Tetrahedron 72 (2016) 7263–7267

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Identification of a novel oxygenase capable of regiospecific hydroxylation of D-limonene into (+)-*trans*-carveol

M. Groeneveld [a], H.L. van Beek [a], W.A. Duetz [b], M.W. Fraaije [a,*]

[a] Molecular Enzymology Group, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
[b] Enzyscreen BV, Tingietersweg 127, 2031 ER Haarlem, The Netherlands

ARTICLE INFO

Article history:
Received 30 October 2015
Received in revised form 17 December 2015
Accepted 23 December 2015
Available online 29 December 2015

Keywords:
D-Limone
Carveol
Carvone
Cumene
Oxygenase
Hydroxylation

ABSTRACT

By genome sequencing and analysis, we have been able to identify a gene cluster encoding a four component oxygenase which is able to oxidize D-limonene, L-limonene, cumene, and indole. Heterologous expression of the complete oxygenase system in *Pseudomonas putida* S12 allowed efficient and highly regiospecific biotransformation of D-limonene into (+)-*trans*-carveol. The discovered oxygenase represents a promising biocatalyst for the biotechnological valorisation of D-limonene.

1. Introduction

Terpenes are widely distributed natural compounds with a range of interesting industrial applications. These compounds are classified based on the number of isoprene units, and commonly consist of two to eight units, leading to a high diversity of terpenes. D-Limonene is a cyclic monoterpenoid and is produced as side-product in the orange juice industry. It is attractive to develop a biotechnological process capable to convert D-limonene, which is often regarded as a waste product, into a more valuable product. One possible D-limonene-derived product would be (+)-carvone which theoretically can be formed by merely two oxidations. (+)-Carvone is a cyclic monoterpenoid, mainly found in the seeds of caraway (*Carum carvi*) or dill (*Anethum graveolens*). The compound has various applications in the fragrance, food and feed industries.[1,2] However, the availability, production costs and purity of (+)-carvone have thus far hampered the extensive usage for this application.

Conversion of D-limonene to (+)-carvone can occur via a two-step enzymatic process, with (+)-carveol as an intermediate product.[3] Several reports have appeared on the conversion of D-limonene into carveol by either isolated biocatalysts or the use of various microorganisms.[4–9] Thus far, the conversion of D-limonene to (+)-*trans*-carveol has been shown to take place with high regio- and enantio-selectivity when using some specific microbial isolates.[4–6] Until now, the enzyme(s) involved in this highly selective hydroxylation reaction remained elusive.

In this paper we described a newly discovered enzyme system which is able to hydroxylate D-limonene. The respective oxygenase was identified from the genome of *Pseudomonas* sp. PWD32 and is capable of the desired regioselective hydroxylation of D-limonene. We demonstrate the functional heterologous overexpression of this four component oxygenase in *P. putida* S12 and subsequent biotransformation of D-limonene into a single and enantiopure alcohol, (+)-*trans*-carveol (Fig. 1).

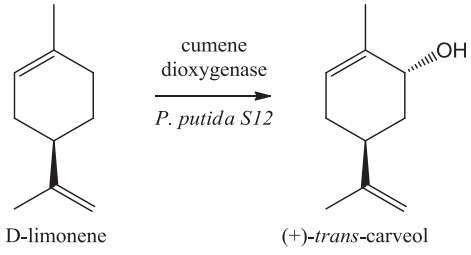

Fig. 1. Conversion of D-limonene into (+)-*trans*-carveol by cumene dioxygenase from *P.* sp. PWD32 expressed in *Pseudomonas putida* S12.

* Corresponding author. Tel.: +31 503634345; e-mail address: m.w.fraaije@rug.nl (M.W. Fraaije).

http://dx.doi.org/10.1016/j.tet.2015.12.061
0040-4020/

2. Results and discussion

2.1. Identification of the oxygenase from the *Pseudomonas* sp. PWD32 genome sequence

Pseudomonas sp. PWD32 was previously shown to exhibit the above depicted limonene-oxygenation activity when grown on toluene or naphthalene (Fig. 1).[5] This led to the hypothesis that the pathway harbouring the enzyme responsible for the formation of (+)-*trans*-carveol was part of the toluene/naphthalene degradation pathway which typically involves a toluene/naphthalene dioxygenase.[10] These four component oxygenases are also known to be able to catalyse hydroxylations and therefore such an oxygenase was considered as a likely candidate.[11,12] As toluene/naphthalene dioxygenases can be recognized at sequence level, we decided to sequence the genome of *Pseudomonas* sp. PWD32.

Toluene/naphthalene oxygenases are composed of four different proteins. Three alpha and three beta protomers form the heterohexameric oxygenase with the alpha subunits containing a Rieske-type iron sulfur cluster and a redox active mononuclear iron. The hexameric oxygenase relies on two other components, a reductase and a ferredoxin, that transfer electrons from NADH to the oxygenase. Since activity and substrate specificity is mainly determined by the alpha subunit, we used the sequence of an alpha subunit of a known toluene dioxygenase from *P. putida* to identify oxygenase homologs in the genome of *Pseudomonas* sp. PWD32. Only one putative dioxygenase gene cluster that contained a gene with significant sequence identity with the alpha subunit (63%) was found in the sequenced genomic DNA of *P.* sp. PWD32. Three genes encoding for the beta subunit, a ferredoxin and a reductase are present directly downstream of the alpha subunit encoding gene. Several other putative dioxygenase-encoding genes were found in the genome, but they all showed less than 30% sequence identity when compared with toluene dioxygenase.

When comparing the identified alpha subunit protein sequence with known alpha subunit sequences, the highest sequence identity is found with the alpha subunits of cumene dioxygenases (Fig. 2). In fact, a few protein sequences are found with an identity of more than 90%. These protein orthologs are present in different *Pseudomonas* species, and among them is the crystallized alpha subunit of the cumene dioxygenase from *Pseudomonas fluorescens* IP01.[13] These proteins are annotated as isopropylbenzene/cumene dioxygenases on the basis of previous experimental evidence.[14–16] All other homologs, with 83% or less sequence identity, are typically annotated as biphenyl or toluene dioxygenases.

The alpha subunit of the discovered cumene dioxygenase shares all essential and conserved residues in the alpha subunit with those of cumene, biphenyl, and toluene dioxygenases. These amino acids are involved in binding the iron sulfur cluster, the non-heme iron or are involved in forming a bridge between these two clusters. The amino acids lining the active site in cumene dioxygenase from *P. fluorescens* IP01 are only strongly conserved in the small subgroup that includes the discovered oxygenase.[13] As already noticed in the crystal structure of cumene dioxygenase, there are noticeable differences in side-chain orientations within the active site when comparing cumene dioxygenase to biphenyl dioxygenase from *R. jostii* RHA1.[17] These differences form the molecular basis for the markedly different substrate specificities.

2.2. Heterologous expression of the oxygenase cluster in *P. putida* S12

Initially, we aimed at expression of all four oxygenase subunits from *P.* sp. PWD32 in *Escherichia coli*, due to the plethora of genetic tools available for the latter organism. However, extensive expression trials using *E. coli* never resulted in cells expressing significant amounts of soluble protein and, as a consequence, they were not able to convert D-limonene. Therefore, we opted for *P. putida* S12 as alternative host strain for both oxygenase expression as well as subsequent biotransformations as *P. putida* S12 has been shown to be ideally suited for conversions of a range of industrially relevant compounds.[18] We used the pBTBX2 plasmid engineered by Prior et al. to overexpress the oxygenase gene cluster in *P. putida* S12.[19] Gratifyingly, under optimized expression conditions we observed good soluble expression of the alpha (predicted molecular weight 52,350 Da) and beta subunits (predicted molecular weight 21,789 Da) (Fig. 3). No additional prominent protein band was observed for either the ferredoxin (predicted molecular weight 11,719 Da) or the reductase (predicted molecular weight 39,635 Da) suggesting that the expression levels of the latter proteins are rather low.

2.3. Biotransformations using recombinant whole cells

To determine whether the recombinantly expressed oxygenase system was capable of converting D-limonene to (+)-*trans*-carveol, *P. putida* S12 cells expressing the oxygenase system were harvested and resuspended in buffer solution containing 1% (w/v) glucose. The cells were incubated overnight in closed scintillation vials, under vigorous stirring, in the presence of 6.2 mM D-limonene. We observed (+)-*trans*-carveol formation only for the induced cells harbouring the plasmid encoding the oxygenase gene cluster (Fig. A.1). No (+)-*trans*-carveol was observed using non-induced cells, or when using cells harbouring an empty pBTBX2 vector. Analysis of the reaction over time led to production of 2.5 mM (+)-*trans*-carveol (40% yield), with a maximum initial reaction rate

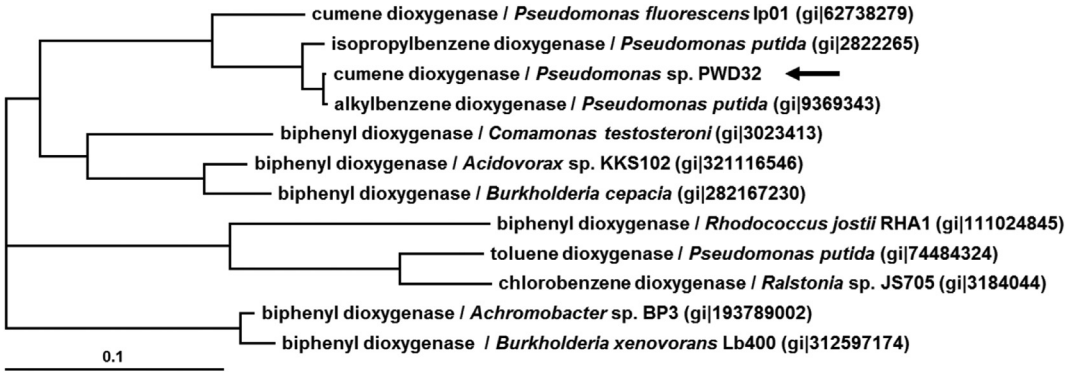

Fig. 2. Phylogenetic tree of the alpha subunit protein sequences of several known four component oxygenase homologs. The alpha subunit of the cumene dioxygenase identified from the genome of *P.* sp. PWD32 is indicated with an arrow.