In vitro expression and activity of lycopene cyclase and β-carotene hydroxylase from *Erwinia herbicola*

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The cyclisation of lycopene to β-carotene and the hydroxylation of β-carotene to zeaxanthin are common enzymatic steps in the biosynthesis of carotenoids in a wide range of bacteria, fungi, and plants. We have individually expressed in *E. coli* the two genes coding for these enzymatic steps in *Erwinia herbicola*. The cyclase and hydroxylase enzymes have apparent molecular weights of 43 kDa and 22 kDa, respectively, as determined by SDS-PAGE. Hydroxylase in vitro activity was obtained only in the cytoplasmic fraction. Cyclase also demonstrated enzyme activity in a crude cell-free lysate, although to a lesser extent.

1. INTRODUCTION

Carotenoids are C_{40} compounds composed of poly-isoprene units, which have been further desaturated to produce a chromophore of six or more conjugated double bonds. Many common carotenoids, such as α- and β-carotenes and their oxidised xanthophyll derivatives, have also undergone cyclisation of their termini into six-membered rings [1]. These coloured compounds typically serve to protect plants as well as some bacteria and fungi from photo-oxidative damage.

The earlier steps of the carotenoid biosynthetic pathway are common to all plant and bacterial systems, and consist of three condensations of isopentenyl pyrophosphate units to form the twenty-carbon geranylgeranyl diphosphate, which is then dimerised to the first C_{40} carotenoid, phytoene. All these reactions utilize soluble, phosphorylated substrates, and the enzyme activities for several of these reaction steps have been isolated in vitro from a variety of organisms [2].

No individual enzymes catalyzing the conversion of phytoene to the more abundant later carotenoids have been isolated and reported to date. The combined carotene desaturation and cyclisation activities which convert phytoene to the end product, β-carotene have been demonstrated in vitro from the collection of membrane bound enzymes solublized from *Narcissus pseudonarcissus* (daffodil) chromoplasts and reconstituted into liposomes [3]. In the fungus, *Phycomyces blakesleeanus*, evidence of an enzyme aggregate containing two cyclases which convert lycopene to β-carotene via γ-carotene has been reported [4].

The hydroxylation of β-carotene utilizes molecular oxygen, as demonstrated by $^{18}O_2$ labelling [5,6]. In vitro experiments indicate that membranes of the cyano-bacterium, *Aphanocapsa*, accomplish the hydroxylation of β-carotene using an O_2 dependent monooxygenase, and that the reaction is stimulated by NADPH [7].

The non-photosynthetic bacterium *Erwinia herbicola* accumulates mono- and di-glucosides of zeaxanthin [8]. All the genes coding for carotenoid production are clustered in *Er. herbicola* and are expressed in *E. coli* [9]. The various enzymatic steps, including cyclisation and hydroxylation, have been assigned to specific loci within the *Erwinia* carotenoid gene cluster via mutagenesis, and this cluster has been sequenced in both *Er. herbicola* (manuscript in preparation) and *Er. uredovora* [10]. The *Er. herbicola* gene product, CrtX, which catalyses conversion of zeaxanthin to its diglucoside has been expressed, and its activity characterised in vitro [11]. This paper describes the individual expression of the two enzymes, lycopene cyclase and β-carotene hydroxylase, in *E. coli* and demonstrates their in vitro activities.

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Abbreviations: HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; IPTG, isopropyl β-ᴅ-thio-galactopyranoside; βME, β-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. Organisms and growth conditions

The *E. coli* strains carrying the *Er. herbicola* genes contained on plasmids pAPU211 (manuscript in preparation), pAPUY (Fig. 1A), and pAPUZ (Fig. 1B) were grown at 37°C in LC medium, by selection for ampicillin resistance using 100 µg/ml ampicillin (Sigma Chemical Co., St. Louis, MO).

2.2. PCR amplification of the crtY and crtZ genes

The *Er. herbicola* lycopene cyclase (*crt*Y) and β-carotene hydroxylase (*crt*Z) genes were amplified separately via polymerase chain reaction (PCR) using *Thermus aquaticus* Amplitaq DNA polymerase (Perkin Elmer Cetus, Emeryville, CA) in 100 µl of the standard buffer [12,13]. The N-terminal primers (*crt*Y-n = 5'-GA-GAGCGTCATATGAGGGATCTGATTTTA, and *crt*Z-n = 5'-CGCGCACCCATATGCTAGTAAATAGTTTAATC, 1 µM) each contained an NdeI restriction site to allow the ligation of the amplified gene directly into the translation start site of the pET-3b expression vector [14]. The two C-terminal primers (*crt*Y-c = 5'-GGCCAC-CAAGATCTGCGCCAATCACAAC, and *crt*Z-c = 5'-TGCCATG-CAGATCTCGGCACCGGGGCAGG, 1 µM) were designed to hybridise to a region immediately downstream of the gene, and to each contain a *Bgl*II site to permit insertion into the *Bam*HI site of pET-3b. Linearised pAPU211 plasmid containing the *Er. herbicola* carotenoid gene cluster was used as the template (0.84 ng or 1.0 pM) for both amplifications. Because insertion of the restriction sites reduced the homology of the primers to the original template, an annealing temperature of 45°C was used for the first five cycles of amplification, with 55°C being used in subsequent cycles. The amplification cycle was as follows: denaturation at 94°C for 1 min; annealing for 1.5 min as described above; extension at 72°C for 2.5 min. Amplifications were performed for 30 cycles, and the products were extracted from an agarose gel slice and precipitated with ethanol.

2.3. Plasmids and cloning techniques

Plasmid pAPU211 was derived from pPL376 [9] and contains the essential *Er. herbicola* genes for carotenoid production [8]. Plasmids pAPUY and pAPUZ (Fig. 1A,B) were constructed by ligating PCR amplified *crt*Y and *crt*Z, into the *Nde*I and *Bam*HI restriction sites, respectively, of the pET-3b vector [14]. The plasmids were each cloned and maintained in *E. coli* DH5α cells. Clones containing pAPUY and pAPUZ were screened by the mini-prep method of Riggs and McLachlan [15]. All nucleic acid and enzymatic manipulations were performed according to standard published procedures [16] or manufacturers' protocols. For overexpression of lycopene cyclase and β-carotene hydroxylase, each plasmid was transformed into *E. coli* BL21(DE3).

2.4. SDS gel electrophoresis

Induced proteins were analysed by a 10% SDS-PAGE gel using the discontinuous buffer system of Laemmli [17]. Intact *E. coli* cells from a 1 ml culture were centrifuged and resuspended in 400 µl 1 × sample buffer per µl. Samples were boiled for 4 min and quenched on ice. Proteins were separated in a Bio-Rad minigel apparatus, and visualised by staining the gel with Coomassie blue R250 and destaining in a solution of 40% methanol, 10% acetic acid, and water.

In order to obtain proteins for N-terminal sequencing, an unstained gel was electroblotted onto an Immobilon membrane, according to the manufacturer's protocol (Immobilon Tech Protocol TP006, Millipore Corp., Bedford, MA). The membrane was stained with Coomassie blue, and bands of interest were excised from the membrane and sequenced.

2.5. Substrate materials

All-*trans*-lycopene, 7,9,9',7'tetra-*cis*-lycopene, 5,5'di-*cis*-lycopene, as well as δ-carotene were kindly provided by Hoffman La-Roche (Basel, Switzerland). All-*trans*-β-carotene was obtained from Sigma Chemical Co.(St. Louis, MO). γ-Carotene was isolated from *Chloroflexus aurantiacus*.

2.6. Preparation of enzyme extract

E. coli BL 21(DE3) cells containing the plasmid pAPUY or pAPUZ were grown to an $OD_{600} = 0.4$ and induced with 0.4 mM IPTG for 30 min at 37°C. In order to account for difficulties in the reproduction of enzyme activities (associated with misfolding of the overproduced proteins, see below), inductions were also performed at 15°C and for shorter (minimal 10 min) and longer (maximal 3.5 h) periods of time. For the preparation of enzyme extracts (Method A), cells were pelleted, frozen in liquid nitrogen, and thawed on ice. All further steps were carried out at 4°C unless otherwise stated. The pellets obtained from 200 ml cultures were washed and suspended in 3 ml Buffer A (0.2 M HEPES, pH 7.0, 1 mM EDTA, 1 mM PMSF), aliquoted (1 ml), and allowed to stand for 30 min after the addition of 100 µl of a lysozyme solution (1 mg/ml). A low-pressure French Press passage (4,000 psi) completed disintegration. This lysate was used directly for incubations with carotene substrates or submitted to fractionation. A 15 min centrifugation at 13 000 × g was used to remove large debris as well as inclusion bodies, and a subsequent centrifugation of the supernatant at 120 000 × g for 1.5 h yielded a soluble fraction and a membrane pellet. The membrane pellet was resuspended in the original volume of buffer, and both fractions were incubated separately. Alternatively (Method B), the induced cells were washed in Buffer B (100 mM Tris-HCl, pH 7.4) and disintegrated using a French pressure cell at 15,000 psi. Fractionation was done as above in Buffer B.

2.7. Enzyme assays

Five nmol of the individual carotene substrate dissolved in 20 µl of acetone was added to each 1 ml enzyme assay. The cofactors examined in the hydroxylase assays were NADPH, NADH, FAD, ascorbate, 2-oxoglutarate (1 ml each), and Fe^{2+} (20 µM). An enzymatic oxygen
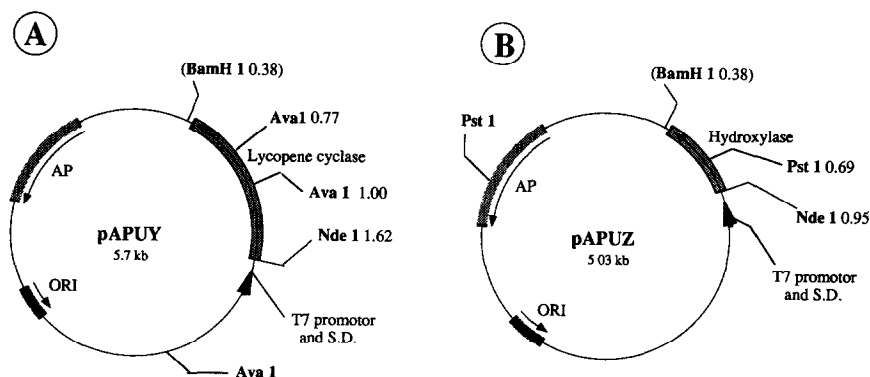

Fig. 1. Ap represents the ampicillin resistance gene. *crt*Y is the *Er. herbicola* lycopene cyclase gene. *crt*Z is the *Er. herbicola* β-carotene hydroxylase gene. Upstream of the inserted gene at an appropriate distance is a T7 promoter and Shine–Dalgarno sequence. Downstream of the insert is a strong T7 terminator. (*Bam*HI) represents restriction sites destroyed by ligation of inserts cut with *Bgl*II.

trap was used according to the method of Lam and Malkin [18], in order to stimulate the reaction, based on experiences with the cyclase reaction in *Narcissus pseudonarcissus* chromoplasts [20]. The reactions were stopped by extracting with chloroform/methanol (2:1, v/v) after an incubation at 28°C, performed for 8 h, if not stated otherwise. The extracts were dried under a stream of nitrogen and analysed by HPLC employing an ET 300/8/4 Nucleosil $5C_{18}$ Column (Macherey-Nagel). The column was developed isocratically with 5% tetrahydrofuran in acetonitrile at a flow rate of 1 ml/min.

3. RESULTS AND DISCUSSION

The plasmid constructs, pAPUY and pAPUZ, are outlined in Fig. 1A,B. PCR-amplified *Er. herbicola* DNA containing *crt*Y or *crt*Z was inserted immediately beyond the T7 promoter and Shine–Delgarno sequence of the pET-3b expression vector. A $\Phi10$ T7 termination signal is located downstream of the inserted DNA. Following an initial screening of colonies by plasmid size, restriction patterns were used to verify the identity and orientation of the inserts (Fig. 2A,B).

These pAPUY and pAPUZ constructs were each maintained in *E. coli* DH5α cells, and the *crt*Y and *crt*Z genes expressed separately in *E. coli* BL21(DE3) via induction with IPTG. The accumulation of a new 43 kDa protein was observed on a 10% SDS-polyacrylamide gel (Fig. 3A) upon induction of pAPUY with IPTG. The calculated molecular weight based on the derived amino acid sequence of the protein is also 43 kDa. The identity of this band as the *crt*Y gene product was confirmed by N-terminal amino acid sequencing. Likewise, the accumulation of a newly formed 22 kDa protein was observed on a 10% SDS-polyacrylamide gel (Fig. 3B) upon induction of pAPUZ with IPTG, although the amount of expressed protein was considerably less than that of the cyclase under the same conditions. The calculated molecular weight based on the derived amino acid sequence of the hydroxylase enzyme is also 22 kDa, and the identity of this band as the *crt*Z gene product was confirmed by N-terminal amino acid sequencing. While the expressed proteins are present as major bands in the induced cells, uninduced cells also showed traces of these proteins due to the basal activity of the T7 RNA polymerase (Fig. 3A,B).

Extracts from *E. coli* strains containing the overexpressed proteins were assayed in vitro for their individ-
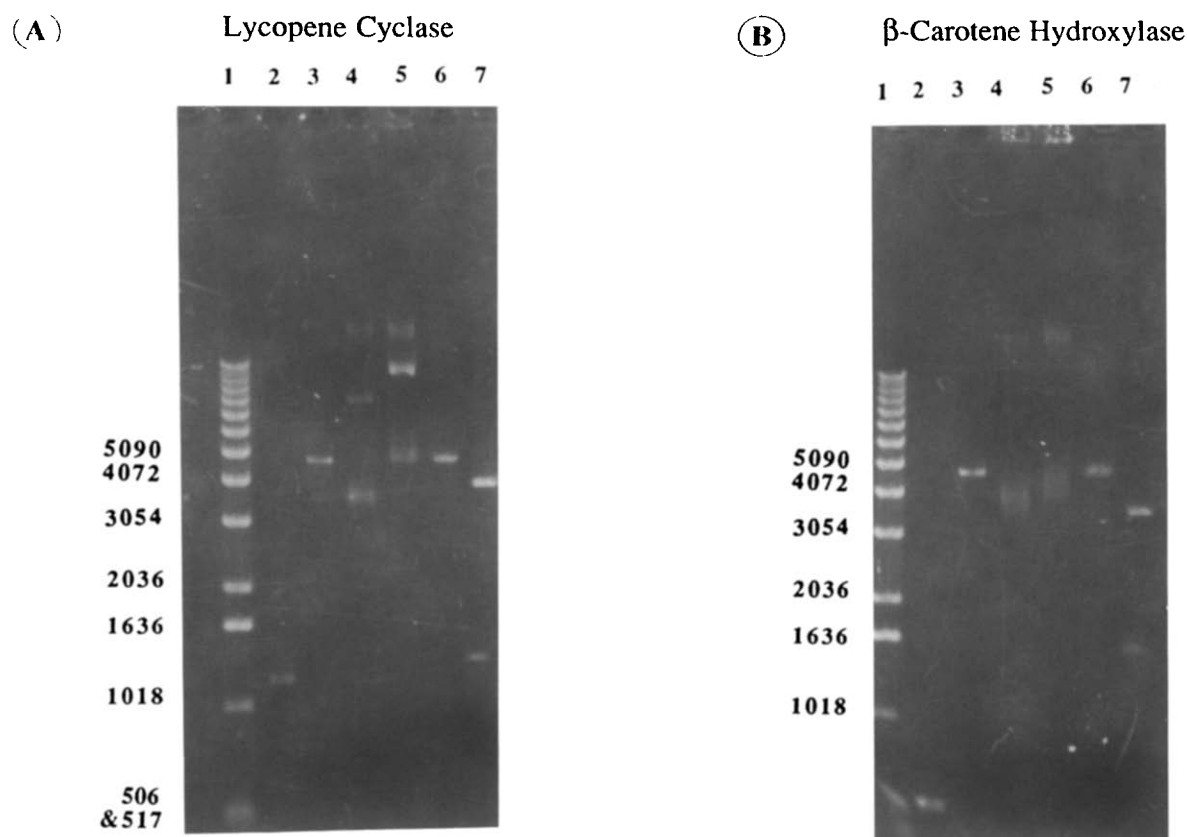

Fig. 2. 0.7% agarose gels showing DNA used in cloning enzymes from *Er. herbicola*: (A), lycopene cyclase (*crt*Y): lane 1, DNA size standards; lane 2, PCR amplification product, digested with *Nde*I and *Bgl*II, used as insert; lane 3, pET3b vector digested with *Nde*I and *Bam*HI; lane 4, uncut pET3b (nicked and supercoiled forms visible); lane 5, uncut pAPUY construct (nicked and supercoiled); lane 6, *Ava*I digest of pET3b; lane 7, *Ava*I digest of pAPUY. (B) β-carotene hydroxylase (*crt*Z): lane 1, DNA size standards; lane 2, PCR amplification product, digested with *Nde*I and *Bgl*II; lane 3, pET3b vector digested with *Nde*I and *Bam*HI; lane 4, uncut pET3b (nicked and supercoiled); lane 5, uncut pAPUZ (nicked and supercoiled); lane 6, *Pst*I digest of pET3b; lane 7, *Pst*I digest of pAPUZ.
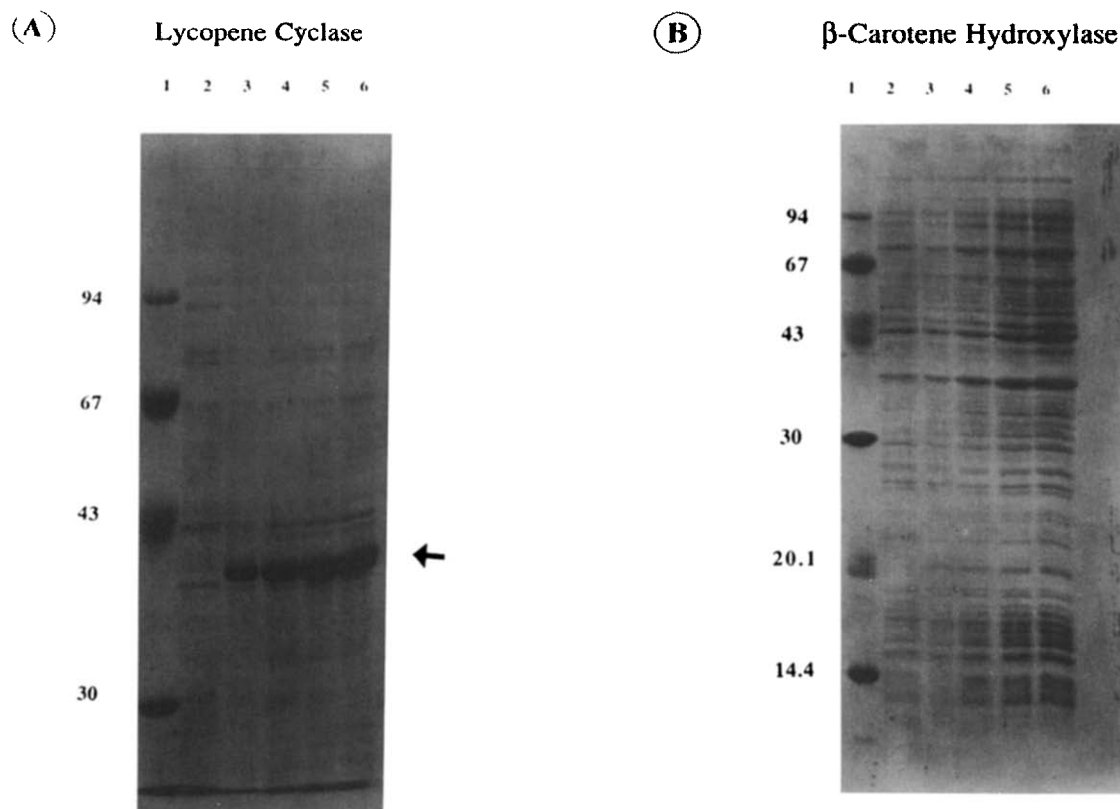
(A)   Lycopene Cyclase   (B)   β-Carotene Hydroxylase

Fig. 3. 10% SDS-PAGE gels showing expression of: (A), lycopene cyclase; and (B), β-carotene hydroxylase. In each gel: lane 1, protein standard markers of molecular mass 94, 67, 30, 20.1, and 14.4 kDa; lane 2, total protein from uninduced whole *E. coli* cells containing the plasmid bearing the gene; lanes 3–6, increasing amounts of total protein from induced cells.

ual activities. In an attempt to optimise enzyme activity, a variety of conditions were employed in both the induction period and the in vitro incubation step for each enzyme. Variables studied in the induction regime include time of induction, growth temperature, and cell density at the onset of induction. In our extensive manipulation of these variables, the enzymatic activities were not always found to be reproducible, probably due to the misfolding of the overproduced proteins. In addition, prolonged incubations also led to significant bleaching of substrates and products. However, despite these problems, in vitro activity was obtained from both of these *Erwinia* carotenoid biosynthesis enzymes, as noted below.

Hydroxylase activity could be obtained when cells were grown at 15°C and induced for 1 h at the same temperature. Fig. 4 shows an incubation of the lysate (Method A), performed in the absence of any externally added cofactors. The formation of zeaxanthin, as well as the formation of some intermediate β-cryptoxanthin was observed. Structural identity was demonstrated by HPLC, both by co-elution with the authentic reference, and with the aid of congruent spectra taken with a photodiode array detector. In fractionation experiments, the enzyme activity was found in the supernatant of the 120,000 × g centrifugation, whereas the mem-

brane fraction was inactive. This result was not expected since it has been generally accepted that the late steps of carotenoid biosynthesis are catalyzed by membrane-bound enzymes. Also, a hydropathy plot (not shown) of the amino acid sequence of this enzyme indicates the presence of several regions of considerable hydrophobicity, consistent with an enzyme which is likely to be membrane bound. Moreover, the membranes were actually found to be inhibitory, since remixing of the supernatant with increasing amounts of membranes abolished the original activity completely. This is qualitatively shown in Fig. 5. This result may indicate that complicated redox reactions could be severely disturbed when localized in an inappropriate topological relation to another dominating redox phenomenon, such as the respiratory chain. The β-carotene hydroxylase reaction proceeded in the absence of externally added cofactors. However, electron donors, such as NAD, NADPH, and ascorbate were able to stimulate the reaction. This is consistent with a mixed-function oxygenase mechanism in carotene hydroxylation.

Although massively overproduced (Fig. 3A), and more hydrophilic based on its amino acid composition, the lycopene cyclase exhibited less enzymatic activity than the β-carotene hydroxylase. In addition, the cyclase activity was less reproducible. This could not be
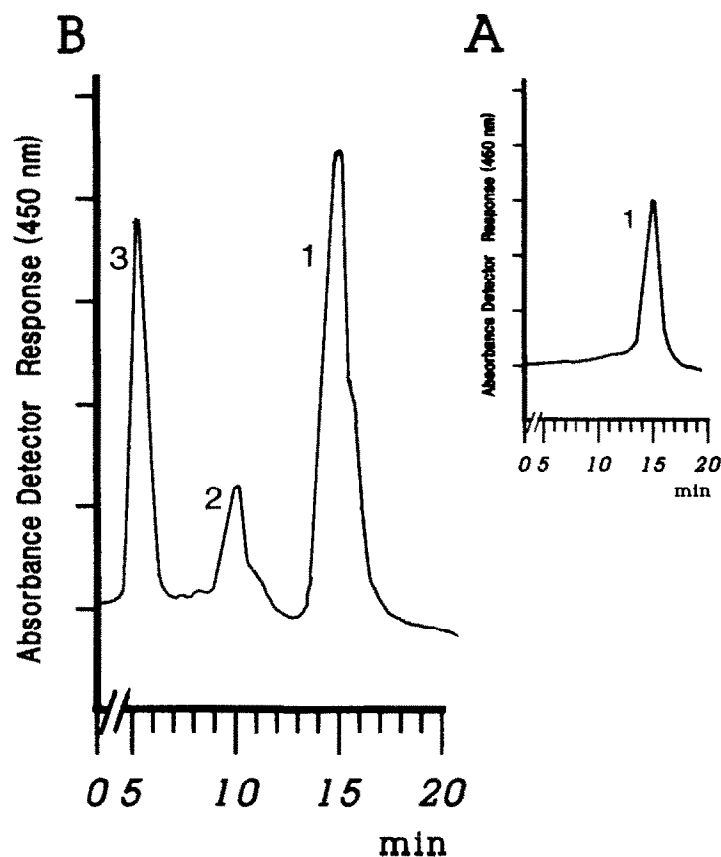
Fig. 4. HPLC analysis of an incubation of an *E. coli* cell lysate (Method A) containing the overexpressed β-carotene hydroxylase with β-carotene as the substrate. Refer to materials and methods for the incubation conditions. 1, β-carotene; 2, β-cryptoxanthin; 3, zeaxanthin. (A) Substrate (β-carotene) co-incubated in the presence of denaturing amounts (2 vols.) of $CHCl_3$/MeOH (2:1, v/v) as a control. (B) Analysis of the enzymatic conversion.

improved upon, either by numerous variations in the induction regime, or by fractionation of the cells. All-*trans*-lycopene was found to be the only acceptable substrate for this cyclase in vitro. In contrast to the findings in *Narcissus pseudonarcissus* chromoplasts [19], no conversion of prolycopene or other *cis*-isomers of lycopene could be obtained. Anaerobic conditions (administered by the use of an enzymatic oxygen trap) which are essential for the function of the *Narcissus pseudonarcissus* cyclase in vitro [20], had no stimulative effect. An example of the conversion of all-*trans*-lycopene into all-*trans*-β-carotene in a cell lysate (Method B), obtained after 20 min induction at 37°C, is given in Fig. 6.

In conclusion, both the lycopene cyclase and β-carotene hydroxylase from *Er. herbicola* could be overexpressed and shown to exhibit enzymatic activity in vitro. The *E. coli* system used, although very well suited for overproduction, does not seem to be as well suited for biochemical investigations of the cyclase and hydroxylase reactions in vitro. Therefore, the development of more sophisticated strategies as well as additional incubation systems will be pursued, in order to better elucidate the enzyme mechanisms.
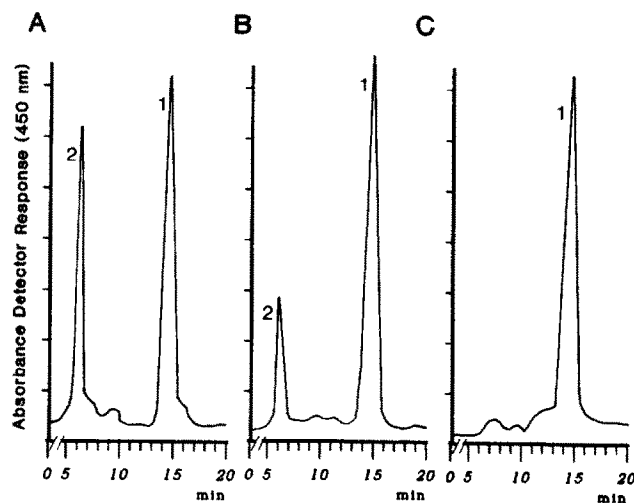

Fig. 5. HPLC analysis of an incubation of an *E. coli* supernatant (A), derived from a cell lysate (Method B) with β-carotene; (B), conversion after re-addition of 25% of the original membrane content; and (C), after re-addition of 50% of the original membrane content. 1, β-carotene; 2, zeaxanthin. Cofactors are NADPH, NADH, FAD, ADP, P_i, 1 mM each.
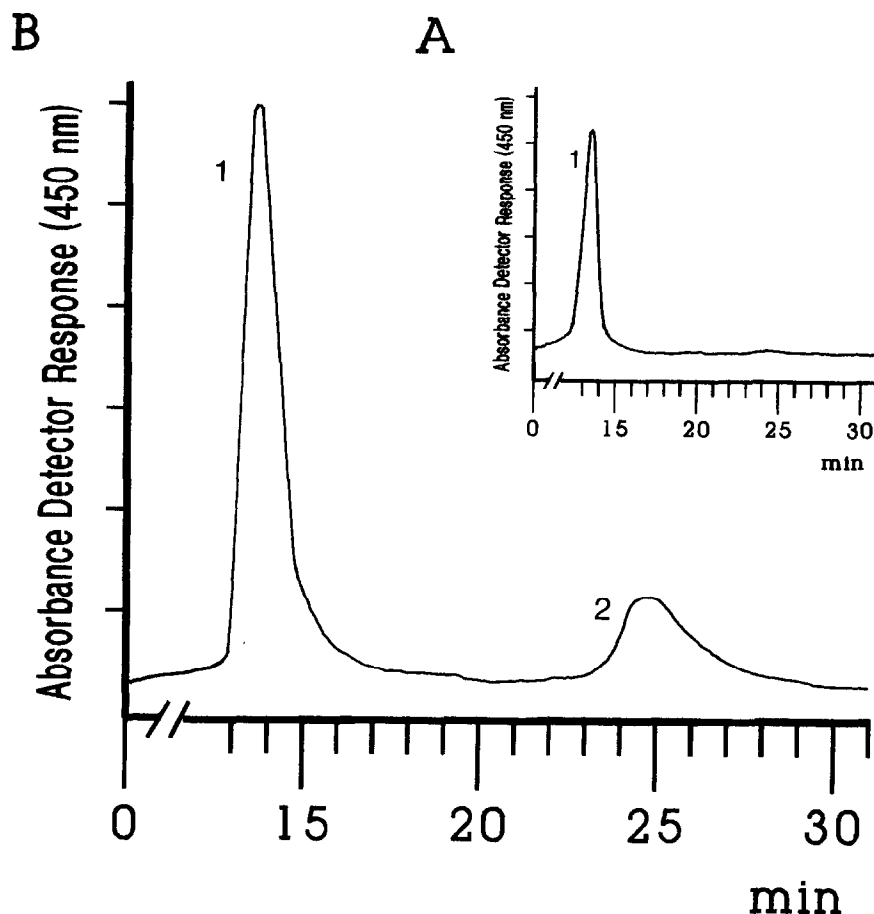
Fig. 6. HPLC analysis of an incubation of an *E coli* cell lysate (Method B) containing the overexpressed lycopene cyclase. (A) Substrate (all-*trans*-lycopene) co-incubated in the presence of denaturing amounts (2 vols.) of CHCl₃/MeOH (2:1, v/v) as a control. (B) Analysis of the enzymatic conversion: 1, all-*trans*-lycopene; 2, β-carotene.

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