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Development of high-performance liquid chromatographic systems for the separation of radiolabelled carotenes and precursors formed in specific enzymatic reactions

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ABSTRACT

High-performance liquid chromatographic (HPLC) systems were developed to separate radiolabelled carotenes and precursors formed in the following carotenogenic enzyme reactions: geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase(s), lycopene cyclase and β-carotene hydroxylase. Separations were carried out on reversed- (C_{18}) and normal-phase columns, using mobile phase mixtures of acetonitrile, methanol and hexane with appropriate modifiers. An isocratic mode of elution was employed throughout, although in several instances isocratic steps were necessary to achieve the desired resolution. The methods developed are specific for each enzyme reaction, resolving substrate from its reaction products and any interfering radiolabelled compounds, thus permitting reliable and accurate determination of enzyme activities. The new separation systems will facilitate further studies on the characterization of these proteins.

INTRODUCTION

Carotenoids are naturally occurring pigments responsible for many of the yellow, red and orange colours distributed throughout nature [1]. Commercially they have found uses as natural colourants [2] and medically their antioxidant properties have been exploited in the treatment of certain cancers [3]. Inhibition of carotenoid biosynthesis in plants is also an important target of bleaching herbicides [4].

The biosynthesis and properties of carotenoids have been studied extensively over the past decade and general aspects of their formation are now well elucidated [5]. More recently, carotenoid genes from a variety of carotenogenic organisms have been isolated and characterized (see ref. 6 for a review). However, despite these notable advances in the field, our understanding of the biosynthetic enzymes and their regulation has not progressed with the same moment. A contributing factor to this void in our knowledge is the difficulties associated with assaying the enzymes involved. For example, their substrates are not available commercially and they are also unstable and insoluble in water, complicating their effective use in aqueous solution. One approach that circumvents some of these difficulties is the use of common isoprenoid substrates directly or provided by a coupled assay system [7,8] which typically exploits the ability of a cell extract usually from a carotenoid biosynthetic fungal mutant to produce the desired carotenoid *in situ*. This experimental approach has over-

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come many practical difficulties but is not ideal and necessitates rigorous purification of the reaction products and substrates to ensure radiochemical purity from radiolabelled compounds formed as a consequence of the initial heterogeneous substrate.

HPLC has become the analytical procedure of choice in the separation of carotenoid mixtures, although TLC separations must not be ignored. Papers describing such separations are relatively common and have recently been reviewed [9–11]. However, the problems associated with the separation of radiolabelled carotenes from their precursors have not been addressed to the same degree. When separating labelled carotenes formed in enzyme reactions, the routine separations typically used in analysis rarely fulfil the rigorous requirements necessary for the purification of radiolabelled reaction products. This paper describes HPLC systems developed for the separation of reaction products formed in specific carotenoid enzyme assays, permitting the reliable determination of different reaction products as a measure of specific enzyme activity.

EXPERIMENTAL

Carotenogenic enzyme assays

(*R*)-[2-^{14}C]Mevalonic acid lactone was converted into sodium mevalonate [8] and used as the initial substrate from which specific carotenogenic substrates were formed *in situ* using cell extracts prepared from a variety of fungal mutants. These mutants and their respective coupled substrates included the *Neurospora crassa* al-3 forming FPP, *Gibberella fujikuroi* SG4 producing GGPP and the *Phycomyces blakesleeanus* C5 *carB10(−)*, C9 *carR21(−)* and C115 *carS42mad-107(−)* mutants yielding radiolabelled phytoene, lycopene and β-carotene, respectively. The precise experimental details concerning these assays have been extensively described [7,8,12].

Extraction of radiolabelled isoprenoids

Incubations were terminated with 6% KOH–methanol (1.5 ml) and heating for 20 min. The radiolabelled isoprenoids formed were then partitioned into 10% diethyl ether in light petroleum (b.p. 35–80°C) (3 × 2.5 ml). To the pooled epiphases authentic unlabelled carotene standards were added in amounts ranging between 0.1 to 1 μg and the extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in acetone (20 μl) and any particulate material removed by centrifugation (2000 *g* for 3 min).

HPLC

An LKB Model 2150 HPLC pump (Pharmacia Biosystem, Freiburg, Germany) was used to pump the mobile phase at a constant flow-rate of 1.0 ml/min. Mobile phases were premixed to alleviate any problems resulting from irregularities in the pump mixing and degassed extensively by sonication prior to use.

The HPLC columns used to perform the separations included a normal-phase 3-μm Spherisorb silica column (250 × 4.5 mm I.D.), reversed-phase 5-μm ODS-1 and ODS-2 columns (250 × 4.6 mm I.D.), all obtained from Gynkotek (Germering, Germany), and a 3-μm Nucleosil C_{18} reversed-phase column (250 × 4.6 mm I.D.) from Macherey–Nagel (Düren, Germany). After prolonged use the columns were routinely washed with methanol. Further details of the specific separation systems used are provided under Results and Discussion.

Identification and determination of the radiolabelled carotenes separated by HPLC

An on-line radiodetector (Ramona LS, Raytest, Straubenhardt, Germany) was connected to detect radioactivity eluted from the column. A 1.5-ml flow cell together with Flo Scint A from Packard (Frankfurt, Germany) was used as a liquid scintillator. Integration of the peak areas and calibration by simultaneous collection of several eluted fractions and their counting in a liquid scintillation spectrometer permitted direct determination. Radiolabelled carotenes were identified by co-chromatography with authentic carotenes prepared using methods described previously [13]. The characteristic absorbance of the standards in the column eluate was monitored continuously with a Jasco Model 8201 programmable UV–visible spectrophotometer (Biotronik, Maintal, Germany). The spectral

properties and relative polarities were also used to aid the identification of carotenoid isomers [14]. The radiochemical purity of the eluted compounds was determined by separation using several HPLC and TLC systems as described previously [15].

Chemicals

(*R*)-[2-^{14}C]Mevalonic acid lactone was purchased from Amersham Buchler (Braunschweig, Germany) and farnesol from Sigma (Munich, Germany). The following HPLC-grade solvents were used: acetonitrile (from Lab-Scan, Stillorgan, Ireland), methanol, 2-propanol, ethyl acetate and hexane (all from Merck, Darmstadt, Germany) and water. Carotenes were isolated from natural sources as described [13].

RESULTS AND DISCUSSION

The water-soluble substrates mevalonic acid and isopentyl pyrophosphate can be metabolized to a wide range of isoprenoids [16]. As a consequence, numerous isoprenoids in addition to the specific substrates used in the carotenogenic assays are formed. Typically sterols and their precursors, a range of prenyl alcohols and carotenoid oxidative products are formed by the fungal extracts used in coupled assays. Such compounds share similar physical chemical properties with the carotenoids of interest and are therefore partitioned into the crude lipid extract and possess similar chromatographic properties. In this paper we focus on the HPLC systems developed during our studies on carotenogenic enzymes that can be utilized to achieve radiochemical purity of the substrates and their products formed. For convenience the chromatographic conditions of these systems are comprehensively summarized in Table I and are arranged and described below according to the reactions of the biosynthetic pathway.

TABLE I

HPLC SEPARATION OF RADIOLABELLED CAROTENES FORMED IN CAROTENOGENIC ENZYME ASSAYS

Abbreviations: RP = reversed phase; NP = normal phase; ACN = acetonitrile; EA = ethyl acetate; MeOH = methanol; 2-PrOH = 2-propanol; Bdh-Lycop. = bisdehydrolycopene; Car. = carotene; Lycop. = lycopene; Neurosp. = neurosporene; Phyt. = phytoene; Phytofl. = phytofluene; Zeax. = zeaxanthin; *t-* = *trans-*; *c-* = *cis-*.

Enzymatic assay	Separation system		Substrate	Retention time (min)	Products	Retention time (min)
	Mobile phase	Support				
(A) GGPP synthase	Isocratic: MeOH–H_2O (90:10)	5-μm Spherisorb ODS-1 (RP)	FPP[a]	6.5	GGPP[b]	11.0
(B) Phytoene synthase	Isocratic + solvent change: (A) ACN–MeOH (98:2), 10 min; (B) ACN–MeOH–2-PrOH (85:10:5)	5-μm Spherisorb ODS-1 (RP)	GGPP[b]	4.5	*c*-Phyt. *t*-Phyt.	28.5 30.0
(C) Phytoene desaturase(s)						
(I) Phyt. to ζ-Car.	Isocratic: ACN–MeOH–2-PrOH (85:10:5)	3-μm Nucleosil C_{18} (RP)	*c*-Phyt.	40	*t*-ζ-Car. *c*-ζ-Car. *c*-ζ-Car. *t*-Phytofl. *c*-Phytofl.	27.5 28.0 30.0 36.0 37.5

(Continued on p. 268)

TABLE I *(continued)*

Enzymatic assay	Separation system		Substrate	Retention time (min)	Products	Retention time (min)
	Mobile phase	Support				
(II) Phyt. to Lycop.	Isocratic: EA–ACN–H_2O (35:60:5)	3-μm Nucleosil C_{18} (RP)	*c*-Phyt.	32	Bdh-Lycop.	13.0
					t-Lycop.	15.5
					t-ζ-Car.	21.0
					c-ζ-Car.	22.0
					c-ζ-Car.	23.0
					t-Phytofl.	25.5
					c-Phytofl.	26.5
	Isocratic + solvent change: (A) ACN–MeOH (98:2) 0–10 min; (B) EA–ACN–H_2O (25:70:5), 10–20 min; (C) MeOH	3-μm Nucleosil C_{18} (RP)	*c*-Phyt.	55	Lycop.	25.0
					ζ-Car.	35.0
(III) Phyt. to Neurosp.	Isocratic + solvent change: (A) ACN–MeOH–H_2O (94:2:4), 0–20 min; (B) ACN–MeOH–2-PrOH (85:10:5), 20–40 min; (C) MeOH	3-μm Nucleosil C_{18} (RP)	*c*-Phyt.	72	*t*-Neurosp.	50.0
					c-Neurosp.	52.0
					t-ζ-Car.	57.0
					c-ζ-Car.	59.0
					t-Phytofl.	66.0
					c-Phytofl.	68.0
(D) Lycopene cyclase	Isocratic: ACN–MeOH–2-PrOH (85:10:5),	5-μm Spherisorb ODS-2 (RP)	*t*-Lyc.	21	*t*-β-Car.	37.0
					c-β-Car.	40.0
	Isocratic: EA–ACN–H_2O (35:60:5)	3-μm Nucleosil C_{18} (RP)	Lycop.	17	γ-Car.	22.0
					t-β-Car.	28.0
					c-β-Car.	30.0
(E) β-Carotene hydroxylase	Isocratic + solvent change: (A) hexane, 0–14 min; (B) EA–hexane (20:80), 14–34 min; (C) EA–hexane (50:50)	3-μm silica (NP)	β-Car.	4	Monohydrox. products	32–38
					t-Zeax.	48.0
					c-Zeax.	52.0

[a] Farnesyl pyrophosphate (FPP) determined as alcohol.
[b] Geranylgeranyl pyrophosphate (GGPP) determined as alcohol.

Geranylgeranyl pyrophosphate synthesis

The enzyme geranylgeranyl pyrophosphate (GGPP) synthase condenses isopentenyl pyrophosphate (IPP) to farnesyl pyrophosphate (FPP) (C_{15}) yielding the C_{20} product GGPP. The substrates for the reaction can be supplied by the al-3 *Neurospora crassa* mutant [12]. These diphosphate compounds can be converted into their alcohol derivatives by acid hydrolysis or naturally as a consequence of inherent phosphatase activity. The principle compounds posing interference to the purification of FOH and GGOH are other prenyl alcohols and their respective isomers of differing chain lengths. To separate such compounds, a methanol-based mobile phase containing 10% of water to alter the polarity was employed [Table I (A)]. Development on a reversed-phase 5-μm ODS-1 column separates these compounds on the basis of their aliphatic chain length, with increases in chain length prolonging their retention on the column. Such a system has proved ideal in the determination of GGPP synthase activity in chloroplast stromal extracts and more recently in an *Escherichia coli* strain expressing the *Erwinia uredovora crtE* gene [17].

Phytoene synthesis

Phytoene synthesis is the first specific reaction in the carotenoid biosynthetic pathway and the enzyme is a characteristic marker of the chloroplast stroma. A typical trace of a chloroplastic phytoene synthase assay is illustrated in Fig. 1A. In this instance the *Gibberella fujikuroi* SG4 mutant was used to provide the substrate GGPP and the solvents consisted of two acetonitrile-based mixtures. After good separation of the prenyl alcohols from each other, a switch from the first to the second solvent was carried out in order to obtain a good and fast resolution of the two phytoene isomers. When the assay mixture is separated on a 5-μm C_{18} reversed-phase column the alcohol derivative of the substrate is detected at a retention time of 4.5 min. The products 15-*cis*-phytoene and all-*trans*-phytoene eluted at 28.5 and 30 min, respectively [Table I (B)]. These compounds of interest are clearly separated from interfering radiolabelled compounds

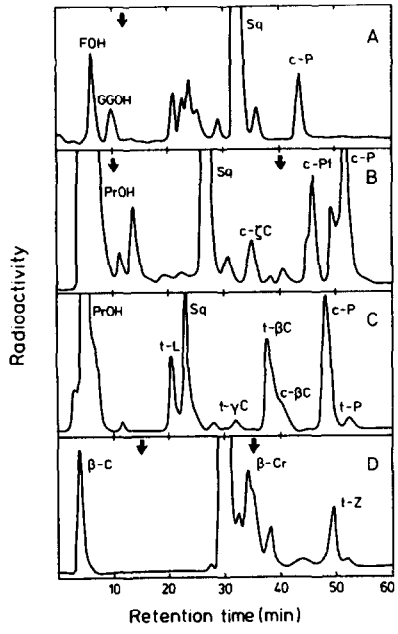

Fig. 1. Selected HPLC traces of radiolabelled carotenoids formed *in vitro* along the biosynthetic pathway. (A) Conversion of FPP to phytoene (chloroplast extract); (B) conversion of phytoene to ζ-carotene (*E. coli*/pds); (C) conversion of lycopene to β-carotene (*Synechococcus*); (D) conversion of β-carotene to zeaxanthin (*Synechococcus*). The HPLC systems used were as follows: (A) 5-μm Spherisorb ODS-1 with methanol–water (90:10) for 12 min, then acetonitrile–methanol–2-propanol (85:10:5); (B) 3-μm Nucleosil C_{18} with acetonitrile–methanol (98:2) for 10 min, then ethyl acetate–acetonitrile–water (25:70:5) until 40 min, then methanol; (C) 5-μm Spherisorb ODS-2 with acetonitrile–methanol–2-propanol (85:10:5); (D) 3-μm Spherisorb silica with hexane for 14 min, then ethyl acetate–hexane until 34 min, then methanol.

derived from the biosynthetic capabilities of the fungal extract.

Phytoene desaturation reactions

During carotenoid biosynthesis, phytoene undergoes a series of desaturations, the enzyme or enzymes responsible for this conversion being phytoene desaturase. Several functionally diverse desaturases form different reaction products and intermediates [18]. The complete resolution of all carotenes formed, including their isomers, is difficult to achieve with a single system. Therefore, it has been necessary to develop specific separation methods based on the

characteristic reaction products formed by each desaturase. One of the principal problems in such separations is the presence of squalene, a sterol precursor. This compound is an example of a molecule whose behaviour in numerous chromatographic systems does not adhere to that predicted for its relative polarity. As a consequence, it co-migrates with desaturase products on many typical carotenoid-separating systems. The incorporation of radioactivity by the fungal coupling extract into squalene is relatively substantial (Fig. 1). This has a secondary effect of masking any adjacent compounds poorly resolved which are present at low levels.

The numerous systems that have been developed for specific desaturase types are listed in Table I (C). For example, the products of a desaturase-converting phytoene (supplied by the C5 *Phycomyces* mutant) to ζ-carotene can be resolved on a 3-μm Nucleosil column using the mobile phase of Märki-Fischer *et al.* [19], *i.e.*, acetonitrile–methanol–2-propanol (85:10:5, v/v/v), as indicated in section I in Table I (C). This separation has been illustrated in a recent publication [20] and is ideal for analysing the typical desaturases from photosynthetic organisms such as the pds of *Synechococcus*. An even better separation of all the reaction products and their isomers as well as the substrate can be obtained in a more complicated system which involves three different solvents which are changed after a certain period as indicated in Fig. 1B. With this procedure the setting up of a gradient system can be avoided. This technique of changing solvents during the HPLC separation of carotenoids has already been successfully employed [21]. The 3-μm Nucleosil C_{18} column used in this and in the following desaturase assays gave good separations of *cis–trans* carotene isomers in shorter runs than described for HPLC separations on Zorbax ODS columns [22].

The *Erwinia uredovora* desaturase is typical of a desaturase that converts phytoene into lycopene (*i.e.*, the introduction of four double bonds). To achieve the effective separation of the reaction products the inclusion of ethyl acetate and water as modifiers of an acetonitrile-based solvent and the use of a 3-μm support were necessary [Table I (C), section II]. Such a system permits the separation of lycopene from squalene, which is an important achievement in the accurate determination of such desaturase activity; this system has been illustrated recently [23]. The migration of the products in this system can be altered slightly to suit more specific requirements by keeping the water content constant and altering the level of ethyl acetate. Another advantageous feature of the system is the speed of the separation, *ca.* 35 min, in which excellent resolution is achieved. One disadvantage of this system, however, is the presence of an unknown compound, believed to be an epoxide from its chromatographic behaviour, whose presence in close proximity to the lycopene can mask low lycopene levels. To overcome this problem, the absence of water from the initial acetonitrile-based mixture can alleviate such an occurrence and the three-phase system described in section II in Table I (C) has to be used. This particular system is also suited to the separation of the ζ-carotene desaturase reaction where only small amounts of lycopene are found owing to the limiting ζ-carotene level. An example of such a system is shown in Fig. 1B where the *Synechococcus* pds was assayed.

The final type of desaturase found in some photosynthetic bacteria such as *Rhodobacter* is one responsible for the desaturation of phytoene to neurosporene. As discussed previously, the main prerequisite in achieving an effective system is the resolution of neurosporene, the principal desaturase product from squalene. Using a system described in Table I (C), section III, this has been achieved and recently illustrated [24].

Lycopene cyclization

The cyclization of lycopene to β-carotene can be assayed using the C9 *Phycomyces* mutant to prepare [^{14}C]lycopene *in situ*. In general, HPLC systems that separate desaturase products are also applicable to the cylase [*e.g.*, the three-step system described in section II in Table I (C)]. However, two less complicated isocratic HPLC systems which basically differ in the stationary phase material and the particle size [Table I (D)] are more convenient for lycopene cyclase assays.

Both systems give a good example of how diverse reversed-phase supports can also be used to attain an efficient system. A very detailed study of the chromatographic behaviour of carotenoids on different reversed-phase columns has recently been published [25].

A typical trace of a cyclase assay developed on an ODS-2 column with an acetonitrile mixture is illustrated in Fig. 1C. The substrate lycopene (21 min) is resolved adequately from squalene (25 min), with the monocyclic products of the reaction eluting at 33 min and the bicyclic β-carotene at 38 min. Also illustrated clearly is the resolution of *cis*-phytoene (48 min) from *trans*-phytoene (53 min).

β-Carotene hydroxylation

The final enzyme-catalysed reaction in most carotenogenic organisms is the hydroxylase. This enzyme introduces two hydroxy moieties into the β-carotene molecule, forming zeaxanthin, thus altering the polarity of the carotene considerably. Xanthophylls such as zeaxanthin are very weakly adsorbed on reversed-phase columns in the typical solvents used for carotenoid separations. Hence, in order to separate the radiolabelled products and substrates formed in the hydroxylase reaction, it is necessary to use a normal-phase silica column. An example of a typical trace is illustrated in Fig. 1D and the chromatographic conditions are defined in Table I (E). It can be seen that the substrate elutes from the column initially at the start and then after a progressive stepwise gradient the zeaxanthin (49 min) is eluted with ethyl acetate–hexane (50:50).

CONCLUSIONS

This paper has described the separation of radiolabelled carotenes formed in specific carotenogenic enzyme assays by HPLC. It is worth addressing as a separate entity to traditional analyses of carotenoids by HPLC as such systems rarely fulfil the rigorous purification requirements. The systems described for GGPP synthase, phytoene synthase, phytoene desaturase, lycopene cyclase and β-carotene hydroxylase are reproducible and can be used in a routine mode. In addition, they can be used for the separation of the intermediates and products formed by the *Synechococcus in vitro* carotenogic enzyme system in the presence of bleaching herbicides, which provides an excellent insight into their mode of action [26]. Some of the HPLC separation systems described have already been successfully employed for assays of carotenogenic enzymes which have been purified very recently [20,23] and the others will be needed in the near future when more enzymes of this pathway will be functionally characterized after purification.

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