Enantiospecific (*S*)-(+)-Linalool Formation from *β*-Myrcene by Linalool Dehydratase-Isomerase

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The linalool dehydratase-isomerase from *Castellaniella defragrans* strain 65Phen catalyzes in the thermodynamically unfavourable direction the hydration of *β*-myrcene to linalool and further the isomerization to geraniol, the initial steps in anaerobic *β*-myrcene biodegradation. We have now investigated the stereochemistry of this reaction. (*S*)-(+)-Linalool is formed with an enantiomeric excess of at least 95.4%. (*R*)-(–)-Linalool was not detected. This indicates an introduction of the hydroxy group on the *si*-face of *β*-myrcene.

Key words: Stereochemistry, Hydratase, Chiral Chromatography

Introduction

In enzyme-catalyzed processes, the reaction pathway is defined by the enzyme and its complex with the substrate. The active site of an enzyme structure determines the interaction with the substrate which often results in high stereospecificity. Classical examples are the reduction of nicotinamide-adenine-dinucleotides (NAD^+ and $NADP^+$) and of aldehydes, the hydration of fumarate, and aldose-ketose isomerase reactions (Fersht, 1998). Fumarase reversibly catalyzes the formation of (*S*)-malate. The crystal structure reveals a tetrameric protein and the presence of two binding sites for dicarboxylic acids per monomer. One site also contains a water molecule (Weaver and Banaszak, 1996; Weaver, 2005). Enoyl-CoA-hydratases, which act stereospecific on *α*,*β*-unsaturated acyl-CoA thiolesters, contain also a water molecule in the active site (Wu *et al.*, 2000; Bahnson *et al*., 2002).

We discovered recently a novel enzyme in the anaerobic biodegradation pathway of monoterpenes, a linalool dehydratase-isomerase (LDI) (Brodkorb *et al.*, 2010). In contrast to well-characterized enzymes acting on alkenes with adjacent polar groups, *e.g.* fumarate, the substrate *β*-myrcene has no polar group that may serve as anchor to bind the substrate and direct the reaction pathway. Hence, we explored whether the LDI catalyzes its reaction in a stereospecific manner.

Abbreviations: *ee*, enantiomeric excess; LDI/*ldi*, linalool dehydratase-isomerase protein/*gene*.

Material and Methods

Escherichia coli BL21 Star™ (Invitrogen, Darmstadt, Germany) containing the plasmids pET-42a(+)*ldi* or, as control, pET-42a(+) were grown in batch culture on lysogeny broth and induced with isopropyl-*β*-D-thiogalactopyranoside (Brodkorb *et al.*, 2010). Soluble enzyme fractions were obtained by cell disruption (French pressure cell press at 10.3 MPa), centrifugation for 90 min at 150000 x *g* and dialysis against 80 mM Tris-HCl, pH 9.0. Assays contained two phases, 500 *μ*l soluble extract and 500 *μ*l *β*-myrcene (~ 90%; Fluka, Neu-Ulm, Germany), and were performed under anoxic conditions and by horizontal shaking at 25 rpm and 37 °C. The protein content was 10 mg/ml as determined in a 200-*μ*l aliquot by the method of Bradford (1976) with bovine serum albumine as standard protein; concentrations were corrected for the unusual high binding of the Coomassie stain to albumin (Biorad, 1994).

Chiral analyses of the *β*-myrcene phase were performed using a gas chromatograph (Perkin Elmer Auto System XL; Überlingen, Germany) equipped with a flame ionization detector. Separation was achieved on a Hydrodex-*β*-6TBDM-column (25 m x 0.25 mm ID; Macherey-Nagel, Düren, Germany) by the following temperature

program: injection port temperature, 200 °C; column separation, 100 °C for 1 min, increasing to 116 °C at a rate of 2 °C/min, 116 °C for 0.5 min, increasing to 230 °C at a rate of 20 °C/min, 230 °C for 2.5 min; detection temperature, 250 °C. The split ratio was set to 1:30. Retention times were 6.5 min for (*R*)-(–)-linalool, 6.5 and 6.7 min for (*R*,*S*)-(±)-linalool (Fig. 2) and 11.6 min for geraniol.

In all analyses an 1-μl sample from the β-myrcene phase was injected directly into the gas chromatograph. The enantiomerspecific assays were measured as biological triplicates with 5 mM 3-pentanol as internal standard.

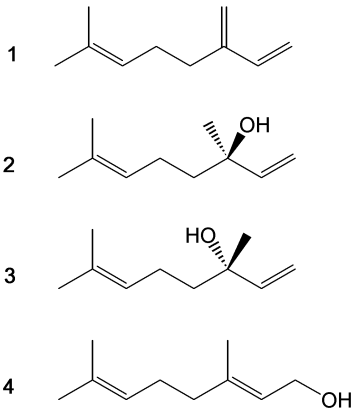

Fig. 1. Chemical structures of β-myrcene (**1**), (*R*)-(–)-linalool (**2**), (*S*)-(+)-linalool (**3**), and geraniol (**4**).

Results

Biotransformation of the acyclic monoterpene β-myrcene (Fig. 1) was studied with the linalool dehydratase-isomerase (LDI) produced in *E. coli* (Brodkorb *et al.*, 2010). The thermodynamically favoured direction is the isomerization of geraniol to linalool and the dehydration reaction to β-myrcene. To enforce the thermodynamically unfavoured direction, we applied a pure β-myrcene phase in a two-phase system. In addition, the organic phase served as solvent for the monoterpenoids (*R*)-(–)-linalool, (*S*)-(+)-linalool, and geraniol. These were sampled dissolved in β-myrcene and identified by chiral gas chromatography (Figs. 2 and 3). The sensitivity of detection in aqueous samples was reduced for geraniol, likely due to reactions of water with the monoterpenoids in the injector of the gas chromatograph (data not shown). Detection limits were 0.013 mM for both linalool enantiomers and 0.007 mM for geraniol in β-myrcene as solvent.

(*S*)-(+)-Linalool and geraniol were formed in the incubation experiment (Fig. 3), but (*R*)-(–)-linalool was not detectable. (*S*)-(+)-Linalool became visible after 1 h of incubation. The concentration reached a steady state of 1.8 mM and increased after 24 h to a level of 11 mM. Based on the detection limit, the enantiomeric excess (*ee*) of the formation of (*S*)-(+)-linalool was 95.4% *ee*. The geraniol concentrations changed accordingly to an early steady state concentration of 0.1 mM and increased after 24 h to 0.5 mM. Protein dena-

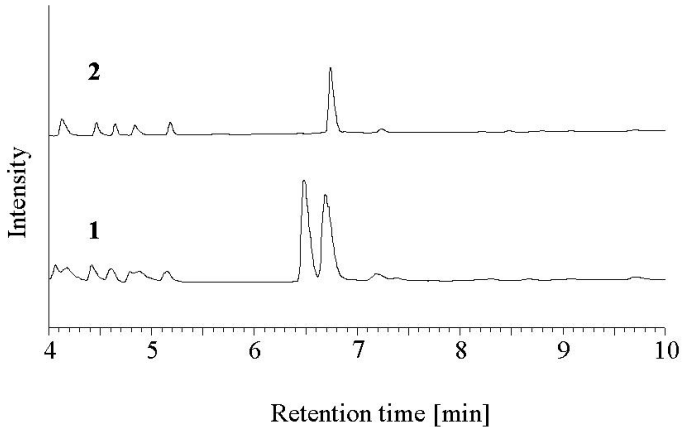

Fig. 2. Separation of linalool enantiomers using chiral chromatography. (1) (*R*,*S*)-(±)-Linalool in β-myrcene phase; (2) (*S*)-(+)-linalool formation after 12 h incubation.

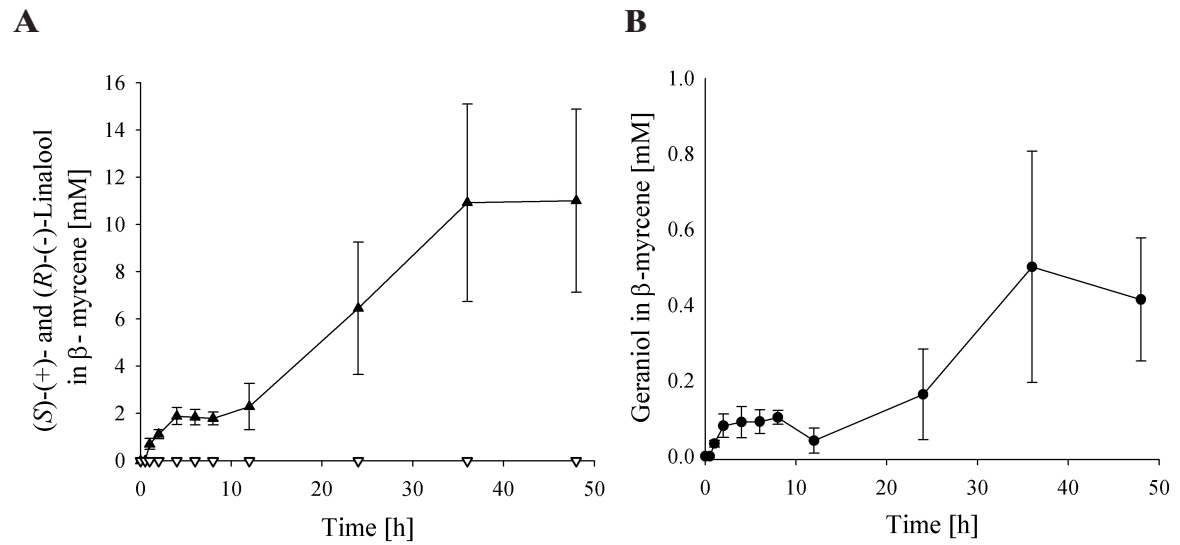

Fig. 3. Time course of LDI activity. Monoterpenes were measured in *β*-myrcene phase with a chiral GC column. (A) (*S*)-(+)-Linalool (▲) and (*R*)-(–)-linalool (▽) in *β*-myrcene; (B) geraniol in *β*-myrcene. Standard deviations are calculated from triplicate measurements.

turation became visible as turbidity in the aqueous phase after 24 h of incubation. Thus, a release of linalool and geraniol that were bound to hydrophobic patches of proteins into the *β*-myrcene phase may explain the late increase in the concentrations of (*S*)-(+)-linalool and geraniol. The observed ratio of geraniol to linalool of 1:18 in the early phase is close to the equilibrium value of 1:10 that was reported for another geraniol isomerase activity (Foss and Harder, 1997). The experiment showed a myrcene to linalool ratio of 8616:1 in the first 10 h of the experiment and of 1410:1 in the late phase. *β*-Myrcene is known to polymerize at room temperature resulting in a higher viscosity (Behr and Johnen, 2009). To take account of this reaction and other potential linalool-forming sources, control reactions with 80 mM Tris-HCl, pH 9.0, as aqueous phase and soluble extracts of *E. coli* pET-42a(+) in the aforementioned buffer were performed. Neither in the abiotic nor in the biotic control the conversion of *β*-myrcene to linalool and subsequent isomerization to geraniol was detectable.

The chemical isomerization of (*R*)-(–)-linalool within 144 h under assay conditions yielded less than 1% (*S*)-(+)-linalool. Thus, the possibility of an unnoticed (*R*)-(–)-linalool formation followed by rapid chemical isomerization to the (*S*)-(+)-enantiomer can be excluded (data not shown).

Discussion

The LDI catalyzes in the absence of oxygen the hydration of an alkene. The addition of the water molecule can occur on one or on both sides of the alkene. This study revealed a reaction on the *si*-face of the prochiral *β*-myrcene resulting in a high enantiospecific hydration reaction to (*S*)-(+)-linalool, with an *ee*-value of at least 95.4%. Previous experiments already exhibited a high substrate specificity of the enzyme: no other acyclic monoterpene or monoterpenoid was transformed (Brodkorb *et al.*, 2010).

Enantioselectivity is often observed in alkene hydrations that are in general activated by a polarization through an electron-withdrawing, adjacent carbonyl group, *e.g.* coenzyme A or acyl carrier protein thioesters (Schwab and Henderson, 1990; Leesong *et al.*, 1996; Wu *et al.*, 2000; Buckel *et al.*, 2005). However, the double bonds in *β*-myrcene are only slightly polarized by hyperconjugation. Furthermore the C-H bonds contribute electron density to the methylene carbon atom by an inductive effect. The resulting polarity is measurable by ^{13}C NMR spectroscopy. The methylene C atom has a chemical shift of 116 ppm. The ternary C_3 atom features a chemical shift of 146 ppm indicating a low electron density at the carbon atom (Honda, 1990). This

difference may direct the water addition according to Markovnikov´s rule.

The formation of linalool has never before been reported for biological *β*-myrcene utilization (Busmann and Berger, 1994; Iurescia *et al.*, 1999; Farooq *et al.*, 2004; Broudiscou *et al.*, 2007; Thompson *et al.*, 2010). The presented enantiospecific reaction may have potential applications, since (*S*)-(+)-linalool, also known as coriandrol, is commercially not available. So far, a selective biological synthesis has only been described with geranyl diphosphate and plant (*S*)-(+)-linalool synthases, with *ee*-values ranging from 85% to 99% (Pichersky *et al.*, 1995; Sitrit *et al.*, 2004; Chen *et al.*, 2010). For a biotechnological application, a detailed characterization of the LDI is highly desirable.

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