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Biosynthesis of psoralens

Psoralen 5-monooxygenase activity from elicitor-treated *Ammi majus* cells

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Microsomes prepared from cultured *Ammi majus* cells that had been challenged for 14 h with an elicitor derived from the cell walls of *Phytophthora megasperma* f.sp. *glycinea* (Pmg) converted psoralen to bergaptol (5-hydroxypsoralen) in the presence of NADPH and oxygen. The enzymatic activity was characterized as an inducible cytochrome-P-450-dependent monooxygenase associated with the endoplasmic reticulum. All of the steps involved in bergapten (5-methoxypsoralen) biosynthesis in *Ammi majus* have now been demonstrated in vitro. The results suggest that bergaptol and not hydroxymarmesin in the precursor of bergapten.

Elicitor enzyme induction; Psoralen biosynthesis; Cytochrome P-450 monooxygenase; Furanocoumarin phytoalexin; (*Ammi majus*)

1. INTRODUCTION

Suspension cultures of *Ammi majus* produce large quantities of linear furanocoumarins upon elicitor treatment and appear, therefore, well suited for enzymatic investigations into their biosynthesis [1,2]. The pathway leading to bergapten (5-methoxypsoralen) biosynthesis suggested on the basis of precursor studies has remained under debate, since, formally, it may involve either 5-hydroxymarmesin or psoralen [3] as an intermediate (fig.1). Furthermore, the respective *O*-methyltransferases (fig.1) reported from *Ruta* [4] and parsley cell cultures [5] show a relatively broad substrate specificity. The formation of psoralen from (+)-marmesin (fig.1), catalyzed by microsomes from elicitor-treated *Ammi majus* cells, was reported on previously [2].

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Abbreviations: Pmg elicitor, elicitor preparation from cell walls of *Phytophthora megasperma* f.sp. *glycinea*; R_f, mobility relative to solvent front; TLC, thin-layer chromatography; DTE, 1,4-dithioerythritol.

Since we have never observed the formation of 5-hydroxymarmesin in incubations employing (+)-marmesin as a substrate, we have investigated whether extracts from these cells can efficiently convert psoralen to bergaptol (fig.1).

2. MATERIALS AND METHODS

2.1. *Chemicals*

Psoralen and bergapten were purchased from Roth, Karlsruhe. Carbon monoxide was from Messer Griesheim, Düsseldorf. All cytochrome P-450 inhibitor chemicals were kindly supplied by BASF, Ludwigshafen [6,7]. *S*-Adenosyl-L-methionine and *S*-[*methyl*-^{14}C]adenosyl-L-methionine (2.17 GBq/mmol) were purchased from Sigma, Deisenhofen, and Amersham-Buchler, Braunschweig, respectively. [3-^{14}C]-Psoralen (approx. 180 GBq/mol) was prepared enzymatically, employing synthetic (±)-[3-^{14}C]marmesin (603.5 GBq/mol) in a mixture with unlabelled psoralen and microsomes from *Ammi majus* cells that had been treated for 14 h with an elicitor derived from the cell walls of *Phytophthora megasperma* f.sp. *glycinea* (Pmg) [2]. [*methyl*-^{14}C]Bergapten (2.17 GBq/mmol) was prepared enzymatically, employing *S*-[*methyl*-^{14}C]adenosyl-L-methionine and an enzyme extract from parsley cells that had been treated for 27 h with Pmg elicitor [5]. The elicitor was isolated as described elsewhere [8]. Bergaptol was prepared from isoimperatorin [9] kindly provided by S.A. Brown, Peterborough, Canada.

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2.2. *Analytical procedures*

Carbon monoxide inhibition studies were carried out as described previously [10] using mixtures of either 10% oxygen in nitrogen or 10% oxygen in carbon monoxide in the dark. In parallel experiments, incubations were irradiated with blue light (450 nm). TLC on silica gel plates (Merck, Darmstadt) was carried out using the following solvent mixtures (by vol.): (I) trichloromethane/methanol (95:5); (II) *n*-hexane/acetic acid/ethyl ester (5:5:1); (III) trichloromethane/*n*-hexane (2:1); (IV) benzene/acetone (9:1). TLC on cellulose plates (Merck, Darmstadt) was carried out in solvent V, water/ethanol/formic acid (88:10:2). Radioactivity on TLC plates was spotted using an LB 2832 automatic linear analyzer (Berthold, Wildbad). Protein determination was carried out according to Lowry et al. [11].

2.3. *Buffers*

The following buffers were used: (A) 50 mM Tris-HCl, pH 7.5, containing 3 mM EDTA, 5 mM KCl, 1 mM DTE, and 250 mM sucrose; (B) 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA; (C) 100 mM Tris-HCl, pH 7.5.

2.4. *Incubations*

Ammi majus L. suspension cultures were treated with Pmg elicitor for various lengths of time and microsomes were isolated from the cells as described previously [2]. For determination of psoralen 5-monooxygenase activity, a small drop of a [3-^{14}C]psoralen solution (approx. 0.6 nmol, equivalent to 9300 dpm) was placed in the tip of an Eppendorf tube, and the solvent was removed in a nitrogen stream. Buffer B (30 μl), the microsomal suspension (20–30 μg protein in 10 μl buffer B), and NADPH (100 nmol in 10 μl buffer B) were added, and the mixture was incubated at 20°C for various periods of time. The incubation was terminated by extraction with ethyl acetate (50 μl), and the extract was separated by cellulose TLC in solvent V. For identification, enzymatically prepared [3-^{14}C]-bergaptol (approx. 1.3 nmol; 20000 dpm) was converted to [3-^{14}C]bergapten by incubation with crude *o*-methyltransferase extracts from either parsley [5] or *Ammi majus* cells and *S*-adenosyl-L-methionine (1.86 nmol) in a total volume of 870 μl buffer C for 1 h at 30°C. The product was identified by silica TLC in solvent III (R_f for bergaptol = 0.04; R_f for bergapten = 0.50). Control incubations were carried out with non-labelled bergaptol (90 nmol) and *S*-[*methyl*-^{14}C]adenosyl-L-methionine (0.9 nmol).

3. RESULTS AND DISCUSSION

Microsomes isolated from suspension-cultured *Ammi majus* cells that had been treated with Pmg elicitor catalyzed the NADPH-dependent conversion of [3-^{14}C]psoralen into a chromatographically distinct, labelled product. This product was identified as bergaptol (fig.1) by co-chromatography with authentic bergaptol on silica gel in solvents I (R_f for psoralen = 0.86; R_f for bergaptol = 0.39) and II (R_f for psoralen = 0.69; R_f for bergaptol = 0.56), as well as on cellulose in solvent V (R_f for psoralen = 0.34; R_f for bergaptol = 0.20). The identity of the labelled product was further confirmed by incubation with *S*-adenosyl-L-methionine and crude *O*-methyltransferase from elicitor-treated parsley cells [5] to give labelled bergapten. Due to the small amount of material available, a bergaptol concentration below the reported K_m [5] had to be used. Nevertheless, approximately 70% of the substrate was converted to bergapten under the conditions employed. Very similar conversion rates were achieved with crude *O*-methyltransferase extracts from *Ammi majus* cells that had been treated for 27 h with Pmg elicitor. These results clearly suggest that, in *Ammi majus*, the biosynthesis of bergapten involves psoralen rather than 5-hydroxymarmesin (fig.1) as an intermediate. Moreover, all of the reactions from umbelliferone to bergapten [1] can now be demonstrated in vitro with *Ammi majus* extracts.

Elicitor-treated *Ammi majus* cells also accumulate small amounts of isopimpinellin (5,8-dimethoxypsoralen), which requires an 8-hydroxylated psoralen as a biosynthetic precursor. However, neither labelled bergaptol nor [*methyl*-^{14}C]bergapten were hydroxylated on in-

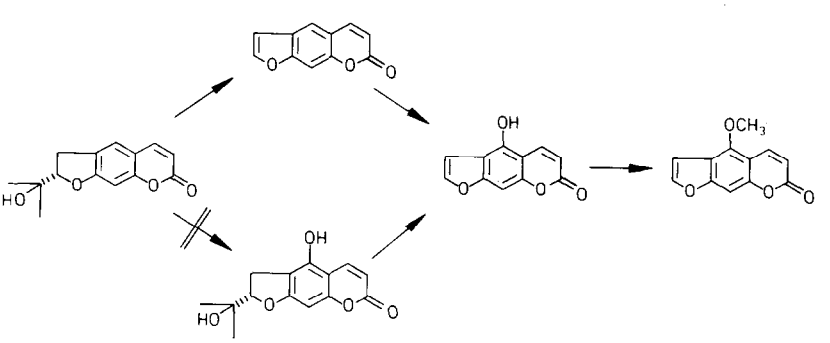

Fig.1. Hypothetical pathways for the conversion of (+)-marmesin to bergapten involving psoralen and 5-hydroxymarmesin as intermediates.

cubation with the microsomal preparations, irrespective of the NADPH concentration employed (up to 10 mM).

The optimal rate of bergaptol formation by *Ammi majus* microsomes was observed in buffer B at a temperature range of 20 to 30°C. Standard incubations were carried out at 20°C for 20 min, where roughly 40–50% of the substrate was converted into bergaptol, although the conversion rate was linear with time up to 30 min and with protein up to 40 μg. K_m values of approx. 12 μM for psoralen and 200 μM for NADPH were determined. The enzymatic activity was maximally induced by the elicitor within about 20–24 h following its addition to the dark-cultured cells.

Inhibition studies with various cytochrome-P-450-specific inhibitor chemicals (table 1) indicated that the formation of bergaptol from psoralen is catalyzed by a cytochrome-P-450-dependent monooxygenase, analogous to the marmesin and psoralen synthases previously described from elicitor-treated *Ammi majus* cells [2]. This was confirmed by the blue light-reversible inhibition by carbon monoxide (table 2). Furthermore, separation of microsomes by sucrose gradient centrifugation and comparison of the psoralen 5-monooxygenase activity distribution with that of marker enzymes [2] pinpoint the membranes of the endoplasmic reticulum as the site of bergaptol synthesis in *Ammi majus* cells. These results complement our previous claim [1,2,10] that dimethylallyl diphosphate:umbelliferone dimethylallyltransferase, marmesin synthase, psoralen synthase and psoralen 5-monooxygenase, the enzymes specific to furanocoumarin biosynthesis, are all located at the endoplasmic reticulum. The elicitor therefore appears to induce a particular set of cytochrome-P-450-dependent enzymes.

Table 1

Effect of different cytochrome-P-450-specific inhibitors on psoralen 5-monooxygenase activity

Inhibitor	Concentration of the inhibitor (μmol·l^{-1})	Enzyme activity (%)
None	–	100
Ketoconazole	5	94
	50	73
Ancymidole	5	85
	50	26
Tetcyclacis	5	20
	50	14
BAS 110	5	86
	50	36
BAS 111	5	96
	50	46
BAS 978	5	82
	50	41

Table 2

Effect of carbon monoxide and light at 450 nm on psoralen 5-monooxygenase activity

Assay conditions	Enzyme activity (%)
N_2/O_2 dark	100
CO/O_2 dark	56
N_2/O_2 light	97
CO/O_2 light	77

The oxygen content of the gas mixture was 10%

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