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Cytochrome P-450 dependent (+)-limonene-6-hydroxylation in fruits of caraway (*Carum carvi*)☆

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Abstract

Microsomal preparations from fruits of annual and biennial forms of caraway (*Carum carvi* L.) catalyze the C-6 hydroxylation of (+)-limonene to (+)-*trans*-carveol, the key intermediate in the biosynthesis of carvone. The enzyme activities from both caraway forms had similar properties (pH optimum, K_m, cofactor requirements, etc). Both activities were dependent on NADPH and O_2, and were inhibited by N-substituted imidazoles, metyrapone, cytochrome *c* and CO, and thus meet many of the established criteria for cytochrome P-450-dependent mixed function oxygenases. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Introduction of oxygen, via hydroxylation, into the olefinic terpene skeletons derived from the ubiquitous precursors geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}) is an important reaction in the formation of the wide variety of different terpenoid derivatives found in the plant kingdom. Most of the enzymatic hydroxylations of terpene substrates described so far involve cytochrome P-450 dependent monooxygenases, regardless of the size of the substrate, including hydroxylation of the monoterpene sabinene (Karp, Harris, & Croteau, 1987), hydroxylation of the sesquiterpene 5-epi-aristolochene (Hoshino et al., 1995), and hydroxylation of the diterpene taxa-4(5),11(12)-diene (Hefner et al., 1996).

Oxygenated terpenes are major components of the essential oil of many important aromatic plants. For example, the essential oil of caraway fruits (*Carum carvi* L.) contains the monoterpenes (+)-carvone and (+)-limonene. Because of the culinary importance of caraway and the recent introduction of (+)-carvone extracted from caraway fruits as an effective sprouting inhibitor of potatoes (Oosterhaven, Poolman, & Smid, 1995), we have begun a detailed study of the biosynthesis of (+)-carvone in caraway. In previous work (Bouwmeester, Gershenzon, Konings, & Croteau, 1998), we have shown that carvone formation in caraway proceeds in an analogous fashion to the biosynthesis of (−)-carvone in *Mentha spicata* (Gershenzon, Maffei, & Croteau, 1989) (Fig. 1). In this process, a monoterpene cyclase, (+)-limonene synthase, cyclizes GPP to (+)-limonene. Then, the (+)-limonene formed is hydroxylated to (+)-*trans*-carveol, which is subsequently oxidized by an NAD^+ utilizing terpenol dehydrogenase to (+)-carvone (Bouwmeester et al., 1998). Studies of the developmental regulation of the activities of these three enzymes suggested that (+)-limonene-6-hydroxylation is the rate-limiting step in the formation of (+)-carvone, based on its kinetics and the time course of its appearance in development (Bouwmeester et al., 1998). For this reason, and because carvone is the commercially most important component in caraway seed essential oil, we investi-

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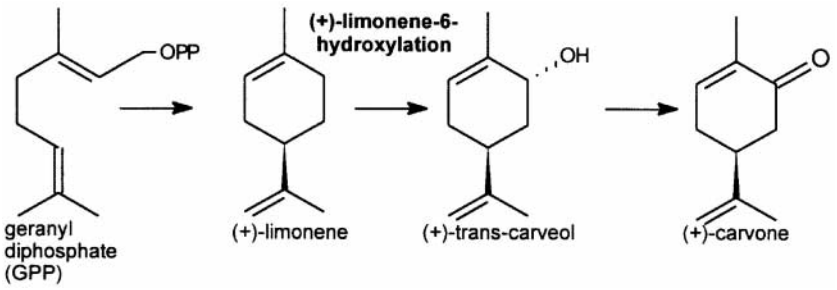

Fig. 1. Biosynthetic route to (+)-carvone.

gated the properties of the (+)-limonene-6-hydroxylase in annual and biennial forms of caraway.

2. Results and discussion

2.1. Enzyme product, extraction conditions and stability

Caraway (+)-limonene-6-hydroxylase exhibits strict regio- and enantiospecificity. When the 150,000*g* pellets of caraway fruit extracts were assayed with (+)-limonene, the product consisted of over 95% (+)-*trans*-carveol. This substance was identified by GC-MS with a chiral column using standards of (+)- and (−)-*trans*-carveol obtained as described by Bouwmeester et al. (1998).

Optimization of the extraction procedure was carried out with annual caraway. Substantial (+)-limonene-6-hydroxylase activity was obtained when frozen fruits were ground on ice in the presence of a cold Hepes buffer, pH 7.5, containing glycerol, polyvinylpolypyrrolidone (PVPP), reducing agents, protease inhibitors and other protectants. When seeds were ground in liquid N_2 (without buffer), there was a complete loss of enzyme activity. Polyvinylpyrrolidone was instrumental in preserving enzyme activity with the insoluble form (PVPP) at equal tissue weight being more effective than the soluble form (PVP-40) at 3–4% (w/v). Among reducing agents, the use of $NaHSO_3$ was more effective in preserving enzyme activity than sodium diethyldithiocarbamate. Omitting leupeptin, EGTA, catalase and glutathione reduced the activity by almost 50% although the effect of the individual compounds of this mixture was not assessed. Following grinding, the extract was treated with polystyrene resin (XAD-4) to adsorb phenolics and terpenes. The amount of XAD-4 used strongly affected the recovery of hydroxylase activity, with maximal activity obtained with 0.5 g XAD-4 per g of tissue. When no XAD-4 was employed, activity was 75% of maximum, while levels of 1 g g^{-1} resulted in only 50% of maximal activity. After XAD-4 treatment, sonication for several minutes on ice improved hydroxylase activity by ca. 60%.

Differential centrifugation at 20,000 and 150,000*g* was routinely used for the preparation of microsomal pellets since almost 90% of the total hydroxylase activity remained in the supernatant upon centrifugation at 20,000*g* but sedimented with the light membrane fraction (the microsomal fraction) upon subsequent centrifugation at 150,000*g*. Although some experiments were carried out with freshly prepared microsomes, freezing the microsomal pellets in liquid N_2, and storage at −80°C under argon seemed to enhance activity, and, therefore, in most experiments frozen pellets were used. These frozen microsomal pellets could be stored for over a year at −80°C without any loss of activity.

2.2. Characterization of limonene-6-hydroxylase activity

The limonene-6-hydroxylase activity of annual caraway was ca. 3-fold higher than that of the biennial form (Table 1). This difference in activity is due to a difference in the age of the fruits used for extraction rather than a fundamental difference between the two forms (Bouwmeester et al., 1998). The hydroxylases from both forms required NADPH and O_2 for catalysis, and NADH did not support activity (Table 1). The presence of an NADPH-regenerating system slightly stimulated hydroxylation activity, but incubation without O_2, under an Ar atmosphere or with an O_2-scavenging system decreased activity by 85–96% (Table 1). Microsomal NADPH- and O_2-requiring hydroxylation reactions also depend on the activity of an NADPH-cytochrome P-450 reductase for the electron transfer from NADPH to the cytochrome P-450 oxygenase. The flavin moiety of this reductase is often labile and inclusion of FAD and FMN during preparation of microsomal pellets or in the assay generally enhances hydroxylase activity (Mihaliak, Karp, & Croteau, 1993). However, in this study the omission of FAD and FMN from the extraction buffer did not have a negative effect on (+)-limonene-6-hydroxylase activity as long as flavins were present during the assay. When FAD and FMN were omitted from the assay, (+)-limonene-6-hydroxylase activity decreased by over 50% (Table 1).

A number of compounds known to inhibit cytochrome P-450 catalyzed oxygenation reactions were tested to examine the possible involvement of cytochrome P-450 in the hydroxylation of (+)-limonene in caraway. Clotrimazole and miconazole have been previously shown to be effective inhibitors of cytochrome

Table 1
Requirements for caraway (+)-limonene-6-hydroxylase activity. All values are the mean of at least two independent experiments

Assay conditions	Annual caraway[a]		Biennial caraway[b]	
	(pkat g^{-1} fruit (dry wt))	(%)	(pkat g^{-1} fruit (dry wt))	(%)
NADPH + FAD, FMN + O_2 + RS[c] (control)	218	100	75.2	100
Control-RS	206	94	63.3	84
Control-NADPH − RS + NADH	0.0	0	0.71	1
Control-O_2 (Ar atmosphere)	31.9	15	5.67	8
Control-O_2 (O_2 scavenging system[d])	9.78	4	7.69	10
Control-FAD, FMN	89.7	41	30.5	45

[a]Extracted in buffer without FAD, FMN.
[b]Extracted in buffer with FAD, FMN.
[c]RS = NADPH regenerating system (5 mM glucose-6-phosphate, 1 i.u. glucose-6-phosphate dehydrogenase).
[d]O_2 scavenging system (10 i.u. of glucose oxidase, 6 μmol β-D-glucose and 1300 i.u. catalase).

P-450 enzymes (Karp et al., 1987; Karp, Mihaliak, Harris, & Croteau, 1990; Hallahan, Dawson, West, & Wallsgrove, 1992). In caraway, clotrimazole was ca. 200–300-fold more effective in inhibiting limonene-6-hydroxylation than was miconazole (Table 2). Sheet, Mason, Wise, & Estabrook (1986) and Karp et al. (1990) showed that these N-substituted imidazoles may be useful for distinguishing among different forms of cytochrome P-450. For example, enzymes that catalyze the oxygenation of a single substrate at different positions may be differentially affected by the various inhibitors (Karp et al., 1990). The present work shows that these inhibitors can also distinguish among enzymes whose chiral specificity differs. Whereas (−)-limonene-6-hydroxylase from *M. spicata* was inhibited equally by miconazole and clotrimazole (Karp et al., 1990), (+)-limonene-6-hydroxylase from caraway was inhibited 200–300-fold more by clotrimazole than miconazole (Table 2). Another inhibitor often used to demonstrate the involvement of cytochrome P-450 is metyrapone. At low concentrations, metyrapone slightly stimulated limonene-6-hydroxylase activity and high concentrations were required for inhibition (Table 2). This is in agreement with the sensitivity to metyrapone of the (−)-limonene hydroxylases (Karp et al., 1990), geraniol-10-hydroxylase (Hallahan et al., 1992) and naringenin 3′-hydroxylase (Doostdar et al., 1995). Cytochrome *c* inhibits cytochrome P-450 catalyzed reactions by acting as an alternative electron acceptor for reducing equivalents from cytochrome P-450 reductase (Mihaliak et al., 1993). Cytochrome *c* strongly inhibited caraway (+)-limonene-6-hydroxylase activity (I_{50} < 10 μM) (Table 2) suggesting involvement of the P-450 system.

The inhibition of enzyme activity by CO and its reversal by blue light are another indication of the involvement of cytochrome P-450. CO inhibited the limonene-6-hydroxylase activity of biennial caraway in a concentration dependent manner (Table 3), while, in annual caraway, a 100% CO atmosphere completely inhibited limonene-6-hydroxylation (data not shown). However, blue light reversal was not clearly demonstrated because blue light strongly decreased enzyme activity in control assays (Table 3), which can probably be attributed to flavin or chlorophyll-dependent oxygen radical generation (Karp et al., 1987). Nevertheless, at a high CO:O_2 ratio, blue light reduced the enzyme activity less than expected on the basis of photoinhibition alone.

The pH optima for the two hydroxylases in Tris buffer were similar, 7.4 for annual and 7.2 for biennial caraway, with estimated half-maximal activities at a pH of ca. 6.3 and 8.4 for both enzymes. Enzyme activities in Mes and Bis–Tris buffers were similar, but

Table 2
Inhibition of caraway (+)-limonene-6-hydroxylases

Inhibitor	I_{50}[a] (μM)	
	annual caraway	biennial caraway
Clotrimazole	< 1	0.34
Miconazole	47.7	103
Metyrapone	1516	1459
Cytochrome *c*	< 10	2.87

[a]Inhibitor concentration for half-maximal activity determined from measurements of at least four separate concentrations.

Table 3
Carbon monoxide inhibition and blue light reversal of (+)-limonene-6-hydroxylase activity from biennial caraway

CO:O_2	Enzyme activity (pkat g^{-1} fruits (dry wt))	
	dark	blue light
air	53.7	9.13
1:9	56.6	
4:6	37.4	
6:4	22.3	7.96
8:2	6.62	

much lower than in Tris buffer. The shape of the pH curve is typical for cytochrome P-450 enzymes (Mihaliak et al., 1993; Madyastha, Meehan, & Coscia, 1976) and the pH optima found are similar to pH optima reported for other cytochrome P-450 hydroxylases such as 7.4 for sabinene hydroxylase (Karp et al., 1987) and (−)-limonene C-3 and C-6 hydroxylase (Karp et al., 1990), 7.0 for camphor hydroxylase (Funk & Croteau, 1993) and 7.4–7.6 for naringenin 3′-hydroxylase (Doostdar et al., 1995).

Additional evidence for the involvement of cytochrome P-450 can be obtained from spectral data, i.e. by carbon monoxide difference spectroscopy (Omura & Sato, 1964). However, CO difference spectra could not be obtained with microsomal preparations from caraway fruits probably because of interfering pigments. Researchers commonly encounter difficulties doing spectral studies on membrane suspensions from green plant tissues due to the presence of green pigments (Hallahan et al., 1992).

2.3. Kinetics

Plots of limonene-6-hydroxylase activity versus limonene concentration gave rise to typical hyperbolic saturation curves (Fig. 2). Using non-linear regression for kinetic analysis, yielded apparent K_m values of 11.4 μM for annual and 14.9 μM for biennial caraway, similar to values reported for other terpenoid hydroxylases such as geraniol-10-hydroxylase (15 μM (Hallahan et al., 1992)) and (−)-limonene C-3, C-6 and C-7 hydroxylases (18, 20 and 21 μM) (Karp et al., 1990).

3. Conclusion

The present results confirm that (+)-limonene in caraway is hydroxylated at the 6-position to (+)-*trans*-carveol by a cytochrome P-450 dependent monooxygenase in analogous fashion to the 6-hydroxylation of (−)-limonene to (−)-*trans*-carveol in *M. spicata* (Gershenzon et al., 1989; Karp et al., 1990). Cytochrome P-450 dependent hydroxylases have also been implicated in the hydroxylation of other monoterpenoids such as sabinene (Karp et al., 1987), geraniol (Hallahan et al., 1992; Meijer, Souer, Verpoorte, & Hoge, 1993) and camphor (Funk & Croteau, 1993). The enzymes from the biennial and annual forms of caraway are very similar in properties, including K_m, pH optimum, cofactor requirements and the degree of inhibition by cytochrome P-450 inhibitors.

As (+)-limonene-6-hydroxylation was shown to limit carvone formation in caraway (Bouwmeester et al., 1998), gene isolation and over expression may enable an increase in the carvone content of this important spice plant. However, despite progress in the solubilization and purification of the cytochrome P-450 dependent enzymes, it is still usually difficult to purify these proteins and isolate the corresponding gene using amino acid sequence information for the purified protein. Recent successes in the cloning of P-450 genes have resulted from a PCR strategy using primers based on the conserved regions in their sequences (Meijer et al., 1993; Schuler, 1996). As this approach often yields a multitude of P-450 clones, a biochemical characterization of the enzymes using the information presented in this communication will be necessary to identify the gene of interest.

4. Experimental

4.1. Plant materials

Annual caraway (*Carum carvi* L.) var. 'Karzo' was grown in the field, essentially as described (Bouwmeester & Smid, 1995). Biennial caraway var. 'Bleija' was grown in a greenhouse as described (Bouwmeester et al., 1998). Batches of unripe fruits of both caraway forms were harvested, frozen in liquid N_2 and stored at −80°C until further use. Fruit dry matter percentage was determined by weighing a sub sample of ca. 0.5–1.0 g (fr. wt) of fruits before and after drying overnight at 105°C.

4.2. Preparation of microsomes

During enzyme isolation and preparation of the assays, all operations were carried out on ice or at 4°C. Samples of 2 g of the frozen fruits were ground in a pre-chilled mortar and pestle in 10 ml of a pre-chilled buffer containing 50 mM Hepes (pH 7.5), 20% (v/v) glycerol, 50 mM sodium ascorbate, 50 mM $NaHSO_3$, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM EGTA, 5 mM DTT, 5 μM FAD, 5 μM FMN, 0.5 mM glutathione, 2 mg ml^{-1} BSA, 5 μg ml^{-1} leupeptin and 25 i.u. ml^{-1} catalase slurried with 1 g PVPP and some purified sea sand. During grinding, additional aliquots of buffer (without PVPP and sea sand) were added to a total volume of ca. 60 ml. The homogenate was transferred to a small beaker containing 1 g of polystyrene resin (XAD-4), sonicated for 4 min in 10 s pulses (on ice), stirred carefully for 12 min and then filtered through cheesecloth. The filtrate was centrifuged at 20,000*g* for 20 min (pellet discarded) and then at 150,000*g* for 90 min. Microsomal pellets were either used directly for assays or frozen in liquid N_2 and stored under argon at −80°C.

4.3. Enzyme assays

Microsomal pellets were resuspended in a small volume of assay buffer containing 50 mM Tris (pH 7.2

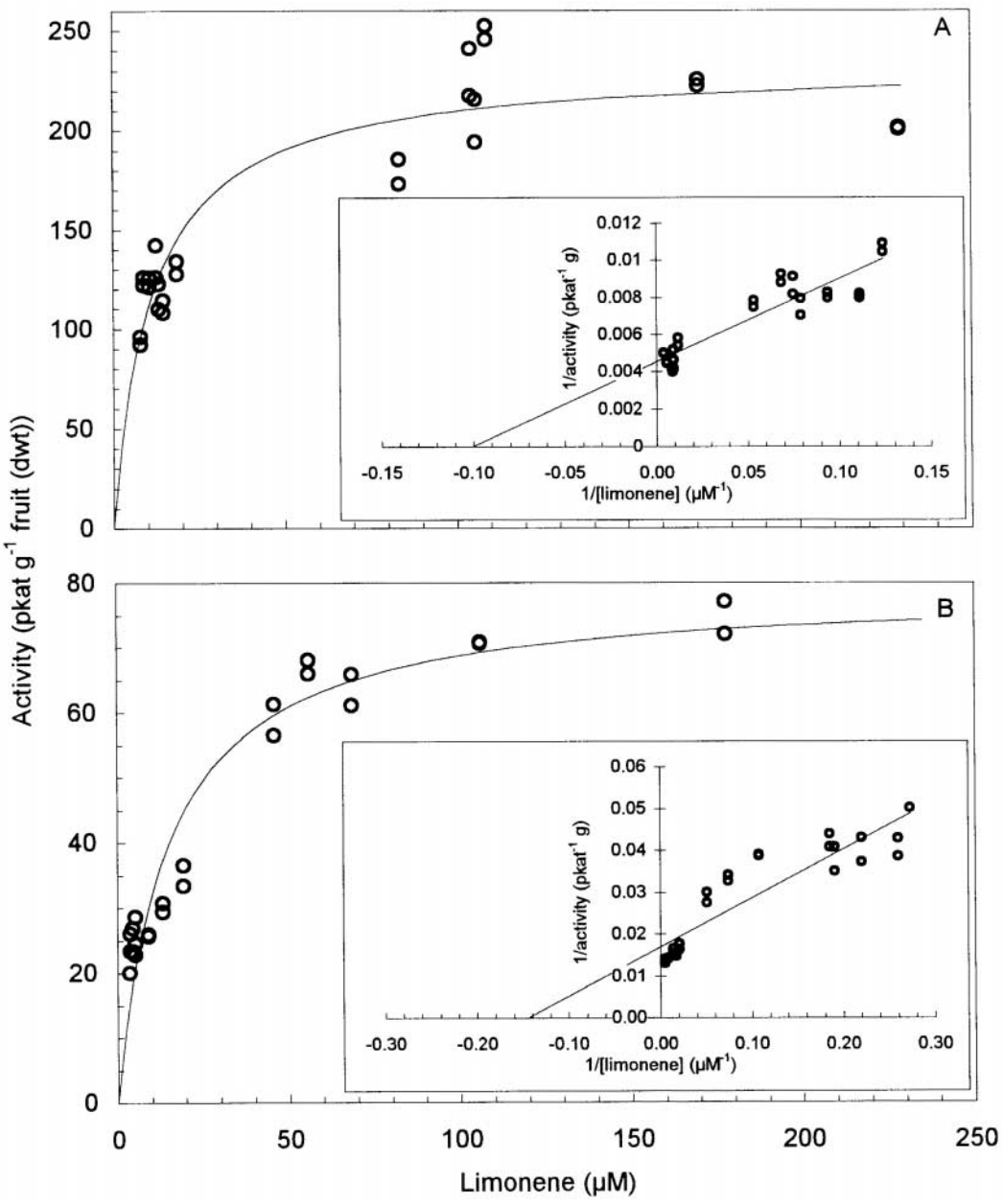

Fig. 2. (+)-Limonene hydroxylase activity as function of substrate concentration and double reciprocal plots of the data (insert) for (A) the annual and (B) biennial form of caraway. (A) $y = 233.26x/(11.35 + x)$, $R^2 = 0.86$ (B) $y = 78.91x/(14.87 + x)$, $R^2 = 0.93$.

for biennial, pH 7.4 for annual), 20% (v/v) glycerol, 1 mM EDTA, 2 mM DTT, 1 μg ml^{-1} leupeptin, 5 μM FAD and 5 μM FMN using a glass rod and teflon potter and then diluted to the desired concentration using the same buffer. To determine pH optima, buffers of 50 mM Mes and 50 mM Bis–Tris were also used. One ml of this microsomal suspension (in standard assays: 6.9–8.3 μg protein) was incubated in a 9 ml teflon-lined screw cap vial and the reaction started by the addition of 1 mM NADPH, an NADPH-regenerating system (5 mM glucose-6-phosphate, 1 i.u. glucose-6-phosphate dehydrogenase) and 200 nmol (+)-limonene (5 μl of a hexane stock). The assays were performed in duplicate. In routine assays, enzyme activities of both caraway forms were linear for over 60 min. Control assays that had been boiled for 5 min showed no enzymatic activity. After incubation for 1 h at 30°C, 1 ml of Et_2O was added and the tube vigorously mixed and then stored at −20°C until further analysis. To assess the levels of limonene and carveols initially present, control assays were run in which the reaction was stopped immediately after substrate addition by adding 1 ml Et_2O and vigorous mixing. For analysis, 25 nmol camphor was added to the reaction mixture as an internal standard. The reaction mixtures were thawed, vigorously mixed and briefly centrifuged to separate phases. The Et_2O phase was then transferred to another vial, and the water

layer re-extracted with another 1 ml portion of Et_2O. The combined Et_2O extracts were decolorized with activated charcoal, washed with 1 ml of water and, after centrifugation, passed over a short column of silica gel overlaid with anhydrous $MgSO_4$. The water phase was re-extracted with another 1 ml portion of Et_2O which was also passed over the $MgSO_4$/silica gel column. After 500 μl of hexane were added, the extracts were concentrated to ca. 500 μl using a centrifugal evaporator and, subsequently, a stream of N_2. Samples were analyzed for limonene, camphor, *cis*- and *trans*-carveol and carvone using GC-MS in the selected ion monitoring mode: for limonene *m*/*z* 68, 93, 136; for camphor: *m*/*z* 81, 95, 152; for *trans*-carveol *m*/*z* 84, 109, 152; for *cis*-carveol *m*/*z* 84, 109, 134; for carvone *m*/*z* 82, 108, 150 on a HP 5890 series II GC and HP 5972A Mass Selective Detector. The GC was equipped with a HP-5MS column (30 m × 0.25 mm id × 0.25 μm df). The injection port (splitless mode), interface and MS source temp. were 175, 290 and 180°C, respectively. The He inlet pressure was controlled by Electronic Pressure Control to achieve a constant column flow of 1.0 ml min^{-1} during the following oven program: initial temp. 45°C for 1 min, ramp of 10°C min^{-1} to 220°C and a final time of 5 min. Ionization potential was set at 70 eV. The compounds were quantified using an external standard mixture with known concentrations of authentic reference compounds that was measured under the same conditions.

To create an O_2-free atmosphere, septum-capped vials were flushed with argon immediately after addition of substrate and cofactor and just before incubation. Alternatively, an O_2-scavenging system (10 i.u. of glucose oxidase, 6 μmol β-D-glucose and 1300 i.u. catalase) was used which was pre-incubated for 15 min before substrate and cofactor were added. Inhibitors, dissolved in 5 μl DMSO (miconazole), EtOH (clotrimazole and metyrapone) or assay buffer (cytochrome *c*), were added to the assay mixtures 15 min before addition of substrate and cofactor. Solvent controls were included.

CO:O_2 mixtures were made from pure CO and O_2 essentially as described (Karp et al., 1987). Assays were incubated in 4.5 ml septum capped vials and the reaction mixture (containing substrate and cofactor) was slowly bubbled before incubation with 45 ml of the gas mixture through a needle inserted through the (vented) septum. Blue light was obtained by passing the beam of a tungsten lamp (250 W) through 10% $CuSO_4$ for a distance of 5 cm. CO-treated and control vials were incubated on a rotary shaker at room temperature either in blue light or, covered with aluminum foil, in darkness.

Microsomal preparations of both caraway forms contained appreciable amounts of residual limonene (approximately 3 μmol g^{-1} seed (dwt) for biennial and 9 μmol g^{-1} seed (dwt) for annual caraway). These could only partially be removed by increasing the amount of XAD-4 used during extraction, which was however undesirable as higher amounts of XAD-4 decreased the yield of limonene-6-hydroxylase activity. Hence, kinetic analyses were carried out by first diluting the enzyme preparation, and then adding additional limonene to obtain a range of substrate concentrations.

4.4. Other analytical procedures

Attempts to obtain CO-difference spectra were carried out according to (Omura and Sato, (1964) with sodium dithionite as reductant. Because BSA was included in the extraction buffer, microsomal protein levels were determined using the micro-assay protocol of the Coomassie Plus Protein Assay (Pierce) and BSA as protein standard after resuspension of the pellet in a buffer without BSA, an additional 150,000 × *g* centrifugation step and resuspension of the resulting pellet in water.

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