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Functional Expression of Regiospecific Cytochrome P450 Limonene Hydroxylases from Mint (*Mentha* spp.) in *Escherichia coli* and *Saccharomyces cerevisiae*[1]

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The oxygenation pattern of the essential oil monoterpenes of commercial mint (*Mentha*) species is determined by regiospecific cytochrome P450-catalyzed hydroxylation of the common olefinic precursor (−)-4*S*-limonene. In spearmint (*M. spicata*), C6-allylic hydroxylation leads to (−)-*trans*-carveol and thence (−)-carvone, whereas in peppermint (*M.* × *piperita*), C3-allylic hydroxylation leads to (−)-*trans*-isopiperitenol and ultimately (−)-menthol. cDNAs encoding the C6-hydroxylase and C3-hydroxylase from spearmint and peppermint, respectively, were isolated by a combination of reverse genetic and homology-based cloning methods (S. Lupien, F. Karp, M. Wildung, and R. Croteau, *Arch. Biochem. Biophys.* 368, 181–192, 1999). Although both hydroxylase genes were confirmed by functional expression using the baculovirus–*Spodoptera* system, too little protein was available by this approach to permit detailed study of the structure–function relationships of these catalysts, especially the substrate binding determinants that underlie the regiochemistry and stereochemistry of the reactions. Therefore, heterologous overexpression systems based on *Escherichia coli* and *Saccharomyces cerevisiae* were developed to produce several N-terminally modified versions of the recombinant hydroxylases. Ancillary methods for the solubilization, purification, and reconstitution (with recombinant spearmint cytochrome P450 reductase) of the limonene hydroxylases were also devised, with which substrate binding behavior and precise regiochemistry and stereochemistry of product formation were determined. © 2000 Academic Press

Key Words: **monoterpene biosynthesis; cytochrome P450 monooxygenase; (−)-limonene hydroxylase; *Mentha* × *piperita; Mentha spicata;* (−)-*trans*-isopiperitenol; (−)-*trans*-carveol.**

Cytochrome P450 monooxygenases are powerful oxidizing agents capable of activating molecular oxygen and concomitantly hydroxylating a very broad range of lipophilic substrates (1, 2). More than 500 cytochrome P450 genes have now been identified and classified into over 70 subfamilies (3). The great versatility of these catalysts is well illustrated by the cytochrome P450 oxygenases of plants, in which they play essential roles in the biosynthesis of terpenoid, alkaloid, and phenylpropanoid natural products, and in the catabolism of herbicides and other xenobiotics (4). In spite of many years of investigation, the structural determinants of cytochrome P450 substrate selectivity, and regio- and stereochemistry, are still only poorly understood (5–8), in part due to the lack of suitable model systems for comparative study.

In the monoterpene family of natural products, cytochrome P450 hydroxylases are responsible for establishing the oxygenation patterns of the various skeletal types (9). For example, in the *p*-menthane monoterpenes of agronomically significant mint (*Mentha*) species, P450-mediated allylic hydroxylation of the simple parent olefin (−)-limonene at either the C3-position (peppermint, *M.* × *piperita*) or the C6-position (spearmint, *M. spicata*) sets in motion a series of subsequent enzymatic redox reactions ultimately leading to (−)-menthol in peppermint and to (−)-carvone in spearmint (Fig. 1) (10). These oxygenated monoterpene derivatives are largely responsible for the characteristic,

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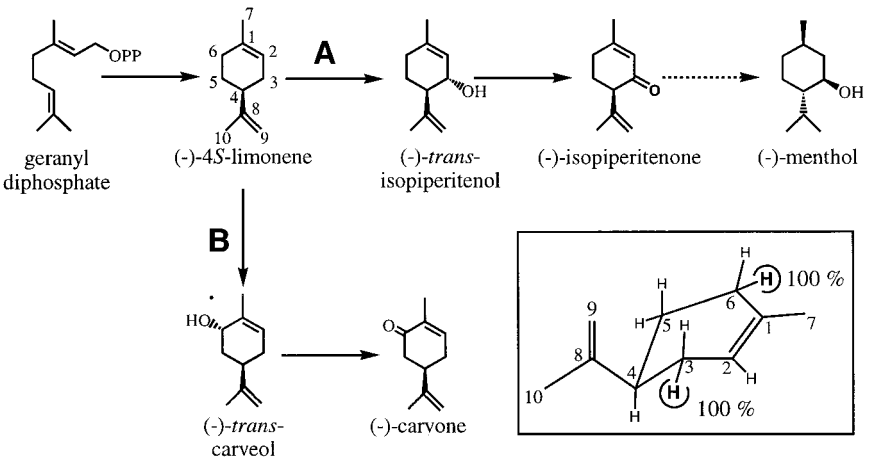

FIG. 1. Pathways for monoterpene biosynthesis in peppermint (A) and spearmint (B). The dotted arrow indicates several enzymatic steps. The inset illustrates the most stable conformation of (−)-4*S*-limonene with the positional specificity of the C3- and C6-hydroxylases indicated.

and distinctive, odors and flavors of these two mint essential oils (11), which, *in planta*, may fulfill communication and defensive functions (12).

The microsomal cytochrome P450 enzymes from peppermint and spearmint responsible for the respective C3-hydroxylation and C6-hydroxylation of (−)-limonene have been well characterized, and both exhibit a high degree of substrate selectivity and regiospecificity in the hydroxylation reaction (13). A reverse genetic cloning approach, based on the purification and microsequencing of the limonene-6-hydroxylase, led to the isolation of the corresponding cDNA (designated SM12) from a spearmint oil gland cDNA library, whereas homology-based cloning, using the spearmint gene, allowed acquisition of two isoforms of the limonene-3-hydroxylase from a peppermint oil gland library (designated cDNAs PM2 and PM17) (14). The two limonene-3-hydroxylases were 95% similar and 93% identical to each other at the deduced amino acid level, and showed ~78% similarity and ~70% identity when compared to the spearmint limonene-6-hydroxylase. The availability of these two closely related hydroxylases differing strictly in regiochemistry (but exhibiting the same relative facial stereochemistry) provides a rare opportunity to investigate active site binding interactions with a simple olefinic substrate that dictate the geometry of oxygen insertion in cytochrome P450 catalysis.

Although both of the hydroxylase genes from *Mentha* were confirmed by functional heterologous expression using the baculovirus-*Spodoptera* system (14), a system generally considered to be highly reliable for plant genes (15), microsomal preparations from these transgenic insect cells yielded too little recombinant cytochrome P450 protein to permit detailed study of the structure–function relationships of these enzymes. Of the many cytochrome P450 genes of plant origin now defined, very few have actually been functionally expressed (1, 4, 16), and the extant literature on general cytochromes P450 overexpression systems (17, 18) provides limited guidance directed specifically to plant proteins of this type. In this paper, we describe the development of bacterial and yeast expression systems for the production of several versions of the limonene hydroxylases, as well as methods for partial purification and reconstitution of activity that have allowed characterization of these recombinant enzymes.

MATERIALS AND METHODS

Construction of E. coli expression vectors for limonene-6-hydroxylase. The cDNA encoding cytochrome P450 (−)-limonene-6-hydroxylase (SM12; CYP71D18) was subcloned from the original pBSSK(+) vector (14) into pSBETa (19) and pCWOri(+) (18) in four different versions bearing alterations of the 5′-terminus. Modifications (described in Table I) were made to either truncate the hydrophobic N-terminus (constructs designated S12-1, S12-3, and S12-5), introduce GCT at the second codon position (S12-2 and S12-5), enrich AT content (S12-5), or (for S12-2) substitute the seven N-terminal residues of bovine 17α-hydroxylase, i.e., CYP17A (18). Each of these modifications also introduced a 5′-*Nde*I restriction site coincident with the ATG start codon. The original full-length cDNA in pBSSK(+) was used as template for PCR[4] amplification with *Pfu* polymerase (Stratagene) and each of the four forward primers S12-1, S12-2, S12-3, and S12-5 (Table I) in conjunction with reverse primer S12-4 (5′-CCAAGATCTTAAGGACTTTTATAGAGTGTGGG-3′), which allowed the introduction of a *Bgl*II restriction site downstream of the stop codon. The resulting amplicons, designated 1-4, 2-4, 3-4, and 5-4, were ligated into the *Sma*I site of pBSSKII(+) to yield vectors S12-1-46, S12-2-46, S12-3-46, and S12-5-46, and their se-

[4] Abbreviations used: BHT, butylated hydroxytoluene; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GC, gas chromatography (chromatograph); IPTG, isopropyl-β-D-thiogalactopyranoside; MS, mass spectrometry (spectrum); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris, tris-(hydroxymethyl)aminomethane; TLC, thin layer chromatography.

TABLE I

N-Terminal Modifications of Cytochrome P450 Limonene Hydroxylases

Construct	Amino acid sequence[a]	Oligonucleotide sequence[b]
NATIVE S12-0	MELDLLSAIIILVATYI	ATGGAGCTCGACCTTTTGTCGGCAATTATAATCCTTGIGGCAACCTACATC[c]
S12-1	M**A**LLSAIIILVATYI	CCACATATGGCACTTTTGTCGGCAATTATAATCCTTGTGG
S12-2	M**ALLLAVFL**SAIIILVATYI	CCACATATGGCTCTGTTATTAGCAGTTTTTTTGTCGGCAATTATAATCCTTGTGGC
S12-3	**M**AIIILVATYI	CCACATATGGGCAATTATAATCCTTGTGGCAACCTACATC
S12-5	M**AIIILVATYI**	CTCGACCTTCATATGGCTATTATAATATTAGTAGCAACCTACATCGTATCCCTCC
NATIVE PM2	MELLQWSALIILV	ATGGAGCTCCTCCAGCTTTGGTCGGCGCTTATAATCCTCGTAG[c]
P2-2	M**ALLLAVF**WSALIILV	CCACATATGGCTCTGTTATTAGCAGTTTTTTGGTCGGCGCTTATAATCCTCGTAG
NATIVE PM17	MELQISSAIIILV	ATGGAGCTTCAGATTTCGTCGGCGATTATAATCCTTGTAG[c]
P17-2	M**ALLLAVF**SSAIIILV	CCACATATGGCTCTGTTATTAGCAGTTTTTTCGTCGGCGATTATAATCCTTGTAG

[a] Modifications to the native enzyme by the indicated PCR primers are shown in bold.
[b] The ATG initiation codon is underlined.
[c] These are the original cDNA sequences.

quences were verified to insure that no errors had been introduced by the polymerase reactions (dyedeoxy terminator sequencing; ABI 373 sequencer). The constructs were then digested with *Nde*I and *Bgl*II and ligated into *Nde*I–*Bam*HI doubly digested pSBETa to give vectors S12-1-47, S12-2-47, S12-3-47, and S12-5-47. The oriented pBSSKII(+) constructs were also subcloned into pCWOri(+) by digestion with *Nde*I and *Hin*dIII and ligation into the identically digested expression vector. These vectors were designated S12-1-48, S12-2-48, S12-3-48, and S12-5-48.

The full-length limonene-6-hydroxylase in pBSSK(+) (14) was modified by the QuickChange protocol (Stratagene) using the forward primer NdePS12+ (5′-CAAAAAAGAACATATGGAGCTCGACC-3′) and the reverse primer NdePS12− (5′-GTCGAGCTCCATATGTTCTTTTTTGTTTTTTCTCG-3′) in order to introduce an *Nde*I site in frame with the ATG initiation codon. The *Nde*I–*Nar*I fragment from this vector was then inserted into S12-2-48 (above), which had been digested with the same restriction enzymes, in order to replace the 5′-terminus of the cDNA. This full-length limonene-6-hydroxylase construct in pCWOri(+) was designated S12-0-48.

Construction of E. coli expression vectors for limonene-3-hydroxylase. The original cDNA encoding limonene-3-hydroxylase (PM2, CYP71D15) in pBSSK(+) (14) was used as a template for PCR amplification using *Pfu* polymerase (Stratagene) with the forward primer P2-2 (Table I), which substituted the 5′-terminus of CYP17A as before and installed an *Nde*I restriction site at the starting methionine, in conjunction with reverse primer P2-4 (5′-AAAGAAGTAAAGCTTGATCATGAGGAAGGATCGTA-3′), which introduced a *Hin*dIII site following the stop codon. The original cDNA encoding isoform PM17 (CYP71D13, also in pBSSK(+) (14)) was amplified using forward primer P17-2 (Table I), in order to substitute the CYP17A terminus and add the *Nde*I site, in conjunction with reverse primer P2-4 as before. The blunt-ended PCR products were ligated into the *Srf*I site of pCR-Script AmpSK(+) (Stratagene) to yield the corresponding vectors P2-2-46 and P17-2-46; sequencing, as before, confirmed that no errors had been introduced by the polymerase reactions. These constructs were then digested with *Nde*I and *Hin*dIII and ligated into *Nde*I–*Hin*dIII doubly digested pCWOri(+) to provide vectors P2-2-48 and P17-2-48.

Expression of cytochrome P450 limonene hydroxylases in E. coli and enzyme isolation. Vectors based on pSBET were propagated in *E. coli* XL1Blue cells, and the purified plasmids obtained were transformed into *E. coli* BL21(DE3)pLysS expression cells (Stratagene) by standard protocol (20). Single colonies were isolated, grown overnight at 37°C in Luria-Bertani medium containing 30 μg chloramphenicol/ml and 34 μg kanamycin/ml, and then used to inoculate 500 ml of Terrific broth (20) containing the same antibiotics. When A_{600} of the suspended culture reached ~0.5, 75 mg/liter of δ-aminolevulinic acid and 1 mM thiamine HCl were added. After 1 h, IPTG was then added to a final concentration of 1 mM and the culture was incubated at 28°C for 24 to 60 h with moderate shaking (200 rpm). The vectors based on pCWOri(+) were amplified in *E. coli* cells as before and transformed into *E. coli* JM109 expression cells (Stratagene) by standard protocol (20). Preparation of the inoculum and expression conditions were identical to those described above except for the antibiotic which in this case was ampicillin (at 50 μg/ml).

After incubation, an aliquot of the cells was retained for analysis, while the remaining cells were harvested, disrupted by lysozyme treatment, cold shock, and final sonication, and the light membranes were isolated according to a published protocol (21). Hydroxylase expression levels obtained with the different constructs and host cells were determined by SDS–PAGE (22) and immunoblotting using rabbit polyclonal antibodies prepared against the native, purified cytochrome P450 limonene-6-hydroxylase from spearmint (14, 23) and by CO-difference spectroscopy (24) using a Perkin–Elmer Lambda 18 spectrophotometer equipped with an end-on photomultiplier tube to minimize the influence of light scattering. Intact cells or membrane preparations suspended in TE buffer (50 mM Tris–HCl (pH 7.4) containing 1 mM EDTA), plus 0.2% (v/v) reduced Triton X-100 and 20% (v/v) glycerol were evaluated, as were solubilized, purified, and reconstituted preparations for which hydroxylase activity assays were also conducted. Protein content was estimated by the method of Bradford (25) following solubilization and/or acetone precipitation when necessary, or by a modified bicinchonic acid method (26).

For solubilization and subsequent purification of the limonene-6-hydroxylase, the isolated microsomes (1-liter scale preparation) were resuspended in 25 mM Tris buffer (pH 7.0) containing 1 mM DTT, 0.1 mM EDTA, 50 μM BHT, and 20% (v/v) glycerol (buffer A) using a Ten-Broeck homogenizer. While stirring on ice, an equal volume of the same buffer containing 3% (v/v) Emulgen 911 was slowly added and stirring was continued for 1 h. The suspension was then centrifuged at 150,000*g* (90 min). To the resulting supernatant (solubilized protein), an equal volume of buffer A containing 60% polyethylene glycol (MW = 3,350, w/v) was slowly added over 15 min while stirring on ice. The mixture was then slowly brought to 50 mM $MgCl_2$ by addition from 1.5 M stock while stirring for an additional 15 min. The suspension was centrifuged at 150,000*g* (90 min) and the resulting pellet was resuspended using a glass homogenizer to a cytochrome P450 concentration of 5 nmol/ml in buffer A containing 0.4% Emulgen 911 without EDTA (buffer B), and then dialyzed exhaustively against this buffer containing 0.2% Emulgen 911.

The resulting reddish-brown material was applied to a column of

O-diethylaminoethyl-Sepharose (20 nmol cytochrome P450 to 10 ml column volume), and the column was slowly eluted with buffer B while monitoring the eluted fractions spectroscopically and by SDS–PAGE. Fractions containing cytochrome P450 that were devoid of other cytochromes (cytochrome b_5 and *d*- and *o*-complexes) and that yielded a ~57-kDa protein by SDS–PAGE were combined and concentrated (Millipore YM-30 membrane). For the limonene-6-hydroxylase expressed from S12-2-48 (pCWOri(+) in JM109 cells) the average yield to this point was ~40% with a specific content of about 10 nmol cytochrome P450/mg protein.

The partially purified material was next applied to a 1.7 ml column of SpectraPore HQ-20 that was previously equilibrated with 5 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTT, 0.2% (v/v) Emulgen 911, and 20% (v/v) glycerol (buffer C). The column was washed with 30 ml of the same buffer and then eluted with a linear gradient from 5 to 250 mM potassium phosphate in the same buffer (20 ml gradient) while monitoring the effluent as before. The appropriate pooled fractions (~30% yield with specific content of about 13 nmol cytochrome P450/mg protein) were next applied to a 1.7 ml column of ceramic hydroxyapatite previously equilibrated with buffer C. After washing the column with 30 ml of the same buffer, protein was eluted with a linear gradient from 5 to 80 mM potassium phosphate in buffer C (30 ml).

The resulting material (eluting at 25–75 mM phosphate, in ~20% yield with specific content of ~15 nmol cytochrome P450/mg protein) appeared to be homogeneous by SDS–PAGE (a single ~57-kDa band); however, in larger scale preparations, a doublet or triplet was observed in the purified material by SDS–PAGE (53–57 kDa range, with pI 6.1–6.2 by isoelectric focusing). Since these multiple species were detected by immunoblotting with the anti-limonene-6-hydroxylase polyclonal antibody preparation (data not shown), and could be observed by Western blot analysis of the initial membrane preparations, they are believed to represent translational truncation products or partially proteolyzed forms resulting from expression in the *E. coli* host.

The use of Emulgen 911 in the solubilization and purification protocol severely compromised the ability to obtain substrate binding spectra with these preparations. Nevertheless, hydroxylase activity could be reconstituted with purified, recombinant NADPH-cytochrome P450 reductase from spearmint (Haudenschild and Croteau, unpublished) by the method described below, although the yields were low (<10%) because of residual detergent.

For the solubilization and purification of the limonene-3-hydroxylase, the corresponding light membranes (1-liter scale preparation) were similarly treated with buffer A (at pH 7.2), with the final Emulgen 911 content brought to 1% (v/v). The polyethylene glycol-$MgCl_2$ precipitation step was eliminated in this case, and the solubilized cytochrome P450 limonene-3-hydroxyalse was applied directly to an *O*-diethylaminoethyl-Sepharose column (10 ml) that was previously equilibrated with buffer A (without EDTA and containing 0.2% Emulgen 911) and washed with the same buffer. Cytochrome P450 limonene-3-hydroxylase was then eluted with this buffer containing 100 mM KCl, and the eluent was concentrated by ultrafiltration (Centriprep-50) and desalted (Bio-Rad Econopac 10 DG) into 5 mM potassium phosphate (pH 5.8) containing 1 mM DTT, 0.2% (v/v) Emulgen 911, and 20% (v/v) glycerol (buffer D).

The concentrate was next applied to a 12-ml column of SP-Sephadex C-50 (Sigma) that was previously equilibrated with buffer D and then washed with 40 ml buffer D followed by 30 ml of buffer D that had been adjusted to pH 7.4. The hydroxylase was then eluted with 240 mM potassium phosphate (pH 7.4) containing 1 mM DTT, 0.2% Emulgen 911, and 20% glycerol. For the PM17 version of the limonene-3-hydroxylase (expressed from P17-2-48), the overall yield from light membranes was about 30% with a minimum specific content of 8 nmol cytochrome P450/mg protein; additionally, the recovery of the C3-hydroxylase in light membranes was substantially less than for the C6-hydroxylase when compared to the levels of the cytochrome present initially in the corresponding *E. coli* cells. As similarly observed with the C6-hydroxylase, SDS–PAGE of the purified C3-hydroxylase revealed the presence of a doublet (55 and 53 kDa) that could not be resolved by subsequent chromatography on hydroxyapatite or HQ-20 matrices. As with the limonene-6-hydroxylase, the presence of Emulgen 911 prevented the recording of useful binding spectra; however, hydroxylase activity could be reconstituted in low yield (<10%) using the recombinant NADPH-cytochrome P450 reductase from spearmint as described below for routine measurement.

Routine measurement of limonene hydroxylase activity expressed in E. coli. Limonene hydroxylase activity was generally measured *in vitro* by functional reconstitution of membrane preparations with the purified, recombinant NADPH-cytochrome P450 reductase from spearmint. Briefly, 125 μl of 100 mM Tris–HCl buffer (pH 7.4) containing 200 mU cytochrome P450 reductase, 250 mM KCl, 50 mM $MgCl_2$, and *E. coli* membranes containing 5 to 100 pmol cytochrome P450 (by CO-difference spectra) were incubated at room temperature for 30 min with gentle agitation. The same buffer (875 μl), containing the remaining reactants, was then added to obtain final concentrations of 300 μM (−)-limonene, 500 μM NADPH, 2 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase, and 5 μM each FAD and FMN. After 2 h incubation in a sealed tube at 31°C with gentle agitation (the time was reduced to 10 min in kinetic assays to insure linearity), the reaction was stopped on ice, followed by the addition of 25 nmol (+)-camphor (as internal standard) and extraction three times with 1.5 ml of diethyl ether. The combined ether extracts were then dried by passage through a short column of silica gel and $MgSO_4$, concentrated under N_2, and the sample was analyzed by combined capillary GC (cool, on-column injection)-MS (electron impact, 70 eV full spectrum, Hewlett-Packard 6890 GC-MSD) using a 0.25 mm i.d. by 30 m fused silica column coated with AT-1000 (Alltech, Deerfield, IL) programmed from 35 to 45°C at 50°C/min (5 min hold) and then to 230°C at 10°C/min with H_2 as carrier at 13.5 psi. The identity of the products was confirmed by coincidence of retention time and matching of full mass spectrum with the authentic standard; quantification was by electronic integration based on the internal standard. The product distribution generated by each enzyme was determined at saturating levels of substrate, and with sufficiently large preparations to insure accuracy by GC-MS peak integration of total ion current.

(−)-4*S*-Limonene, (−)-*cis*-carveol, (−)-*trans*-carveol, and (+)-camphor (internal standard) were obtained from Aldrich Chemical Co. (Milwaukee, WI), and (−)-*cis*-isopiperitenol and (−)-*trans*-isopiperitenol were synthesized from (−)-limonene as previously described (27). The separation of *cis*- and *trans*-monoterpenol isomers and the purification of the substrate and all reference standards (to >99%) were accomplished by argentation TLC (28, 29).

Binding constants for the (−)-limonene substrate were determined spectrophotometrically by standard protocol (30) using the Perkin–Elmer Lambda 18 spectrophotometer. Membrane preparations were diluted to 0.2 mg protein/ml in 0.1 M potassium phosphate buffer (pH 7.4), divided into two cuvettes and the baseline corrected. Difference spectra were recorded sequentially after addition of increasing amounts of limonene (0.5 to 100 μM) diluted in DMSO as solvent, an equal amount of which was added to the control cuvette, and binding constants were deduced from the double reciprocal plot of spectral amplitudes versus substrate concentrations.

Construction of S. cerevisiae expression vectors for limonene-6-hydroxylase. The vector pYeDP60 (31) was utilized for expression of the full-length and N-terminally modified versions of the spearmint cytochrome P450 limonene-6-hydroxylase. This *E. coli–S. cerevisiae* replicative shuttle vector contains the *GAL10–CYC1* hybrid promoter, which permits repression of gene expression in the presence of glucose and allows high level expression in the presence of galactose. For selection of transformants, this vector also contains an *ADE2* marker gene (for adenine auxotrophy) and a *URA3* marker

gene (for uracil auxotrophy). The previously described pBSSKII(+) constructs that provide the different 5′-terminally modified sequences (S12-1-46, S12-2-46, S12-3-46, and S12-5-46) were doubly digested with *Bam*HI and *Kpn*I and ligated into pYeDP60 that was similarly restricted (partial *Bam*HI digestion was required for this purpose due to the presence of an internal *Bam*HI site in the hydroxylase cDNA). The vectors were designated, correspondingly, S12-1-49, S12-2-49, S12-3-49, and S12-5-49. The native, full-length limonene-6-hydroxylase cDNA in pYeDP60 (designated S12-0-49) was obtained by simply replacing the *Bam*HI fragment corresponding to the 5′-terminus of construct S12-2-49 with the *Bam*HI fragment of the original SM12 cDNA, thus leaving the 20 original untranslated nucleotides upstream of the initiating ATG.

Construction of S. cerevisiae expression vectors for limonene-3-hydroxylase. The original PM2 clone in pBSSK(+) (14) was digested with *Not*I and *Eco*RV and blunted with the Klenow fragment of DNA polymerase I (20). This blunt-ended, full-length version was then ligated into the *Sma*I site of pYeDP60 to yield P2-0-49. The original PM17 limonene-3-hydroxylase clone in pBSSK(+) (14) was digested with *Bam*HI and this full-length fragment was ligated into the *Bam*HI site of pYeDP60 to yield P17-0-49.

Expression of cytochrome P450 limonene hydroxylases in S. cerevisiae and enzyme isolation. Recombinant pYeDP60 plasmids were transformed into *S. cerevisiae* by the lithium chloride method (32). The yeast host cells employed were WAT11U and WAT21U kindly provided by D. Pompon (Gif-sur-Yvette, France). The expression cells contain genomic insertions, into the endogenous NADPH-cytochrome P450 reductase (*CPR1*) locus, of *ATR1* or *ATR2*, the two NADPH-cytochrome P450 reductase genes from *Arabidopsis thaliana* (33). Transformants were plated on SGI medium [20 g glucose/l, 1 g bactocasamino acids (Difco)/l, 6.7 g yeast nitrogen base without amino acids (Difco)/l, and 40 mg DL-tryptophan/l], and individual colonies were grown overnight in 30 ml of the same liquid medium. The culture was then diluted 1 to 50 into YPGE complete medium [5 g glucose/l, 10 g yeast extract (Difco)/l, 10 g bactopeptone (Difco)/l, and 3% (v/v) ethanol], and grown at 28°C with agitation (300 rpm) until a cell density of $\sim 8 \times 10^7$ cells/ml was reached. The cells were then induced by the addition of 10% (v/v) of a sterile aqueous solution of 20% galactose. The cells were harvested 15 h after induction (at 28°C) and the microsomes were prepared by established protocols (31). Briefly, the cultures were cooled at 4°C for 1 h following the induction period, then the cells were harvested by centrifugation and washed with 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 100 mM KCl. Following a second centrifugation, the cells were resuspended in a minimum volume of TES buffer (50 mM Tris–HCl (pH 7.4), containing 1 mM EDTA and 0.6 mM sorbitol) and mechanically disrupted by shaking with glass beads at 0–4°C.

Following low speed centrifugation (10,000*g*, 10 min) to remove dense material, microsomes were harvested by ultracentrifugation (140,000*g*, 90 min) and then resuspended in the previously described TE buffer additionally containing 20% (v/v) glycerol. Expression levels resulting from the various constructs were evaluated by SDS–PAGE and immunoblotting, and by CO-difference spectroscopy as before, either directly with intact cells or with the yeast microsome preparations suspended in TE buffer.

Measurement of limonene hydroxylase activity expressed in S. cerevisiae. In vitro assays of limonene hydroxylase were performed directly with the isolated yeast microsomes that contain the recombinant *A. thaliana* NADPH-cytochrome P450 reductase. Activities were determined in a 1-ml assay mixture containing microsomal membranes (50 to 200 pmol cytochrome P450), 0.4 mM NADPH, 300 μM (−)-limonene, and 5 μM each FAD and FMN, as well as a regenerating system comprised of 2 mM glucose-6-phosphate and 0.5 U of glucose-6-phosphate dehydrogenase, in TE buffer. After 2 h incubation in sealed tubes at 31°C under gentle agitation (the time was reduced to 10 min in kinetic assays to insure linearity), the reactions were stopped, internal standard was added, and the mixtures were extracted with ether as before, followed by GC-MS analysis as described above.

RESULTS AND DISCUSSION

Expression of Limonene-6-Hydroxylase in E. coli

Two vectors were used to evaluate the expression of cytochrome P450 limonene-6-hydroxylase from spearmint in *E. coli*. The first, pCWOri(+), employs the *LacUV5-Tac* promoter and has been used successfully to functionally express high levels of eukaryotic cytochrome P450 (18). The second, pSBET, employs the T7 promoter and additionally contains the *arg*U gene for the tRNA that allows use of the arginine codons AGA and AGG (19). The latter vector has been employed successfully to functionally express plant genes encoding enzymes of terpenoid biosynthesis, especially those that specify rare arginine codon usage (for *E. coli*) (34).

N-Terminal modifications of limonene-6-hydroxylase were designed based on literature precedent for the successful expression of eukaryotic cytochrome P450 genes in *E. coli* (18, 21, 35, 36). A total of nine constructs were prepared, five in pCWOri(+) and four in pSBET, all of which involved replacement of the second codon of the original cDNA with GCT (specifying alanine) preferred for expression of the *LacZ* gene from the *LacZ* promoter in *E. coli* (Table I). Constructs S12-1-47 (pSBET) and S12-1-48 (pCWOri(+)) correspond to truncation of the three amino acids (ELD) following the initiating methionine of the native form (S12-0-47/48), in order to remove charged residues in this hydrophobic stretch that could alter solubility (37, 38). Constructs S12-2-47 and S12-2-48 correspond to deletion of five residues (aa2–aa6) of the native form, with substitution of the N-terminal nine residues of the CYP17A bovine 17α-hydroxylase (net change of seven residues) such that the distance between the first amino acid and the beta sheet breaker [(K)R, localized at position 25 in CYP17A] was conserved (18). Constructs S12-3-47 and S12-3-48 correspond to more substantial truncation of the largely hydrophobic terminus (six residues), thus leaving only the 13 residues of the predicted membrane anchor. The same truncation was employed in constructs S12-5-47 and S12-5-48 but the nucleotide sequence was modified by silent mutations to increase the AT content.

Preliminary evaluation of cytochrome P450 production, by CO-difference spectra, consistently showed much higher expression levels for the same coding sequence (in pCWOri(+)) in *E. coli* host JM109 compared to XL1Blue cells; therefore, all subsequent expression studies were carried out in *E. coli* JM109 cells. Time-course studies revealed that maximum production of cytochrome P450, again by evaluation of CO-difference spectra, was obtained after 30 to 40 h of induction, and that maximum production was reduced

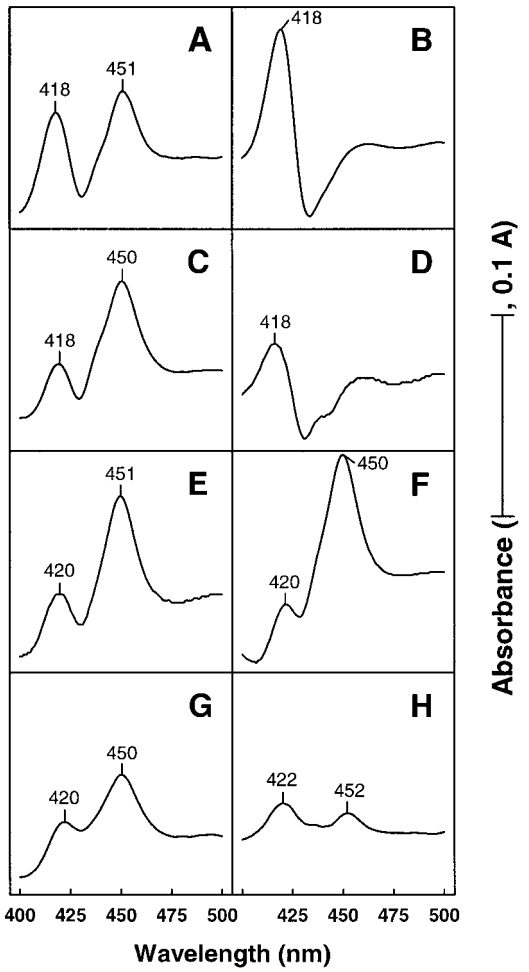

FIG. 2. CO-difference spectra of intact cells and membrane fractions for the limonene hydroxylases expressed from different constructs. (A) pCWOri(+)-S12-2-48-JM109 intact cells; (B) pCWOri(+)-empty vector-JM109 intact cells; (C) pCWOri(+)-S12-2-48-JM109 membranes; (D) pCWOri(+)-S12-0-49-JM109 membranes; (E) pCWOri(+)-P2-2-48-JM109 intact cells; (F) pCWOri(+)-P2-2-48-JM109 membranes; (G) pYeDP60-S12-0-49 WAT11U membranes; (H) pYeDP60-P17-0-49 WAT11U membranes. Membrane fractions were diluted to 0.5 mg protein/ml and pelleted intact cells were resuspended in the same volume of Tris buffer as described in Materials and Methods.

by half after 65 h (data not shown). Accordingly, all subsequent expression studies were carried out with an induction period of 36 h. The pCWOri(+)-based constructs S12-1-48, S12-2-48, S12-3-48, and S12-5-48 all yielded a readily measurable cytochrome P450 absorbtion peak (450 nm) in the CO-difference spectrum of the corresponding intact cells (see, for example, Fig. 2A), whereas the full-length, unmodified native sequence (S12-0-48) in the same vector and host failed to produce a detectable cytochrome P450 signal. The most efficient expression, determined by this means, was obtained with construct S12-2-48 (substitution of the bovine 17α-hydroxylase N-terminus), with maximum spectrally measurable cytochrome P450 production of 350 nmol/l culture. Lower expression levels were observed with constructs S12-1-48 and S12-3-48, with substantially less with construct S12-5-48 (Table II). The absorbance peak at about 420 nm observed in the difference spectra (see Fig. 2A) was not indicative of degraded cytochrome P450, as this signal was also observed in the difference spectrum of control *E. coli* JM109 cells transformed with the empty pCWOri(+) vector (Fig. 2B). Subsequent results (see below) suggest that the species absorbing at ~420 nm represent other heme proteins.

Cytochrome P450, as measured by CO-difference spectra (see Fig. 2C), was also recovered in the membranes isolated from *E. coli* JM109 cells that expressed constructs S12-2-48 (810 pmol/mg protein), S12-1-48 and, to a lesser extent, S12-3-48 (Table II), whereas no detectable cytochrome P450 was observed by this means in the membrane preparations of these cells that expressed constructs S12-5-48 and S12-0-48 (Fig. 2D), as might be expected from the prior results. Consistent with these spectrophotometric measurements, the expression in *E. coli* of all constructs except S12-0-48 and S12-5-48 yielded protein extracts in which a protein species of the appropriate size upon SDS–PAGE (~57 kDa) was recognized by rabbit polyclonal antibodies directed against the native spearmint limonene-6-hydroxylase (Fig. 3). The calculated yield for the cytochrome expressed from S12-2-48 (120 nmol/l culture) and from S12-1-48 (55 nmol/l culture) that was recovered in membrane preparations was 20 to 30% of that present in intact cells. These recovery levels are in the range of previous observations made with similar bacterial expression systems for cytochrome P450 (21). Significantly, the difference spectra of membrane preparations showed little absorbance at 420 nm (see Fig. 2C for example), indicating minimal conversion of the cytochrome to the inactive, low spin, P420 form, and suggesting that the 420 nm peak observed in spectra of intact cells (Fig. 2A) was due to other heme-containing proteins synthesized by the bacteria in the presence of excess δ-aminolevulinic acid.

Although the identical constructs were prepared in pSBET (i.e., the 47-series), little or no expression from this vector was observed in *E. coli* BL21(DE3)pLysS host cells when evaluated by CO-difference spectra (intact cells or membranes), immunoblotting, or activity assay following reconstitution with NADPH-cytochrome P450 reductase (see below). Even for construct S12-2-47, which was successfully expressed from pCWOri(+), little expression from pSBET was observed (<10 nmol P450/l), with or without induction with IPTG, in either the 140,000*g* supernatant or microsomal pellet, or under a range of induction conditions from 0 to 60 h (with intact cells and membrane preparations).

TABLE II

Expression of Modified Versions of the Limonene Hydroxylases in Different Vectors and Hosts

Vector–Host–Construct	P450 Content		Hydroxylation[a]	
	Intact cells (nmol/l)	*Microsomes* (pmol/mg protein)	*Rate* (nmol/h/mg protein)	*Spec. act.* (min^{-1})
pCWOri(+)-JM109-S12-0-48	0	0	0	0
pCWOri(+)-JM109-S12-1-48	280	400	247 ± 36	10.3 ± 1.5
pCWOri(+)-JM109-S12-2-48	350	810	369 ± 15	7.6 ± 0.3
pCWOri(+)-JM109-S12-3-48	150	150	88 ± 13	9.8 ± 1.5
pCWOri(+)-JM109-S12-5-48	trace	0	0	0
pCWOri(+)-JM109-P2-2-48	500	1400	2260 ± 400	26.9 ± 4.7
pCWOri(+)-JM109-P17-2-48	265	420	391 ± 5	15.5 ± 0.2
pYeDP60-WAT11U-S12-0-49	n.d.	527	1510 ± 41	47.9 ± 1.3
pYeDP60-WAT21U-S12-0-49	61	328	1675 ± 26	85.1 ± 1.3
pYeDP60-WAT11U-P2-0-49	0	50	31.8 ± 3.3	10.6 ± 1.1
pYeDP60-WAT11U-P2-0-49	0	44	36.2 ± 7.1	13.7 ± 2.7
pYeDP60-WAT11U-P17-0-49	14	66	570 ± 26	143.8 ± 6.2
pYeDP60-WAT21U-P17-0-49	n.d.	110	763 ± 28	115.6 ± 4.2

Note. n.d., not determined.

[a] For the hydroxylases expressed in *E. coli*, functional reconstitution of activity was obtained by the direct addition of recombinant NADPH-cytochrome P450 reductase to membrane preparations.

Purification and Reconstitution of Recombinant Limonene-6-Hydroxylase

Construct S12-2-48 (bearing the 17α-hydroxylase N-terminus), when expressed from pCWOri(+) in JM109 cells, consistently afforded the highest production yields of recombinant limonene-6-hydroxylase, and therefore was used for purification and reconstitution experiments. Following the standard preparation of membranes from the transformed, induced cells, the cytochrome was solubilized with Emulgen 911, precipitated with polyethylene glycol, and purified to apparent homogeneity, with good yield and specific content, by anion-exchange and hydroxyapatite chromatography (Table III). Binding spectra could not be evaluated with these preparations due to the presence of residual detergent; however, C6-hydroxylation activity could be reconstituted with NADPH-cytochrome P450 reductase (in yields approaching 10% of starting material).

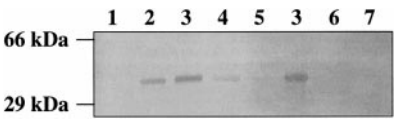

FIG. 3. Immunoblot analysis of membrane fractions from *E. coli* expressing (from pCWOri(+)) different limonene hydroxylase constructs. The lanes are the protein samples corresponding to S12-0-48 (1), S12-1-48 (2), S12-2-48 (3), S12-3-48 (4), S12-5-48 (5), P2-2-48 (6), and P17-2-48 (7). Membrane proteins (5 μg) were loaded in each lane and the blot was probed with polyclonal antibodies raised in rabbits against the purified, native limonene-6-hydroxylase from spearmint.

Characterization of Recombinant Limonene-6-Hydroxylase

Because of the limited utility of solubilized preparations for detailed characterization of the limonene-6-hydroxylase, substrate binding spectra (typical Type I (30)) were evaluated with membrane preparations (Fig. 4A), and the response to concentration thus employed to calculate a dissociation constant of 3.8 ± 0.9 μM. This K_d value compares to a K_m value of 20 μM previously estimated for the native limonene-6-hydroxylase in microsomal preparations from spearmint leaf oil glands (13).

Product distribution by GC-MS analysis (Fig. 5) was also evaluated with this version of the recombinant limonene-6-hydroxylase at saturating levels of substrate using membrane preparations that were functionally reconstituted by direct addition of the recom-

TABLE III

Purification of Recombinant (−)-Limonene-6-Hydroxylase

	Total protein[a] (mg)	P450 (nmol)	Spec. content (nmol/mg)	Yield (%)
E. coli membranes	149	121	0.81	100
Solubilized protein	96	103	1.07	85
PEG-$MgCl_2$ ppt.	52.5	74.5	1.42	62
DEAE-Sepharose	5.15	50.0	9.7	41
SpectraPore HQ-20	2.72	34.8	12.8	29
Hydroxyapatite	1.62	24.3	15.0	20

[a] One-liter preparation.

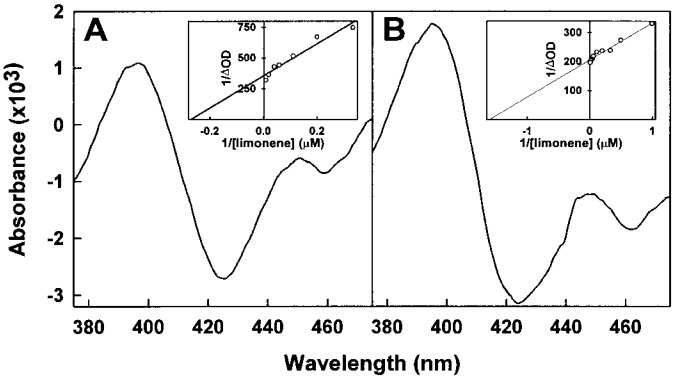

FIG. 4. Limonene-induced binding spectra obtained with membranous pCWOri(+)-S12-2-48-JM109 at 104 pmol/ml (A) and pCWOri(+)-P2-2-48-JM109 at 294 pmol/ml (B). Spectra were obtained as described under Materials and Methods at a saturating concentration of (−)-limonene (100 μM). Insets are double reciprocal plots of spectral amplitude versus limonene concentration for the respective hydroxylases. Calculation from the extinction coefficient (39) indicated 30% conversion of the 6-hydroxylase and 14% conversion of the 3-hydroxylase to the high spin state.

binant NADPH-cytochrome P450 reductase from mint. The anticipated product *trans*-carveol was formed at greater than 99% by this enzyme preparation (Fig. 5A), consistent with prior product analyses of the native microsomal enzyme (13) and the recombinant enzyme expressed in the baculovirus-*Spodoptera* system (14).

Expression of Limonene-3-Hydroxylase in E. coli

Based on the successful expression from pCWOri(+) in *E. coli* JM109 cells of the spearmint limonene-6-hydroxylase that had been modified by N-terminal substitution of the bovine 17α-hydroxylase sequence, the two peppermint limonene-3-hydroxylase clones (PM2 and PM17) were identically altered at the 5′-terminus to yield the corresponding plasmids designated P2-2-48 and P17-2-48 (Table I). Both were expressed under the identical conditions employed for the limonene-6-hydroxylase, and the production of cytochrome P450 was monitored as before. CO-difference spectra using the intact cells (Fig. 2E) indicated maximum production levels of 500 nmol and 265 nmol cytochrome P450 per liter of culture for the P2-2-48 and P17-2-48 constructs, respectively; the cytochrome was also readily detected by this means in microsomes (Fig. 2F) prepared from the corresponding transformed bacterial cells (Table II).

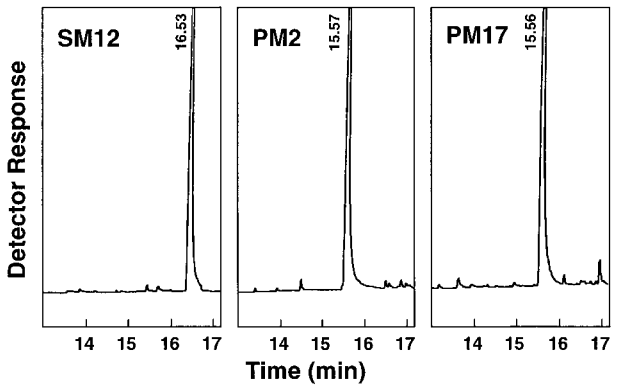

FIG. 5. Capillary GC-MS analysis of product distributions for recombinant SM12 (A), PM2 (B), and PM17 (C) hydroxylases. The pCWOri(+)-S12-2-48-JM109, pCWOri(+)-P2-2-48-JM109, and pCWOri(+)-P17-2-48-JM109 membrane fractions (100 pmol CO-detectable P450) were reconstituted with reductase and incubated for 1 h at 31°C prior to extraction and analysis as described under Materials and Methods. The products identified are (−)-*trans*-isopiperitenol (15.56 ± 0.01 min) and (−)-*trans*-carveol (16.53 min).

TABLE IV

Purification of Recombinant (−)-Limonene-3-Hydroxylase

	Total protein[a] (mg)	P450 (nmol)	Spec. content (nmol/mg)	Yield (%)
E. coli membranes	78.9	33.1	0.42	100
Solubilized protein	35.2	33.1	0.94	100
DEAE-Sepharose	19.6	23.9	1.22	72
SP-Sephadex	1.12	9.56	8.53	29

[a] One-liter preparation.

Based on the high level of sequence similarity between the spearmint limonene-6-hydroxylase and the peppermint limonene-3-hydroxylases, immunoblotting was attempted of the electrophoretically separated proteins extracted from transformed cells expressing the P2-2-48 and P17-2-48 constructs. Although sufficiently high levels of spectrally detectable limonene-3-hydroxylase were present (to have easily produced a signal for the limonene-6-hydroxylase), no protein could be detected with the polyclonal antibodies raised against the limonene-6-hydroxylase (Fig. 3), indicating that these antibodies did not significantly cross-react with the limonene-6-hydroxylase.

Purification and Reconstitution of Recombinant Limonene-3-Hydroxylase

The recombinant limonene-3-hydroxylase expressed from both P2-2-48 and P17-2-48 could be efficiently solubilized from light membranes of *E. coli* JM109 cells, as with the limonene-6-hydroxylase. The purification of the PM17 version of the C3-hydroxylase was affected by the combination of anion- and cation-exchange chromatography (Table IV). Although binding spectra could not be obtained with these preparations in the presence of Emulgen, activity could be functionally reconstituted in low yield (<10%) with recombinant NADPH-cytochrome P450 reductase from mint.

Characterization of Recombinant Limonene-3-Hydroxylase

As before, substrate binding spectra (Type I (30)) were evaluated with membrane preparations (illustrated for P2-2-48 in Fig. 4B), and the response to concentration was employed to calculate a dissociation constant of 0.66 ± 0.07 μM for the recombinant enzyme. This K_d value compares to a K_m value of 18 μM previously determined for the native limonene-3-hydroxylase in microsomal preparations from peppermint leaf oil glands (13).

Product distribution was evaluated with both P2-2-48 and P17-2-48 versions of the recombinant limonene-3-hydroxylase at saturating levels of substrate using membrane preparations that were reconstituted by direct addition of the recombinant NADPH-cytochrome P450 reductase from mint (Figs. 5B and 5C). Like the limonene-6-hydroxylase, the limonene-3-hydroxylase from peppermint is both regio- and stereospecific. Only the C3-equatorial (exo)-hydrogen atom is abstracted to give the equatorial (exo)-isomer (−)-*trans*-isopiperitenol as the sole product from (−)-limonene.

Expression of Limonene-6-Hydroxylase in S. cerevisiae

The unaltered form and the N-terminally modified versions of spearmint limonene-6-hydroxylase were expressed in *S. cerevisiae* using the vector pYeDP60 (31). Exactly the same N-terminal modifications were made as used previously for expression in *E. coli*, to yield the plasmids designated S12-0-49 (unaltered full length), S12-2-49, S12-3-49, and S12-5-49 (Table I). These constructs were tested in two yeast strains, WAT11U and WAT21U, which expressed two different isoforms of the *A. thaliana* NADPH-cytochrome P450 reductase. CO-Difference spectra were obtained using microsomal preparations from *S. cerevisiae* that expressed each version of the enzyme. All constructs yielded readily detectable levels of the hydroxylase that were very similar for both WAT11U (Fig. 2G) and WAT21U cells (not shown). Whereas S12-2-49 and S12-3-49 gave approximately the same yields of microsomal cytochrome P450 as S12-0-49 (~400 pmol/mg protein) (Table II), the microsomes from cells that expressed S12-5-49 consistently afford 2-3 times less cytochrome P450. To confirm expression based on CO-difference spectra, the above microsomal proteins were electrophoretically separated and probed with the anti-limonene-6-hydroxylase antibody to demonstrate the presence of immunoreactive protein, e.g., a band at 57 kDa for the unmodified S12-0-49 recombinant form (data not shown).

Limonene-6-Hydroxylase Activity in Recombinant S. cerevisiae Microsomes

Microsomes obtained from *S. cerevisiae* WAT11U and WAT21U cells that individually expressed the S12-2-49, S12-3-49, S12-5-49, and S12-0-49 constructs were tested for catalytic capability with (−)-limonene as substrate using the capillary GC-based assay. Since the WAT11U and WAT21U yeast cells express genomic versions of an *Arabidopsis* NADPH cytochrome P450 reductase, direct assay is possible without need for reconstitution. The highest rates of NADPH-dependent conversion to (−)-*trans*-carveol were observed with microsomes derived from construct S12-3-49, followed by S12-2-49 and the unmodified form S12-0-49 (Table II), and the lowest conversion rates were noted in microsomes derived from construct S12-5-49; specific activities for these enzymes were in the same range. The catalytic activities correlated well with the levels of cytochrome P450 determined by CO-difference spectra for the various constructs, and the constructs that were most suitable for expression in *E. coli* also proved to be suitable for expression in *S. cerevisiae*. Little difference in catalytic capacity was observed whether yeast strain WAT11U or WAT21U was used as host, indicating that both *Arabidopsis* NADPH-cytochrome P450 reductases are compatible with the mint hydroxylase. Detailed product analysis, as before, revealed the production of only *trans*-carveol by microsomal preparations containing all versions of the C6-hydroxylase.

Expression of Limonene-3-Hydroxylase in S. cerevisiae

Both of the limonene-3-hydroxylases of peppermint (PM2 and PM17) were expressed in unmodified form (P2-0-49 and P17-0-49, respectively) from pYeDP60 using both the WAT11U and WAT21U yeast strains. The level of cytochrome P450 determined to be present by CO-difference spectra in microsomes of WAT11U and WAT21U cells was approximately 80 pmol/mg protein for the expression of P17-0-49 (Fig. 2H) and about 50 pmol/mg protein for the expression of P2-0-49 (Table II).

Limonene-3-Hydroxylase Activity in Recombinant S. cerevisiae Microsomes

Although the levels of expression of the limonene-3-hydroxylases in the yeast system were low, the specific activity for the PM17 version was quite respectable (Table II), and catalysis by these microsomal preparations was readily observed in the capillary GC-based assay. As previously observed with the limonene-3-hydroxylase expressed in *E. coli*, the enzyme expressed

in yeast is absolutely regio- and stereospecific in yielding (−)-*trans*-isopiperitenol as the only product.

Conclusion

Heterologous overexpression of the peppermint and spearmint cytochrome P450 limonene hydroxylases in *E. coli* and yeast has provided relatively large amounts of protein for structure-function studies, and with which the precise product distributions and dissociation constants of these regiospecific enzymes could be readily determined. Comparison of production levels obtained from the different constructs revealed that minimal alteration in the N-terminal coding sequence can markedly influence expression in *E. coli*, that the full-length forms expressed from pYeDP60 in yeast WAT11 and WAT21 were compatible with the *Arabidopsis* NADPH-cytochrome P450 reductases present in these cells, and that the limonene-6-hydroxylase appears to possess an inherently lower specific activity than the limonene-3-hydroxylase. The ease of expression in *E. coli*, coupled to successful reconstitution with the mint cytochrome P450 reductase, makes this an attractive system for *in vitro* studies. However, the direct use of yeast microsomes harboring recombinant hydroxylase and the *Arabidopsis* reductase is very convenient, particular for evaluating chimeras and other mutant proteins constructed in an attempt to define binding interactions that underlie regiospecificity, and primary structural features responsible for differences in specific activity.

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