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In vitro Assays of Three Carotenogenic Membrane-bound Enzymes from Escherichia coli Transformed with Different crt Genes

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In vitro assays have been developed for three membrane-bound carotenogenic enzymes, phytoene desaturase, lycopene cyclase and β-carotene hydroxylase, expressed in Escherichia coli. Transformants of E.coli containing different deletion constructs of the Erwinia herbicola carotenogenic gene cluster were employed, allowing the estimation of enzyme activities without interference from subsequent reactions. New HPLC systems were developed to separate substrates and reaction products enabling the determination of radioactivity on-line. The newly developed assays facilitate the purification of these enzymes which have never been isolated before.

Certain species of the Enterobacteriacea Erwinia herbicola are able to synthesize yellow pigments (1). These compounds were identified as cyclic carotenoids and their biosynthetic pathway leading to zeaxanthin and its glucosides has been established (2, 3).

Some years ago, a 12.4 kb gene cluster was cloned from E. herbicola which after transformation into Escherichia coli led to pigmented E. coli cells (4). On this fragment, six genes have been mapped and identified (5, 6). One approach used to characterize this fragment was the generatiion of deletion mutants and subsequent HPLC analysis of the different accumulating carotenoids. They were identified as phytoene, lycopene, and zeaxanthin (6). Three of these deletion mutants are used in the present study to perform in vitro carotenogenesis. Cell-free assays have been developed for all enzymes involved in the pathway from phytoene to zeaxanthin. They include phytoene desaturase, lycopene cyclase, and B-carotene hydroxylase. These enzymes are present in E. coli transformed with E. herbicola carotenoid genes. The use of the transgenic E. coli deletion mutants makes it possible to assay single enzymatic reactions of carotenoid enzymes which have never been isolated and purified before.

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MATERIALS AND METHODS

E. coli strain LE392 containing the different plasmids pPL376del16, pPL376del17, or pPL376del22 (6) was cultured on LB media (7). The C5 *carB10* (-), C9 *carR21* (-) and C115 *carS42mad-107* (-) mutant strains of *Phycomyces blakesleeanus* were maintained and grown as previously described (8).

E. coli cells were harvested from a dense overnight culture (10 ml) by centrifugation at 6000 g for 5 min. Pelleted cells were resuspended in ice cold 0.1 M Tris-HCl buffer pH 8.0, containing 5 mM DTT and 1 mM EDTA (1 ml). The resuspended cells were ruptured in a precooled French press using a hydraulic pressure of 50 bar. The resultant homogenate was treated with DNase (10 µg) for 20 min on ice, then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was discarded and the pigmented membrane fraction resuspended in 0.1 M Tris-HCl pH 8.0, containing 5 mM DTT, 1 mM EDTA (300 µl). An aliquot of this fraction was used to assess carotenogenic enzyme activity. *Phycomyces* mycelia were harvested after 4 days growth by filtration through two layers of muslin and subsequently lyophilized. Cell-free extracts were obtained by rubbing dried mycelia through a sieve (mesh size 355 µm) and the fine powder produced, stirred into a thick paste with the addition of 0.4 M Tris-HCl pH 8.0, containing 5 mM DTT (6 volumes). The resultant supernatant after centrifugation at 10,000 g was used as the cell-free preparation.

The assay mixture for phytoene desaturase contained 5 µmol ATP, 1 µmol NAD, 1 µmol NADP, 1 µmol FAD, 6 µmol $MnCl_2$, 4 µmol $MgCl_2$, 5 mM DTT, 5 µl 3-(R)-[2-^{14}C] sodium mevalonate (equivalent to 0.25 µCi 3-(R)-[2-^{14}C] MVA), buffered in 0.4 M Tris-HCl pH 8.0, *Phycomyces* C5 extract (150 µl; 2 mg protein) and an aliquot (150 µl) of the *del*16 mutant membrane fraction giving a final volume of 0.5 ml. 3-(R)-[2-^{14}C] Mevalonic acid (MVA) lactone with a specific radioactivity of 53 mCi/mmol was purchased from Amersham. The MVA lactone was converted to the sodium salt prior to use by addition of 50 µl of a 10 mM NaOH solution.

An identical assay mixture to that described for phytoene desaturase was used to determine lycopene cyclase activity, except a *Phycomyces* C9 extract and *del*22 membrane fraction were used. The β-carotene hydroxylase assay mixture contained 5 µmol ATP, 6 µmol $MnCl_2$, 4 µmol $MgCl_2$, 5 µl 3-(R)-[2-^{14}C] sodium mevalonate, 5 mM DTT, and either 0.25 mM 2-oxoglutarate, 0.25 mM $FeSO_4$, and 25 mM ascorbate, or a combination of NADPH, NAD and FAD, 1 µmol each, in 0.4 M Tris-HCl pH 8.0, *Phycomyces* C115 extract (150 µl) and the *del* 17 membrane fraction (150 µl), in a final volume of 0.5 ml. All assay mixtures were incubated at 35°C in dim light for 3 h.

Reactions were terminated with methanol (1.5 ml), and may be stored at -20°C, if necessary. Alternatively, petroleum ether (b.p. 40-60°C; 3 ml) and authentic carotenoid standards were added to the incubation mixture, mixed thoroughly and then partitioned into two phases by centrifugation at 3000 rpm for 3 min. The lipid-containing epiphase was removed and the aqueous hypophase re-extracted twice. The combined epiphases were evaporated to dryness under nitrogen. Dried extracts were resuspended in acetone (20 µl) and separated by HPLC. Carotenes were purified with a reversed-phase Nucleosil C_{18} 3 µm column (250 x 4.6 mm) using an isocratic mobile phase comprised of ethyl acetate/acetonitrile/water (35:60:5 by vol). Xanthophylls were separated on a normal-phase Spherisorb silica 3 µm column (250 x 4.6

mm), eluted stepwise with hexane for approximately 14 min, followed by 20% (v/v) ethyl acetate in hexane and finally after 34 min 50% (v/v) ethyl acetate in hexane. A flow rate of 1 ml min^{-1} was used. The absorbance of the standard carotenes was recorded with a programmable Jasco UV/visible photospectrometric detector model 8201. Radioactivity was monitored and quantified by an on-line radioactivity flow detector (Raytest Ramona LS). Comparison between optical absorbance and radioactivity traces enabled effective identification by co-chromatography.

RESULTS AND DISCUSSION

Several bacterial *in vitro* carotenogeic systems have been described (9) of which *Flavobacterium* has been studied most comprehensively. Using a crude preparation an overall overall conversion of ^{14}C-labeled mevalonic acid through the whole biosynthetic chain to the xanthophyll zeaxanthin was reported (10). In the present study, conditions were established for the assay of the three terminal enyzmes involved in this reaction sequence. In order to determine the activity of single enzymatic reactions, the ^{14}C-labeled substrates phytoene, lycopene, and B-carotene were provided in a coupled assay by which the radioactive substrate carotenes were generated *in situ* by extracts from the different *Phycomyces* mutants C5, C9, and C115 which utilised ^{14}C-MVA as the initial substrate. The principle of this coupled assay procedure has been described previously (11, 12).

To facilitate the separation of reaction products from their substrates and circumvent interference from other ^{14}C-compounds produced during substrate formation new HPLC conditions were established. Figure 1 shows the HPLC separation of carotene extracts from terminated phytoene desaturase and lycopene cyclase assay on the reversed-phase system. The separation of unlabeled standard carotenes used in the identification of radioactive peaks is shown in trace A. The all-*trans* isomers of lycopene, γ-carotene, ξ-carotene, and B-carotene chromatograph at 21, 27, 29, and 34 min, respectively. The absorbance wavelength on the detector was descreased from 440 nm to 350 nm after 35 min, and after 40 min to 285 nm. This allows the detection of phytofluene at 37 min and of 15-*cis* and all-*trans* phytoene at 44 and 47 min, respectively.

In a control experiment of the phytoene desaturase reaction, the membranes from mutant *del*16 were omitted. In this trace only the radioactivity peaks of the substrate phytoene (both isomers) together with the peak of squalene at 23 min, which is a contamination produced by *Phycomyces* C5 and prenylalcohols resulting from the hydrolyses of

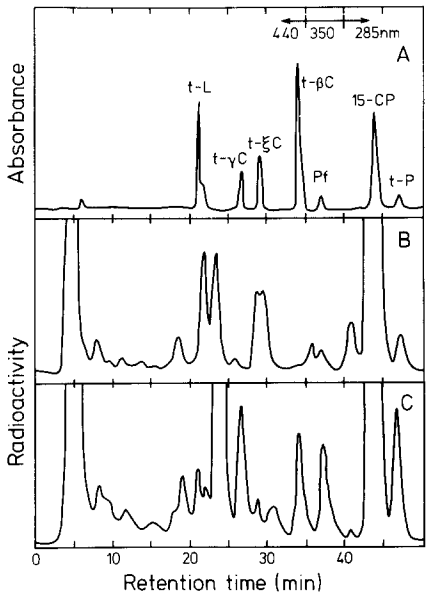

Fig. 1.
HPLC separation of radiolabeled carotenes from the phytoene desaturase (B) and the lycopene cyclase assay (C). Trace A shows the position of all marker carotenes relevant for identification.
t = all-trans, 15c = 15-cis, L = lycopene, γC = γ-carotene, ξC = ξ-carotene, ßC = ß-carotene, Pf = phytofluene, and P = phytoene.

prenylphosphates were present (data not shown). The new compounds detected in the complete phytoene desaturase assay were identified as phytofluene, ξ-carotene, and lycopene (Fig. 1B). Trace C shows the carotenes found in the lycopene cyclase assay with membranes from del22. The additional radioactivity peaks corresponded to γ-carotene and ß-carotene. The HPLC conditions used enables routine and effective separation of lycopene from squalene, a problem that is often encountered with isocratic systems.

It has recently been shown by genetic complementation in E. coli that phytoene desaturase from Erwinia introduces 4 double bonds into the phytoene molecule (13). The end product was predominantly all-trans lycopene. This carotene isomer was also found as the most desaturated product in our assay (Fig. 1B). In addition, the intermediates of this desaturation sequence carried out by one gene product, phytofluene with one additional double bond and ξ-carotene with two additional double bonds, were also detectable in the in vitro assay. Lycopene cyclase is an enzyme which forms two ß-ionone rings at each side of the symmetrical lycopene molecule (14). In accordance to the function of this enzyme, the cell-free assay results in the formation not only of the bicyclic ß-carotene as end product, but also in the formation of monocyclic γ-carotene.

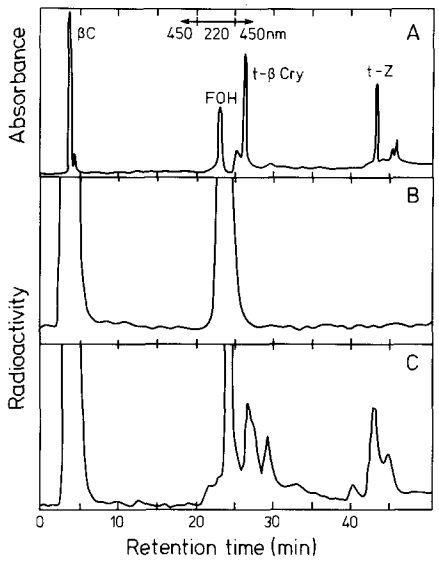

Fig. 2.
HPLC separation of radiolabeled carotenoids from the ß-carotene assay with NADPH/NAD/FAD (B) or oxoglutarate/Fe^{2+}/ascorbate (C) as co-factors. Trace A shows the position of all terpenoids relevant for identification.
FOH = farnesol, ßC = ß-carotene, t-ßCr = all-trans ß-cryptoxanthin, t-Z = all-trans zeaxanthin.

The radioactive compounds accumulating in the ß-carotene hydroxylase assay were separated on a normal-phase HPLC system (Fig. 2). This allows for the separation of all carotenes according in one peak at 3 min as indicated by the ß-carotene marker and at prenylalcohols at 23 min indicated by farnesol, from monohydroxylated all-trans ß-cryptoxanthin (27 min) and dihydroxylated all-trans zeaxanthin (43 min), as shown in trace A. It has been demonstrated for the cyanobacterium Aphanocapsa that the introduction of hydroxy groups into ß-carotene is catalyzed in this species by a NADPH-dependent monooxygenase reaction (15). Therefore, our assay of ß-carotene hydroxylation was supplemented with the cofactor NADPH. Trace B indicates that under these conditions no activity was observed. The only radioactivity peaks found were that of substrate ß-carotene and of prenyl alcohols originating from Phycomyces C115. This trace resembles completely the corresponding control experiment from which the membranes of del17 were omitted. The combination of cofactors necessary for hydroxylation by the Erwinia enzyme consisted of 2-oxoglutarate/Fe_2SO_4/ascorbate. Their presence resulted in the formation of all-trans ß-cryptoxanthin and an additional peak at 29 min which can be regarded as a cis isomer as well as all-trans zeaxanthin together with a cis isomer at 45 min (Fig. 2C). The two-step

Table I
Activity of phytoene desaturase, lycopene cyclase and ß-carotene hydroxylase indicated by the incorporation of radioactivity into intermediates and end products from their respective substrates.

	Enzyme assays of		
	Phyt. Desaturase (del16)	Lycop. Cyclase (del22)	ß-Car. Hydroxylase (del17)
Phytoene	**88652**	n.d.	n.d.
Phytofluene	4955	n.d.	n.d.
ξ-Carotene	13120	n.d.	n.d.
Lycopene	11082	**8503**	n.d.
γ-Carotene	0	12712	n.d.
ß-Carotene	0	8257	**11098**
ß-Cryptoxanthin	0	0	12248
Zeaxanthin	0	0	8902
Conversion rate	25%	71%	66%

Values in bold type indicate the residual radioactivity in susbtrate carotenes after the incubation; n.d. means not determined because these carotenes were formed by the fungal mutants in the coupled assay. Radioactivity of ß-carotene as the substrate in the ß-carotene hydroxylase assay was determined in a parallel HPLC run in the reversed-phase system, all the other conditions were as described in Materials and Methods.

hydroxylation observed in the cell-free reaction corresponds to results from studies on the ß-carotene hydroxylase gene which provided evidence that its gene product is responsible for the formation of both ß-cryptoxanthin and zeaxanthin (14). The requirement of oxoglutarate together with ascorbate and Fe^{2+} is well known for two different hydroxylases which introduce hydroxy groups into gibberellins GA_1 and GA_9 (14). It indicates that hydroxylation catalyzed by the Erwinia enzyme is a dioxygenase reaction differing from the corresponding Aphanocapsa enzyme which exhibits the properties of a NADPH requiring monoxygenase (15).

Table 1 shows the quantitation of the carotenes formed in vitro by the three investigated enzymes. The carotenoids are arranged in descending order as they occur in the biosynthetic pathway and the values for the substrate carotenes converted by the single reactions are shown in bold type. As an indicator of the cell-free activity conversion rates were determined. They were calculated as the sum of radioactivity found after termination of the reactions in intermediates and end products of the reaction divided by the sum of radioactivity in residual substrate, intermediates and end products. Very high conversion rates were found for the lycopene cyclase reaction (71%) and the ß-carotene hydroxylase reaction (68%). The phytoene desaturase activity was lower (25%), but it is known that in various organisms this reaction is the

rate-limiting step of the biosynthetic pathway and also the least active reaction in cell-free assays (14).

The availability of the del mutants in which either phytoene desaturase (del16), lycopene cyclase (del22), or ß-carotene hydroxylase (del17) are the last active enzymes in the carotenoid biosynthetic pathway, the use of different Phycomyces mutants for the generation of their ^{14}C-labeled susbtrates and development of HPLC separation has made it possible to obtain an in vitro assay for all three enzymes without interference by other (e.g. subsequent) reactions. The activities obtained are comparatively high. Therefore, three cell-free assays, which are essential for the isolation and purification of these Erwinia herbicola carotenogenic enzymes expressed in E. coli are now available. This type of work is already in progress.

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