A New Non-Radioactive Assay of Phytoene Desaturase to Evaluate Bleaching Herbicides

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A non-radioactive cell-free assay was developed to quantitatively determine inhibition of plant-type phytoene desaturase by bleaching herbicides. An active desaturase was prepared from an appropriately cloned *E. coli* transformant. Another *E. coli* transformant was used to produce the required phytoene. Phytofluene and ζ-carotene, the products of the desaturase reaction, were either determined by HPLC or optical absorption spectra. Enzyme kinetics and inhibition data for the bleaching tetrazole herbicide WL110547 are presented as an example.

Introduction

Most bleaching herbicides inhibit the membrane-integrated enzyme phytoene desaturase of the carotenoid biosynthetic pathway present in algae and plants (Sandmann and Böger, 1989). The enzyme assays used today in cell-free mode of action studies employ ^{14}C-labelled phytoene (Sandmann, 1993) which is not commercially available. One way to provide this radioactive substrate is to generate it from [^{14}C]mevalonic acid by fungal enzyme preparations. A disadvantage of the radioactive assay is its difficult handling so that only a few laboratories were able to establish this test. Nevertheless, there is a need for an easy phytoene desaturase assay either for basic research on carotenoid biosynthesis or for modern screening of bleaching compounds as herbicides. It should be noted that preparation of an active plant-type phytoene desaturase directly from thylakoids is possible in certain cases only and often does not yield reproducible activities. A transformed *E. coli* strain is the better choice and is applied here.

Genes for the plant-type phytoene desaturase have been cloned from cyanobacteria (Chamovitz *et al.*, 1991; Martínez-Férez and Vioque, 1992) and higher plants (Bartley *et al.*, 1991; Pecker *et al.*, 1992), and were successfully expressed in *E. coli*. The phytoene desaturase from cyanobacteria and plants catalyzes the formation of phytofluene and ζ-carotene from phytoene. We have now used *E. coli* transformants both as a production source for unlabelled phytoene or for an active phytoene desaturase. A phytoene desaturase assay was developed and the conditions optimized for general use. Furthermore, the usefulness of this assay was demonstrated by applying it for enzyme kinetic studies to determine I_{50} (and k_i) values for a tetrazole bleaching herbicide WL110547 as example (Kerr and Whitaker, 1987; see Fig. 3 for structure).

Materials and Methods

E. coli JM 101 was grown in LB medium (Sambrook *et al.*, 1989) with either chloramphenicol (35 μg/ml) or ampicillin (100 μg/ml), overnight at 37 °C. One of the plasmids used was pG-pds which carries the wild-type pds gene from *Synechococcus* PCC 7942 encoding phytoene desaturase. It was cloned from a 5-kb library of this cyanobacterium by complementation with the plasmid pACCRT-BE in *E. coli* (Pecker *et al.*, 1992) in a similar manner as previously described for the ζ-carotene de-

Abbreviations: PDS, phytoene desaturase; pds gene, phytoene desaturase gene from *Synechococcus*; I_{50}, value for 50% inhibition.

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saturase *(zds)* gene (Linden *et al.*, 1993). The latter plasmid was also used to produce phytoene in *E. coli*. *Synechococcus* PCC 7942 was grown for 2 days as previously described (Sandmann, 1992) in the presence of WL110547.

Carotenes and precursors were extracted from cyanobacterial cells with hot methanol containing 6% KOH (65 °C, 20 min) or from the cell-free incubations by addition of 3 ml of petrol ether (b.p. 40–80 °C). Then, either absorbance at 445 nm was measured or absorption spectra were recorded. In some cases, ζ-carotene and phytoene were determined by HPLC using a 25 cm Spherisorb ODS 1 column and acetonitrile/methanol/2-propanol (85:10:5 v/v). For details see Pecker *et al.* (1992). The extinction coefficients used to quantitate phytoene and ζ-carotene from their absorbance at 286 and 424 nm, respectively, were E1%/1cm = 757 and 2500, respectively (Davies, 1976).

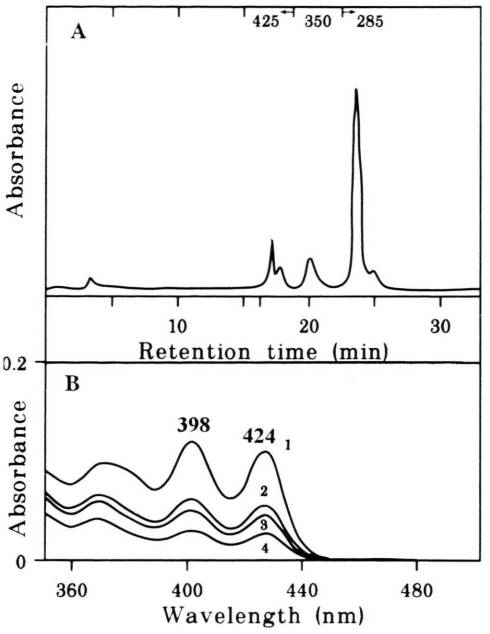

Fig. 1. HPLC separation (**A**) and optical absorption spectra (**B**) of carotenoid extracts from cell-free phytoene desaturase assays. To (**A**): (a) ζ-carotene, (b) phytofluene, (c) phytoene. To (**B**): 1 = control, 2 = addition of 2 μM, 3 = addition of 6 μM, 4 = addition of 10 μM of WL110547 to the assay. Two maxima for ζ-carotene absorption are noted at spectrum 1.

Results and Discussion

To obtain an active enzyme preparation of phytoene desaturase, homogenization of the *E. coli* cells is crucial. The best results were obtained by resuspending pelleted *E. coli* cells in 1/10 of the culture volume in 0.1 M tris(hydroxymethyl)aminomethane(Tris)/HCl buffer, pH 8.0, containing 5 mM DTT and breaking the cells in a French pressure cell at 20 MPa. This procedure was followed either for the *E. coli* transformant which is the production source of phytoene or for *E. coli* cells carrying the gene for the expression of PDS. If the resulting homogenate is very viscous 1 μl/ml of a DNase solution (10 mg/ml) should be added followed by a 15-min incubation on ice. The phytoene-containing homogenate can be frozen for several days. For the enzyme preparation, it is better to freeze aliquots of the harvested *E. coli* cells and homogenize them immediately prior to use. For the enzyme assay 0.5 ml of homogenate was mixed with the herbicides added as methanol or acetone stock solution not exceeding a volume of 10 μl of organic solvent in the assay volume, and incubated at 37 °C with shaking in an incubator. The reaction was terminated by addition of 2 ml of methanol.

Formation of products in the phytoene desaturase assay can either be determined by HPLC or by recording optical absorption spectra. Both procedures are exemplified here and compared. Figure 1A shows a HPLC trace of a carotene extract from this assay. In addition to the substrate phytoene at a retention time of 23.9 min., three compounds with a retention time of 17.4, 18.2 and 20.3 min. were seen, which are two ζ-carotene isomers and phytofluene, respectively. They were identified by reference compounds and also by their typical spectra. For their HPLC determination a detector with programmable wavelengths is essential. Depending on the absorbance of all three carotenes and their retention time, recording was first done at 424 nm and was then changed to 350 nm and to 285 nm. The advantage of using HPLC is the accurate determination of both products phytofluene and ζ-carotene. The absorption spectra of the same carotene extract (spectrum 1) is shown in Figure 1B together with spectra from carotenes formed during incubation in the presence of different concentrations of WL110547 (spectra 2–4). Two absorption maxima of ζ-carotene are found at 398 and 424 nm. The peaks of

Table I. Determination of ζ-carotene (μg per assay) formed in a cell-free assay in the presence of a bleaching herbicide by either optical density (OD) or HPLC.

Concentration of WL110547 [μM]	ζ-Carotene (by OD_{424})	Phytofluene	ζ-Carotene (by HPLC)
0	0.46	0.40	0.59
2	0.28	0.46	0.45
6	0.22	0.22	0.19
8	0.20	0.21	0.15
10	0.15	0.15	0.12

phytofluene around 330, 350, and 370 nm are obscured somewhat by the UV-absorbing background. So, phytofluene cannot be determined spectroscopically.

Table I compares the results of *in-vitro* experiments with different concentrations of WL110547. The recorded spectra allow to calculate the amounts of *in vitro* synthesized ζ-carotene using the absorbance at the maximum at 424 nm. The same samples were then used to determine by HPLC the formation of ζ-carotene and phytofluene. Although the values for ζ-carotene differed somewhat, decreasing amounts were formed with increasing herbicide concentration. I_{50} values were found quite identical when calculating them either from the ζ-carotene amounts obtained from the spectrum or by the sum of ζ-carotene and phytofluene obtained by HPLC, which both are the reaction products of the enzyme. These results demonstrate that the spectroscopic determination is sufficient to obtain reliable results which certainly is of advantage when a greater number of assays should be run. Finally, we evaluated our new assay with two other bleaching herbicides, norflurazon and fluridone.

Routinely the procedure including HPLC was used. Figure 2 shows a time course of the enzyme reaction. Formation of the intermediate phytofluene and the end-product ζ-carotene were determined by HPLC. It is evident that enzyme activity prevails for 400 min. A 4-h incubation time is sufficient for reliable quantitation of ζ-carotene and phytofluene. However, if ζ-carotene is determined in a spectrophotometer, incubation over 6 hours ensures maximum formation giving better results. Longer incubations cause some degradation of the ζ-carotene formed.

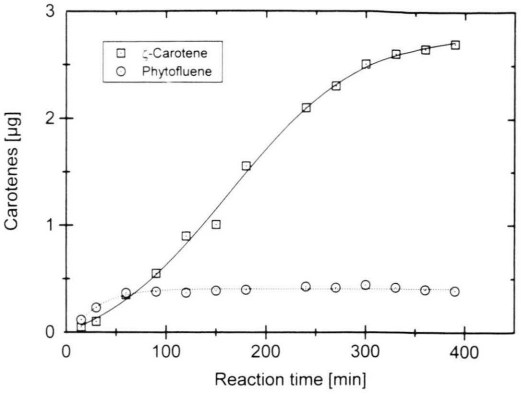

Fig. 2. Time course of the formation of phytofluene and ζ-carotene from phytoene catalyzed by phytoene desaturase.

The I_{50} value for inhibition of WL110547 was determined in cell-free assays (Fig. 3A). For comparison, also the I_{50} value for formation of colored carotenoids in intact herbicide-treated *Synechococcus* cells was measured (Fig. 3B). The reciprocal values of either phytofluene and ζ-carotene formed *in vitro* or total colored carotenoids of

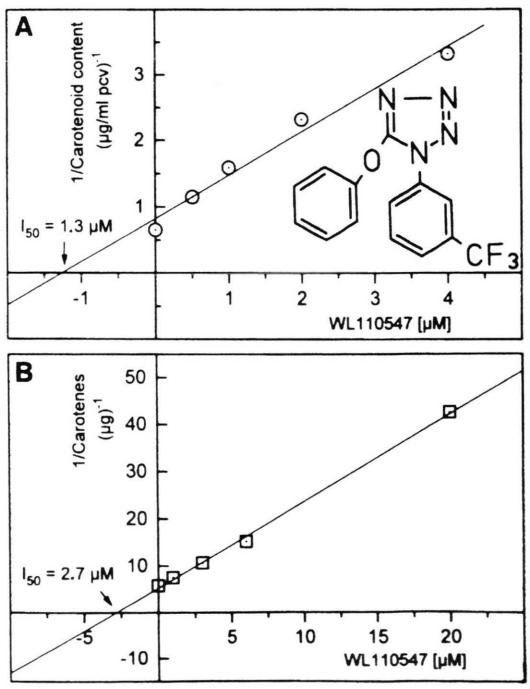

Fig. 3. Determination of I_{50} values for WL110547 with intact *Synechococcus* cells (**A**) and with the phytoene desaturase reaction in a cell-free assay (**B**).

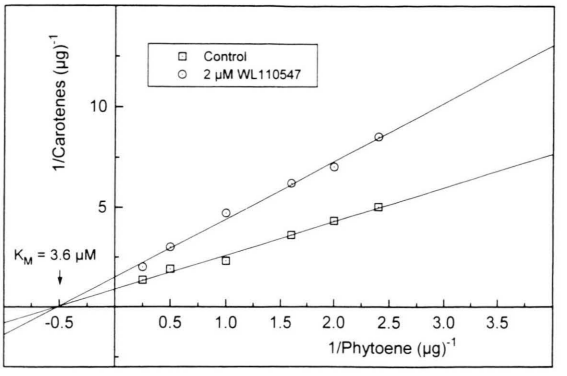

Fig. 4. Lineweaver-Burke plot of reciprocal values of synthesized carotenes (phytofluene plus ζ-carotene) and applied phytoene amounts in an untreated control and with 2 µM WL110547 present.

whole cells were plotted in a Dixon plot against the herbicide concentration (Dixon, 1953). In both cases, straight lines were obtained and the I_{50} values calculated from the intersection with the abscissa. Both values were in the same range differing by a factor of about two. An I_{50} value of 1.3 µM for intact cells versus 2.7 µM in the enzyme assay indicates that the *Synechococcus* cells are able to take up the inhibitor and enrich it to a certain extent in the cell. The regression coefficient obtained for Figs. 3 and 4 was above 0.98.

The potential of the new *in-vitro* assay can also be exploited for enzyme kinetic studies to reveal the nature of inhibition of WL110547 and other bleaching herbicides which directly interfere with phytoene desaturase. Figure 4 is a Lineweaver-Burke plot of two sets of experiments in which the substrate concentration is varied and phytoene desaturase activity measured. Both the control and the activity determinations in the presence of 2 µM inhibitor concentration gave straight lines intersecting through one point on the abscissa. This defines a non-competitive inhibition of phytoene desaturase by WL110547. Reversibility of inhibition of phytoene desaturase was demonstrated by loading enzymatically active membranes with WL110547 and re-establishing activity by subsequent washing. Before and after washing enzyme activity was determined (data not shown). Therefore, we can conclude from Figure 4 that the tetrazole WL110547 is a non-competitive inhibitor of phytoene desaturase. The same characteristic was also found for norflurazon (Sandmann *et al.*, 1989) and fluridone (Kowalczyk-Schröder and Sandmann, 1992). The plot in Fig. 4 was also used to calculate the K_M value for the substrate phytoene as 3.6 µM. This is the first value for a membrane-integrated carotenogenic enzyme determined by adequate enzyme kinetics.

Conclusion

The newly developed non-radioactive assay for phytoene desaturase can be used in a simple spectroscopic variation to elucidate the mode of action of a potential bleaching herbicide. It is also feasible to determine I_{50} values for enzyme inhibition. If high accuracy is needed, e.g. to carry out enzyme kinetic studies, the reaction products formed in the cell-free assay have to be determined by HPLC.

Acknowledgements

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