the presence of an *Eco*RI site in the β-carotene hydroxylase gene (*crt*Z) of *Erwinia uredevora*, no direct cloning into vector pPRcDNA1 was possible. Therefore, overlapping PCR was performed in which the P*gpd* sequence was connected to the *crt*Z cDNA with pPR2TN and pACCAR25 (Misawa et al. 1990), respectively as templates and the primer shown in Table 1. Subsequently, the P*gpd-crt*Z fusion product was ligated in the *Not*I and *Xho*I sites of vector pPRcDNA1 resulting in plasmid pPRcDNA1crtZ.

For the construction of the astaxanthin synthase gene disruption plasmid, vector pPR1T (Visser et al. 1999) was used as backbone. The first 1,381 bp of the 1,671 bp open reading frame, deleting 290 bp of its 3′-end, were obtained as an *Eco*RI fragment. This fragment was inserted into the *Eco*RI-digested and dephosphorylated pPR1T resulting in plasmid FGHV108. The 5′ end of the ORF (475 bp) along with 225 bp of pPR1T vector sequence were removed from FGHV108 using restriction enzymes *Nde*I and *Sal*I. After blunting of the ends of the

Table 1 Primers used for the PCR amplification of various DNA fragments

Steps	Primers
asy Genomic walking	TAGAGAGAAGGAGGGGTACCAGATGC (5′-adjacent primer) CCCCGGATTGTGGAGAAACT (3′-adjacent primer)
Amplification of asy gene	ATGTTCATCTTGGTCTTGCT (forward) ACGTAGAAGTCATAGCGCCT (reverse)
Construction of pPR2TN2BPAT	P1Prom: CTA GTA ACG AAA GCT TGG TAC P2Prom: CAA, GAT, GAA, CAT GAT GGT AAG AGT GTT AGA G P3Ast: CTC TTA CCA TCA TGT TCA TCT TGG TCT TGC BP4AST: GGA GAG ATC CTC ATT CGA CCG GCT TGA BP5Term: CGG TCG AAT GAG GAT CTC TCC AAA CCC TC P6Term: CAG CTA TGA CCA TGA TTA CGG
pPRcDNA1	PGPD1: 5′-ATATTGCGGCCGCTGGTGGGTGCATGTATGTAC-3′ PGPD2: 5′-CTCGAGAGTCAGCTGAATTCGATGGTAAGAGTGTTAGAGA-3′ TGPD1: 5′-GAATTCAGCTGACTCTCGAGCTCCAAACCCTCTC-3′ TGPD2: 5′-AGCTAAGCTTCTTGATCAGATAAAGATAGAGAT-3′
pPRcDNA1crtZ	PGPDCRTZA:CCAAATCCACAACATGATGGTAAGAGTGTTAGAGA PGPDCRTZS: AACACTCTTACCATCATGTTGTGGATTTGG PCRTZSTOP: TATACTCGAGTTACTTCCCGGATGCGGG
pPRcDNA1bkt830	ATATGAATTCATGCACGTCGCATCGGCACT (forward) TATACTCGAGTCATGCCAAGGCAGGCACCAG (reverse)
pBBR-asy	GTA TCG ATA TTA AGA TAT C (forward) M13 reverse primer (reverse)
pUC8-1-YPR	CCCGGGATGCCGTTTGGAATAGACAA (forward) GCGGCCGCTTACCAGACATCTTCTTGGT (reverse)

linear plasmid, it was closed by selfligation. The resulting plasmid contains a truncated version of the astaxanthin synthase ORF, missing 475 and 290 bp at the 5′- and 3′-ends, respectively. It was named pPR1TΔastNC and used as a linear *Sfi*I digested vector to disrupt the astaxanthin synthase gene by intergration in the *X. dendrorhous* endogenous *asy* gene.

Plasmid pBBR-asy for complementation in *E. coli* was constructed by PCR amplification of the astaxanthin synthase cDNA creating a *Hin*dIII and *Sac*II restriction site. Both were used for an in-frame ligation of the cDNA into the *Hin*dIII/*Sac*I-digested plasmid pBBR1-MCS2 (Kovach et al. 1995). Plasmid pUC8-1-YPR contained the cDNA of the NADH-cytochrome P450 reductase from yeast (Yabusaki et al. 1988; Accession No. P16603). The latter was amplified from *Saccharomyces cerevisiae* (strain BJ2168, Nippon Gene) DNA by PCR. The resulting 2.09 kb fragment was ligated into *Pst*I/*Not*I-digested pBluescript SK (−). After *Pst*I/*Sac*I digestion of the latter, the recovered insert was ligated into pUC8-1 (Hanna et al. 1984). pUC8-1 was first *Hin*dIII-digested, blunted and then *Pst*I-digested. All primers used in plasmid constructions can be found in Table 1. The pACYC184-derived plasmid pACCAR16ΔcrtX was previously described (Misawa et al. 1995).

Transformation of *X. dendrorhous*

It was necessary to employ two different transformation procedures depending on the experiment. Biolistic transformation with 0.9 μm gold particles according to the method described by Johnston and DeVit (1996) was advantageous for library screening due to the high transformation efficiency of up to 4,000 transformants per μg of DNA. Specific transformations, e.g. to accomplish gene disruption or to introduce foreign genes were conducted by means of electroporation (efficiency close to 1,000 transformants per μg of DNA). The details on an optimized electroporation protocol were recently published by Visser et al. (2005). The β-carotene producing mutant ATCC96815 was advantageous for the library screening whereas mutant PR-1-104 with the same phenotype was routinely used for genetic modification of the carotenoid pathway.

Carotenoid extraction and HPLC analysis

Carotenoids were extracted from freeze-dried cells by heating in DMSO for 15 min at 60°C (Visser et al 2005). They were partitioned first into ether and then into 10% petrol in ether. After drying in a stream of N_2, carotenoids were re-suspended in acetone and separated by HPLC. The system used involved a HyPurity C18 5 μm column with acetonitrile/methanol/2-propanol (85:10:5, by volume) as the mobile phase for isocratic elution at a column temperature of 32°C (Steiger and Sandmann 2004). Detection was with a Kontron DAD 440 photodiode array detector with on-line registration of the spectra. Peaks were identified with authentic standard which were generated by multiple expression of carotenogenic genes in *E. coli* (Sandmann 2002). Carotenoids were quantitated by integration of the corresponding HPLC peaks and calibration. Details of the analytical methods are given in Visser et al. (2005). Polar carotenoids from the *E. coli* complementation experiment were separated from β-carotene and enriched on a silica gel column after absorption in 0.3% acetone and elution with 100% acetone (Steiger et al. 1999).

Results and discussion

Isolation and characterization of the astaxanthin synthase gene and cDNA sequences

An *X. dendrorhous* genomic library was screened for a gene involved in the biosynthetic pathway from β-carotene to astaxanthin. The screening strategy used was to complement the biosynthetic conversion in a yellow β-carotene accumulating *X. dendrorhous* mutant strain ATCC96815 (Schroeder and Johnson 1995) towards astaxanthin formation. After transformation of this host with the genomic library, several thousands of geneticin-resistant transformants were obtained. Most of these transformants exhibited the yellow color of the recipient mutant but three of them were red-pigmented by astaxanthin (data not shown). These colonies were isolated and the inserted DNA was rescued. All red transformants had the same insert, yielding 0.7 and 2.7 kb DNA fragments after *Eco*RI digestion. The nucleotide sequence of the gene of 3,166 bp that restores the phenotype of wild type *X. dendrorhous* is is deposited in the data base under accession number AX034666. We named the gene *asy* for astaxanthin synthase.

The 2.7 and 0.7 kb *Eco*RI fragments of the genomic clone described above were used as probes to screen 6000 plaques from the λ ZAP cDNA library. The plasmid DNA from one plaque that strongly hybridized to these probes was isolated. The nucleotide sequence of the cDNA present in this plasmid was determined and analyzed. A 1,674 open reading frame (ORF) related to the genomic sequence was found. A sequence consisting of 557 amino acid corresponding to a molecular mass of 62.63 kD can be deduced. Comparison of the cDNA sequence to that of the genomic copy of the putative *asy* gene revealed that the *asy* gene contains 18 exons and 17 introns. They are more or less equally distributed within the gene. Their arrangement is outlined in Fig. 1a. This high number of introns is typical for the genes of *X. dendrorhous* including genes involved in carotenogenesis (Verdoes et al. 1999a, b). After PCR amplification of this gene from the yellow mutant ATCC96815, a single base exchange was found in the splicing sequence of the eighth intron as indicated in Fig. 1b. This suggests that the improper splicing of the astaxanthin synthase

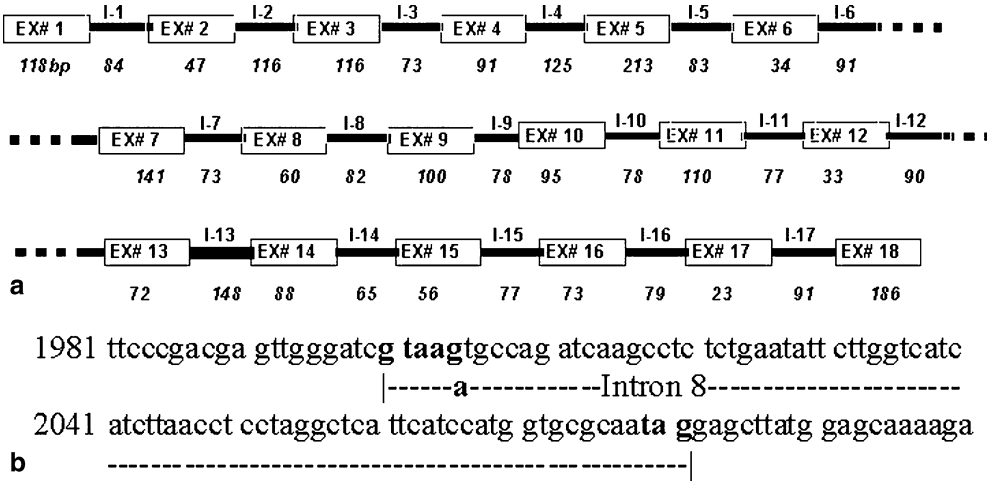

Fig. 1 Arrangement of exons and introns in the astaxanthin synthase gene from *Xanthophyllomyces dendrorhous* (**a**) and localization of the point mutation g –> a in the splicing region of intron 8 of the *β*-carotene mutant ATCC 96815 (**b**). The splicing region is shown in **bold**

mRNA resulting in an inactive enzyme is responsible for the *β*-carotene phenotype. A blast search using the amino acid sequence indicated that the *X. dendrorhous* astaxanthin synthase is a cytochrome P450 belonging to the cytochrome P450 3A subfamily. The best similarities to other proteins were for fungal cytochrome P450s from *Cryptococcus neoformans* (36% identity) and *Ustilago maydis* (35% identity).

Several highly conserved regions can be found in comparison with various animal cytochrome P450s (Fig. 2). They include helices D, E, I, J K and L especially in the C-terminal region (Gotoh and Fujii-Kuriyama 1989). The heme binding site FISGPRA of a cytochrome P450 can also be identified within the L-helix (Fig. 2) as well as the oxygen binding pocket (Danielson et al. 1997) DGVVT close to the D-helix (both highlighted in bolt). The N terminal region is highly hydrophobic which is characteristic for membrane-bound P450 proteins. Its function is discussed as a membrane-anchor signal (Sakaguchi et al. 1987). The sequence of *asy* cDNA is deposited under accession no. AX034665.

Functional assignment of Asy as a cytochrome P450 enzyme

The isolated cDNA was used to construct vector pPR2TN2BPAT for integration into and complementation of the *β*-carotene-accumulating mutant PR-1-104 (Girard et al. 1994). The carotenoid compositions of the recipient strain and the transformant PR-1-104[pPR2TN2BPAT] are shown in Fig. 3. PR-1-104 accumulates mainly the all-*trans* form and 9-*cis* and 13-*cis* isomers of *β*-carotene. In addition, small amounts of neurosporene, torulene and *γ*-carotene were present. The carotenoid pattern of the transformant PR-1-104[pPR2TN2BPAT] was completely changed (Fig. 3b). The major carotenoid was astaxanthin together with substantial amounts of phoenicoxanthin, 3-HO-4-keto-torulene and 3-HO-echinenone. Also traces of echinenone and *β*-carotene were detectable. This carotenoid composition generally resembles the carotenoids in *X. dendrorhous* wild type cultures (Fig. 3c, Andrewes et al. 1976). The restoration of astaxanthin biosynthesis in PR-1-104 (and also in ATCC96815, data not shown) demonstrates the involvement of the cloned gene in the pathway from *β*-carotene to astaxanthin as an oxygenase converting *β*-carotene as its substrate. As expected, an inactivation of the Asy cytochrome P450 encoding gene in wild type *X. dendrorhous* resulted in a *β*-carotene producing phenotype (Fig. 3f). However, it is not possible to draw a conclusion from these experiments regarding the type of oxidation reaction that modifies *β*-carotene. Depending on which genes are additionally involved, this Asy cytochrome P450 enzyme could function in three different ways in the wild type as outlined in Fig. 4. As one possibility, cytochrome P450 may function as a *β*-carotene 3-hydroxylase. Then, the putatively formed zeaxanthin (3,3′-dihydroxy-*β*-carotene) in the wild type should be converted by another ketolase. Alternatively, the P450 enzyme may act as a *β*-carotene ketolase forming canthaxanthin (4,4′-*β*-carotene), which is then metabolized to astaxanthin by an additional hydroxylase. As a third possibility, this Asy P450 enzyme may catalyze both the hydroxylation and the ketolation steps. All three alternatives can be tested by transformation of the yellow *β*-carotene accumulating strain PR-1-104 with either a hydroxylase or ketolase gene and analysis of the accumulating carotenoid.

Transformant PR-1-104[bkt] carries the integrated *β*-carotene ketolase gene from *H. pluvialis* (Breitenbach et al. 1996). HPLC analysis of the carotenoids (Fig. 3d) identifies the presence of ketolated carotenoids canthaxanthin, 4-ketotorulene and echinenone together with torulene and *β*-carotene. Transformant PR-1-104[crtZ] (Fig. 3e) carrying a bacterial *β*-carotene hydroxylase gene (Misawa et al. 1990) synthesized the hydroxy derivatives zeaxanthin and *β*-cryptoxanthin. Both transformants of PR-1-104 with either the *β*-carotene ketolase or hydroxylase gene were unable to synthesize astaxanthin. Therefore, we can exclude that the Asy

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ast        118GDGVVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGES
rat           GKAVSVAKDEEWKRYRALLSPTFTSGRLKEMFPIIEQYGDILVKYLKQEAET--------
rabbit        KKAVSISEDEDWKRVRTLLSPTFTSGKLKEMLPIIAQYGDVLVKNLRQEAEK--------
dog           KSAISLSEDEEWKRMRTLLSPTFTTGKLKEMFPIIGQYGDVLVNNLRKEAEK--------
sheep         KNAVSVAEDEQWKRIRTLLSPTFTSGKLKDMFPIIGKYGDVLVRNLRKEAEK--------
human3A3      KSAISIAEDEEWKRLRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNLRRERET--------
human3A4      KSAISIAEDEEWKRLRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNLRREAET--------
human3A7      KNAISIAEDEEWKRIRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNLRREAET--------
human3A5      KSAISLAEDEEWKRIRSLLSPTFTSGKLKEMFPIIAQYGDVLVRNLRREAEK--------
               ..:  ::.*  ** * :: *::::  :*.*.**: : *  **  : .:
                       <<<<< D-helix >>>>>

ast           AGEKKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAP
rat           ----------GKPVTMKKVFGAYSMDVITSTSFGVNVDSLNNPKDPFVEKTKKLLR----
rabbit        ----------ASPSTLKEIFGAYSMDVITGTSFGVNIDSLRNPQDPFVKNVRRLLK----
dog           ----------GKAINLKDVFGAYSMDVITSTSFGVNIDSLNHPQDPFVENTKKLLK----
sheep         ----------GKSVNMKDIFGAYSMDVITSTSFGVNIDSLGNPQDPFVENAKKLLR----
human3A3      ----------GKPVTLKDVFGAYSMDVITSSSFGVNVDSLNNPQDPLVENTKKLLR----
human3A4      ----------GKPVTLKDVFGAYSMDVITSTSFGVNIDSLNNPQDPFVENTKKLLR----
human3A7      ----------GKPVTLKHVFGAYSMDVITSTSFGVSIDSLNNPQDPFVENTKKLLR----
human3A5      ----------GKPVTLKDIFGAYSMDVITGTSFGVNIDSLNNPQDPFVESTKKFLK----
                        .   :*. .*  ::**:: :.*. . *** :  : :      :
                                   <<<<<< E-helix >>>>>>

ast           TLDSFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDK
rat           -FDFFDPLFLSVVLFPFLTP----IYEMLNICMFPKDSIEFFKKFVYRMK----ETRLDS
rabbit        -FSFFDPLLLSITLFPFLTP----IFEALHISMFPKDVMDFLKTSVEKIK----DDRLKD
dog           -FDFLDPFFFSILLFPFLTP----VFEILNIWLFPKKVTDFFRKSVERMK----ESRLKD
sheep         -FNILDPFLLSVVLFPFLVP----IFEVLNITMFPKSAVDFLTKSVKRIK----ESRLKD
human3A3      -FDFLDPFFLSITVFPFLIP----ILEVLNICVFPREVTNFLRKAVKRMK----ESRLED
human3A4      -FDFLDPFFLSITVFPFLIP----ILEVLNICVFPREVTNFLRKSVKRMK----ESRLED
human3A7      -FNPLDPFVLSIKVFPFLTP----ILEALNITVFPRKVISFLTKSVKQIK----EGRLKE
human3A5      -FGFLDPLFLSIILFPFLTP----VFEALNVSLFPKDTINFLSKSVNRMK----KSRLND
               :. :..:. ..  :         :     : :  :   .::      :     .  :..

ast           KDVQGRDILSLLVRAN-IAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLS
rat           VQKHRVDFLQLMMNAHNDSKDKESHTALSDMEITAQSIIFIFAGYEPTSSTLSFVLHSLA
rabbit        KQKRRVDFLQLMINSQ-NSKEIDSHKALDDIEVVAQSIIILFAGYETTSSTLSFIMHLLA
dog           KQKHRVDFLQLMINSQ-NSKEMDTHKALSDLELVAQSIIFIFAGYETTSTSLSFLMYELA
sheep         NQKPRVDFLQLMINSQ-NSKETDNHKALSDQELMAQSVIFIFAGYETTSNTLSFLLYILA
human3A3      TQKHRVDFLQLMIDSHKNSKETESHKALSDLELVAQSIIFIFAGYETTSSVLSFIMYELA
human3A4      TQKHRVDFLQLMIDSQ-NSKETESHKALSDLELVAQSIIFIFAGYETTSSVLSFIMYELA
human3A7      TQKHRVDFLQLMIDSQ-NSKDSETHKALSDLELMAQSIIFIFAGYETTSSVLSFIIYELA
human3A5      KQKHRLDFLQLMIDSQ-NSKETESHKALSDLELAAQSIIFIFAGYETTSSVLSFTLYELA
               :    *:*.*:: ::  : :      *.* *: **   ::*****.:*. *:: :: *:
                                          <<<<<<    I-helix    >>>>>>

ast           EDKAVQDKLREEICQIDTD--MPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDED
rat           THPDTQKKLQEEIDRALPNKAPPTYDTVMEMEYLDMVLNETLRLYPIGNRLERVCKKD--
rabbit        THPDVQQKLQEEIDTLLPNKELATYDTLVKMEYLDMVVNETLRLYPIAGRLERVCKKD--
dog           THPDVQQKLQEEIDATFPNKALPTYDALVQMEYLDMVLNETLRLYPIAGRLERVCKKD--
sheep         THPDVQQKLQEEIDATFPNKAPPTYDVLAQMEYLDMVVNETLRMFPIAVRLDRLCKKD--
human3A3      THPDVQQKLQEEIDAVLPNKAPPTYDTVLQMEYLDMVVNETLRLFPIAMRLERVCKKD--
human3A4      THPDVQQKLQEEIDAVLPNKAPPTYDTVLQMEYLDMVVNETLRLFPIAMRLERVCKKD--
human3A7      THPDVQQKVQKEIDTVLPNKAPPTYDTVLQLEYLDMVVNETLRLFPVAMRLERVCKKD--
human3A5      THPDVQQKLQKEIDAVLPNKAPPTYDAVVQMEYLDMVVNETLRLFPVAIRLERTCKKD--
               .  .*.*:::**    .:   .* * :  : **: .::*:**: * .   :* * **
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Fig. 2 Alignment of the deduced amino acid sequence of astaxanthin synthase from *Xanthophyllomyces dendrorhous* with other cytochrome P450s of the CYP3A type. Only the part with well-conserved regions is shown

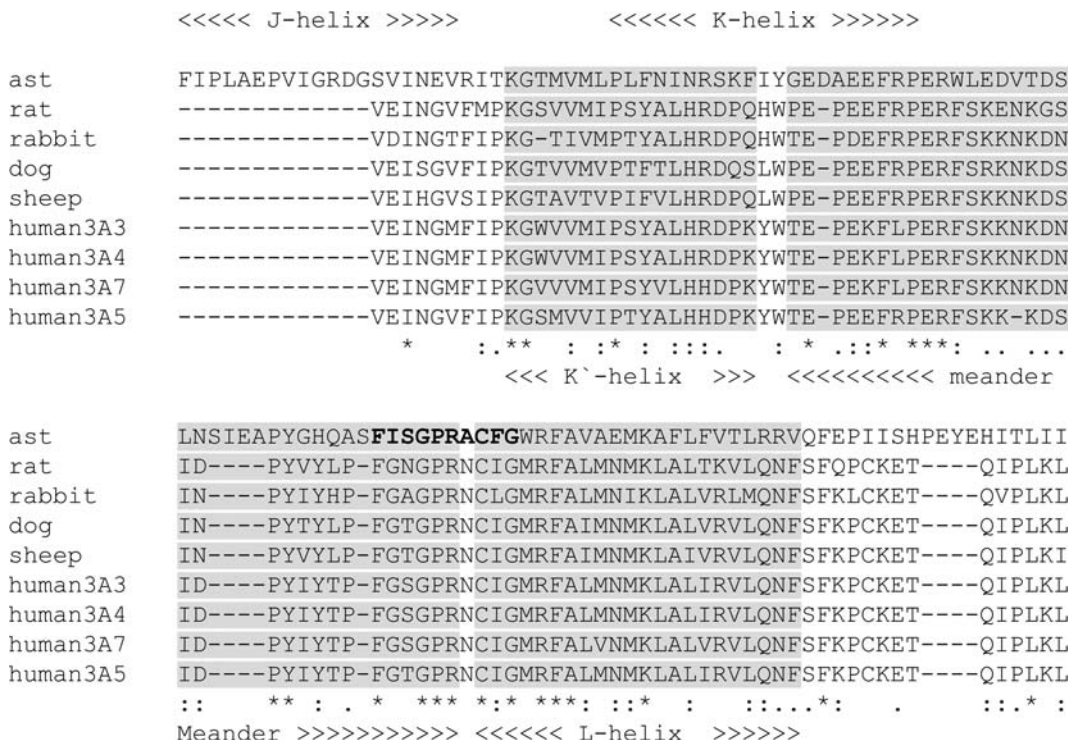

Fig. 2 (Contd.)

cytochrome P450 enzyme is either an individual ketolase or an individual hydroxylase. Since a foreign β-carotene ketolase does not complement a putative hydroxylase, nor a foreign β-carotene hydroxylase a putative ketolase in PR-1-104, Asy must comprise both functions. Therefore, we can define Asy as a β-carotene converting and astaxanthin forming synthase corresponding to alternative three of Fig. 4. All our data exclude the other possibilities.

Reaction sequence and mechanisms of astaxanthin formation in *X. dendrorhous*

The formation of astaxanthin from β-carotene by Asy can either start with hydroxylation or ketolation. The sequence of the reaction steps was investigated by complementation of β-carotene metabolisation in *E. coli*. For this purpose, *E. coli* was transformed simultaneously with three compatible plasmids as explained in Fig. 5a. One plasmid mediates the formation of β-carotene, another carries the *asy* gene from *X. dendrorhous* and the third plasmid the cytochrome P450 reductase from yeast (Yabusaki et al. 1988). Without a hemine and δ-aminolevulinic acid supplementation to the growth medium, no enzymatic conversion of β-carotene was observed (Fig. 5b). The only detectable carotenoid of an enriched polar fraction was β-carotene-5,6-epoxide, a degradation product of β-carotene with spectrum maxima at 425, 450 and 478 nm. The additions mentioned above support the formation of the heme prosthetic group in *E. coli* (Guengerich et al. 1996). When supplemented, *E. coli* carrying the *asy* gene synthesizes canthaxanthin from β-carotene (separation of enriched polar fraction, trace C). It was identified by the spectrum (bell-shaped with a maximum at 475 nm) and the retention time which was identical to a canthaxanthin standard (trace D). However, no hydroxylated keto derivatives including astaxanthin was formed. This may be due to the low astaxanthin synthase activity in *E. coli*. The conversion of β-carotene to canthaxanthin was estimated from the residual β-carotene in the non-polar fraction of around only 1%. This rate is quite low compared to canthaxanthin formation with other ketolase genes in *E. coli* (Breitenbach et al. 1996; Steiger and Sandmann 2004). If the 4-hydroxylation step has a similar affinity, the formed astaxanthin should be below detection. Removal of one of the three plasmids (Fig. 5a) abolished canthaxanthin formation.

Accumulation of low level intermediates of a reaction sequence can be achieved with appropriate inhibitors. Aminobenzotriazol is an inhibitor of cytochrome P450 enzymes (Danielson et al. 1997). We used a concentration of 0.1 mg/ml which inhibited about 90% of astaxanthin formation in wild type *X. dendrorhous* but has little effect on the total amount of carotenoids (Table 2). Nevertheless, the pattern of intermediates between β-carotene and astaxanthin changed. Especially, the relative amounts of non-hydroxylated keto carotenoids like echinenone, canthaxanthin and the

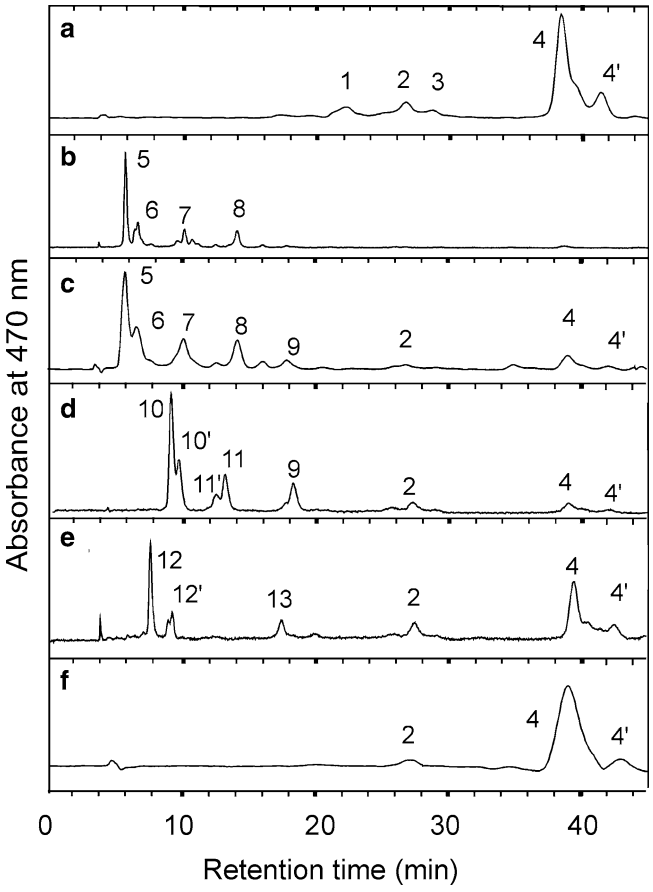

Fig. 3 HPLC analysis of carotenoids in *Xanthophyllomyces dendrorhous* transformants. **a** *β*-carotene mutant PR-1-104, **b** PR-1-104[pPR2TN2BPAT] transformed with *asy*, **c** ATCC96594 wild type, **d** PR-1-104[bkt] with a foreign *β*-carotene ketolase gene, **e** PR-1-1104[crtZ] with a foreign *β*-carotene hydroxylase gene and **f**. ATCC96594[pPR1TΔastNC] with deleted astaxanthin synthase gene. *1*, neurosporene; *2*, torulene, *3*, *γ*-carotene, *4*, *β*-carotene; *5*, astaxanthin; *6*, phoenicoxanthin; *7*, 3-hydroxy-4-ketotorulene; *8*, 3-hydroxyechinenone; *9*, echinenone; *10*, canthaxanthin; *11*, 4-ketotorulene; *12*, zeaxanthin and *13*, *β*-cryptoxanthin. 4′, 10′, 11′ and 12′ are the corresponding *cis* isomers

3-HO-4-ketotorulene precursor 4-ketotorulene increased. This was the case for both the dicyclic (upper part) and monocyclic (lower part) derivatives. Zeaxanthin could not be detected in the original carotenoid analysis of *X. dendrorhous* (Andrewes et al. 1976) nor

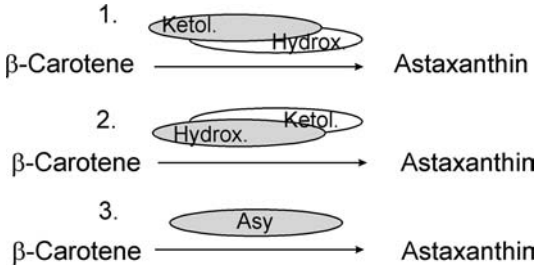

Fig. 4 Putative enzymatic function of the P450 *β*-carotene oxygenase (*shaded*) from *Xanthophyllomyces dendrorhous* as either a hydroxylase, a ketolase or an astaxanthin synthase

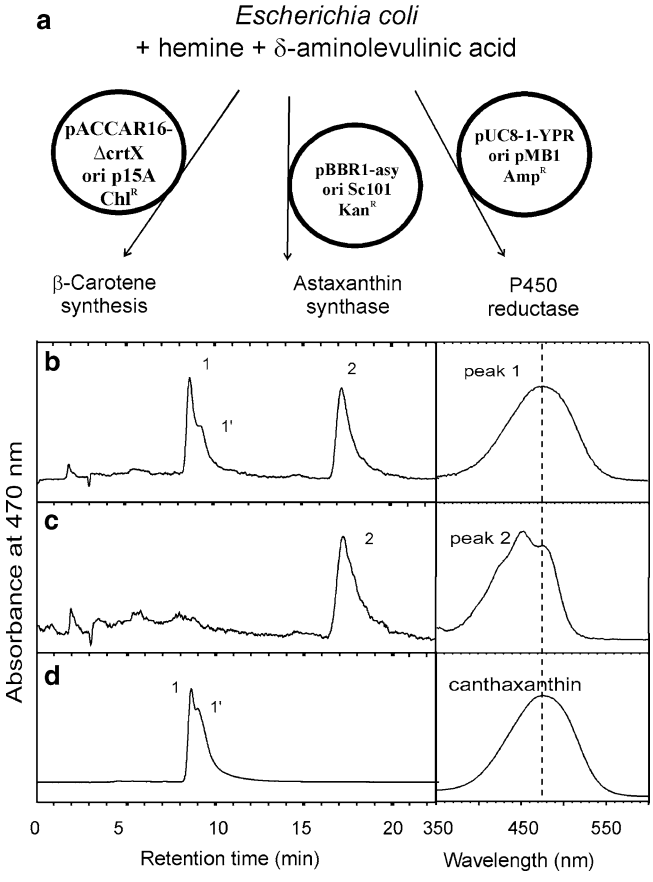

Fig. 5 Genetic complementation of *β*-carotene oxidation in *Escherichia coli*. **a** Combination of three simultaneously expressed plasmids including one with the *asy* gene from *Xanthophyllomyces dendrorhous*, HPLC analysis of enriched polar carotenoid products formed by *E. coli*/pACCAR16Δcrt/pBBR-asy/pUC8-1-YPR, **b** after feeding hemine + *δ*-aminolevulinic acid, **c** without this supplementation, **d** canthaxanthin as standard and spectral properties of the peaks

was it found later by other authors (Girard et al. 1994; Schroeder and Johnson 1995; Visser et al. 2005). It should be pointed out that no zeaxanthin accumulated upon Asy inhibition (Table 2). Its absence together with an increase of non-hydroxylated keto carotenoids like canthaxanthin and echinenone when astaxanthin synthase is strongly inhibited is another indication that *β*-carotene 4-ketolation is the initial reaction catalyzed by astaxanthin synthase.

The results on the function of astaxanthin synthase and the reaction sequence allows to postulate the mechanism for *β*-carotene oxidation to astaxanthin (Fig. 6). Astaxanthin synthase is a cytochrome P450 enzyme which obtains the electron for *β*-carotene oxygenation from a cytochrome P450 reductase (Fig. 6a). The reactions involve a series of hydroxylations of allylic carbons starting at C4. A putative enzyme recognition site for the first step could be the C5,6 double bond. C4 could be hydroxylated twice followed by spontaneous elimination of water forming a keto group (Fig. 6b). A similar reaction mechanism was recently proposed for a

Table 2 Carotenoid distribution (%) in *X. dendrorhous* ATCC96594 treated with aminobenzotriazole (ABT, 0.1 mg/ml)

Carotenoid	Number of oxygen groups		Carotenoid distribution (%)	
	4-O	3-HO	Control	+ ABT
β-Carotene	0	0	14.0	45.4
Echinenone	1	0	5.5	10.2
Canthaxanthin	2	0	0	2.2
3-Hydroxyechinenone	1	1	12.0	8.3
Phoenicoxanthin	2	1	3.4	3.7
Astaxanthin	2	2	42.0	3.3
Torulene	0	0	6.4	14.6
4-KetoT	1	0	0	4.8
3-HO-4-KetoT	1	1	16.8	4.5

The total carotenoid contents were 234 ± 17 for the control and 252 ± 24 μg/g dry weight for the aminobenzotriazole culture

unique 4-ketolase from *Adonis aestivalis* which evolved from a plant type β-carotene 3-hydroxylase (Cunningham and Gantt 2005). Next in the reaction sequence catalyzed by Asy from *X. dendrorhous*, carbon 3 in the 4-keto intermediate which becomes allylic by the keto group is recognized by the enzyme as the subsequent partner for hydroxylation. The reaction sequence outlined here will modify both β-ionone end groups of the symmetrical β-carotene molecule in the same way or the single β-ionone end group in torulene. Although the resulting 3-hydroxy and 4-keto derivatives of *X. dendrorhous* represent different reaction products, they originate from a single type of hydroxylation reaction which modifies similar structural allylic carbon atoms in the β-ionone ring. Thus, 4-ketolation in *X. dendrorhous* is a result of two hydroxylation reactions at carbon 4.

Astaxanthin formation occurs in a stereospecific way resulting in the 3R,3′R enantiomeric form in *X. dendrorhous*. Andrewes and Starr (1976) who determined this chirality suggested that the formation of 3R,3′R or 3S,3′S in other organisms like *H. pluvialis* is determined by different successions of ketolation and hydroxylation. Since in both organisms the ketolation is followed by hydroxylation (Fig. 6a; Breitenbach et al. 1996), it is more likely that the enantiomer formed depends on the type of enzyme involved in catalysis. In lutein, the 3-hydroxy ε-ionone group formed by cytochrome P450 at one end of the molecule and the 3-hydroxy β-ionone group introduced by an ion-containing oxygenase at the other end (Tian et al. 2004) are of opposite chirality (Britton 1988). We take this as an indication that the formation of 3R,3′R hydroxy groups in ionone end groups may be cytochrome P450-specific.

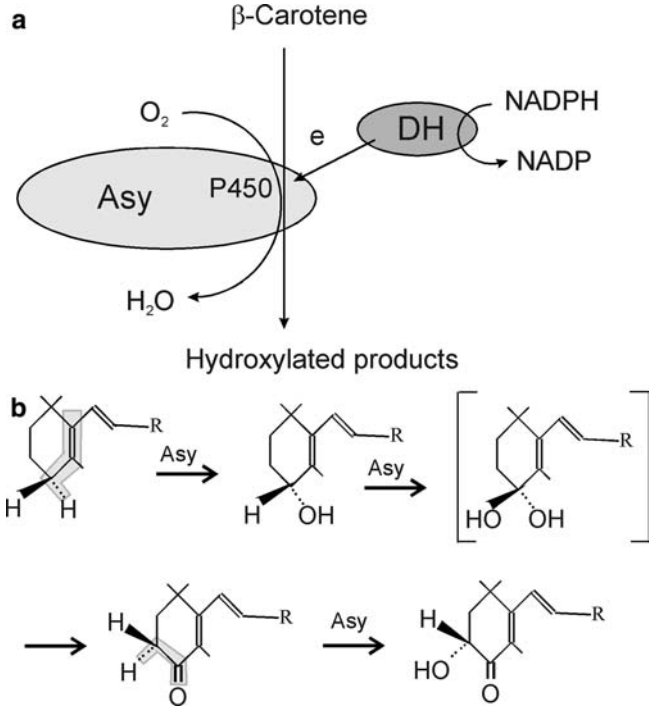

Fig. 6 β-Carotene oxidation by astaxanthin synthase in *Xanthophyllomyces dendrorhous*. **a** P450 mediated conversion (**a**) and mechanism of subsequent 4-ketolation and 3-hydroxylation (**b**). Putative minimum enzyme recognition sites are shaded in *grey*

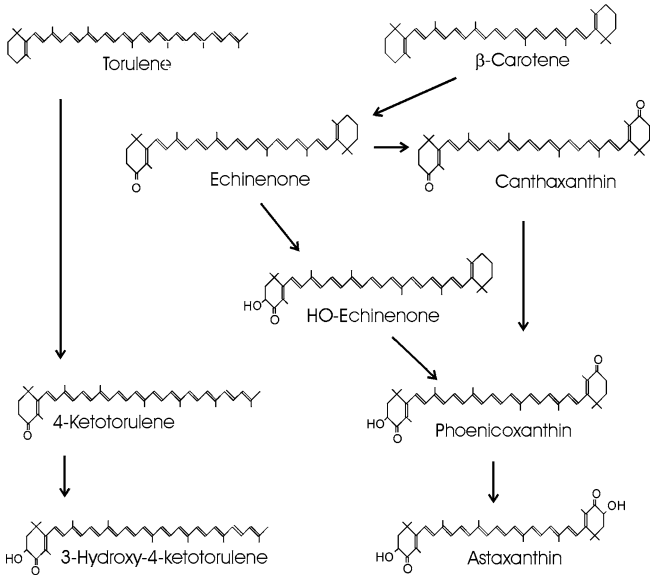

Fig. 7 Biosynthetic pathway from β-carotene to astaxanthin and torulene to 3-hydroxy-4-ketotorulene catalyzed by astaxanthin synthase in *Xanthophyllomyces dendrorhous*

Conclusion

A single gene product catalyses 4-ketolation and 3-hydroxylation of β-carotene to astaxanthin in *X. dendrorhous*. The gene and the cDNA of this unique CYP3A type cytochrome P450 has been cloned and the catalytic function of the enzyme elucidated. The corresponding enzyme is completely unrelated to other 4-ketolases (Kajiwara et al. 1995) and most 3-hydroxylases (Bouvier et al. 1998; Masamoto et al. 1998). The only known P450-type β-carotene hydroxylase is the enzyme from *Thermus thermophilus* (Blasco et al. 2004). Gene and cDNA of astaxanthin synthase are now available for the genetic modification of β-carotene accumulating microorganisms for astaxanthin production.

Based on the identification of keto and hydroxy intermediates (Fig. 3), heterologous complemenation experiments (Fig. 5) and inhibitor studies (Table 2), the biosynthetic pathway from β-carotene to astaxanthin has now been established (Fig. 7). It starts with ketolation of β-carotene to echinenone and can branch depending on 4′-ketolation or 3-hydroxylation as the next step. The resulting canthaxanthin or 3-HO-echinenone will then be hydroxylated or ketolated, respectively, to the same product phoenicoxanthin. This is the direct precursor to astaxanthin. The parallel formation of 3-HO-4-ketotorulene from torulene involves 4-ketotorulene as the only intermediate.

More than ten carotenoids can be identified in *X. dendrorhous*. Their biosynthesis involves only four enzymes, a phytoene synthase, a phytoene desaturase, a lycopene cyclase and a β-carotene oxygenase. These enzymes are encoded in total by only three genes. Thus, *X. dendrorhous* uses the most economical carotenoid biosynthetic pathway in comparison with *H. pluvialis* and astaxanthin forming bacteria.

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