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A novel oxygenase from *Pleurotus sapidus* transforms valencene to nootkatone

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ABSTRACT

A selective and highly efficient allylic oxidation of the sesquiterpene (+)-valencene to (+)-nootkatone was achieved with lyophilisate of the basidiomycete *Pleurotus sapidus*. The responsible enzymatic activity was biochemically characterised and purified by chromatographic and electrophoretic methods. Peptide sequences obtained by mass spectrometry showed homologies to oxygenases from various ascomycetes. Based on the peptide sequences, the encoding cDNA was amplified from a cDNA library of *P. sapidus* by PCR. The cloned sequence consisted of 1309 bp with an open reading frame of 1191 bp. Based on database research, the translated amino acid sequence of 396 amino acids showed on the protein level homologies of ~50% to putative lipoxygenases from *Aspergillus fumigatus* and *Laccaria bicolor* as well as 26% homology to the sequence of lipoxygenase-1 from soy bean (*Glycine max*). A lipoxygenase from a basidiomycetous fungus has not yet been characterised on a molecular level.

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1. Introduction

Terpene hydrocarbons often provide unspectacular odour impressions, while their oxygenated derivatives are widely used as flavours and fragrances. Besides limonene and myrcene, the bicyclic sesquiterpene (+)-valencene is a main component of the essential oil of Valencia oranges (*Citrus sinensis*). Its first description dates back to 1965 [1]. Via allylic oxidation of (+)-valencene, the sesquiterpene ketone (+)-nootkatone is accessible. (+)-Nootkatone was first isolated from the heartwood of Alaska yellow cedar (*Chamaecyparis nootkatensis*) [2], and is present in trace amounts in grapefruits (*Citrus paradisi*) [3] and some other *Citrus* species [4]. (+)-Nootkatone imparts a grapefruit-like flavour, is slightly bitter, and has a low odour threshold of ~1 $\mu g\,L^{-1}$ water [5]. These characteristics turn (+)-nootkatone into a highly demanded product for the fragrance, food, cosmetics, and pharmaceutical industries. Several approaches to produce nootkatone by chemosynthesis have been described so far. All of them include various reaction steps and often require environmentally hazardous reagents, catalysts, and solvents. This quality of (+)-nootkatone cannot legally be marketed as a "natural" flavour. The production of (+)-nootkatone by biotransformation thus represents an interesting alternative. Lyophilisate of the edible fungus *Pleurotus sapidus*, which was previously used for the enzymatic conversion of limonene [6], was found to selectively oxidise (+)-valencene to (+)-nootkatone (Fig. 1).

The aim of the present study was to purify the responsible oxygenase from lyophilisates of *P. sapidus* and to characterise the enzyme on a molecular level.

2. Experimental

2.1. Chemicals

Standard nutrition solution was prepared using chemicals from Merck (Darmstadt, Germany), Riedel-de Haën (Seelze, Germany), and J T Baker (Deventer, The Netherlands). Other fine chemicals were purchased from Fluka (Neu-Ulm, Germany) and Carl Roth (Karlsruhe, Germany). Solvents were obtained from Carl Roth and BASF (Ludwigshafen, Germany), and were distilled before use. High-purity water was prepared with an E-pure water purification system (Barnstead, Dubuque, IA, USA).

Abbreviations: aa, amino acids; CIS, cooled injection system; CV, column volume; DEAE, diethylaminoethyl; FPLC, fast protein liquid chromatography; IEX, ion exchange chromatography; MWCO, molecular weight cut off; TMBZ, tetramethylbenzidine.

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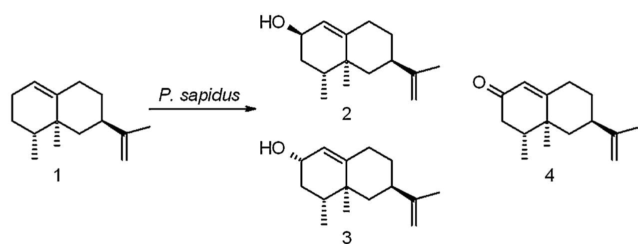

Fig. 1. Biotransformation of (+)-valencene (1) with lyophilisates of *P. sapidus* to α-nootkatol (2), β-nootkatol (3), and (+)-nootkatone (4).

2.2. Microorganism

The filamentous fungus *P. sapidus* was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ 8266), Braunschweig, Germany.

2.3. Cultivation of P. sapidus

Stock cultures were maintained on agar plates containing standard nutrition solution (30.0 g L^{-1} D-glucose·H_2O, 4.5 g L^{-1} L-asparagine·H_2O, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot xH_2O$, 3.0 g L^{-1} yeast extract, and 1.0 mL L^{-1} trace element solution: 5 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 80 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 90 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 30 mg L^{-1} $MnSO_4 \cdot H_2O$, and 0.4 g L^{-1} EDTA; the pH was adjusted to 6.0 with 1 M NaOH prior to sterilisation), and 15.0 g L^{-1} agar agar.

2.4. Enzyme production in submerged cultures

Standard sterile techniques were applied throughout the procedure. Pre-cultures were grown aerobically in standard nutrition solution (*cf.* Section 2.3) after transferring 1 cm^2 agar plugs from the leading mycelial edge of the stock cultures. The mycelium was homogenised using an Ultra Turrax TP18/10 homogeniser (IKA, Staufen, Germany). The submerged cultures (200 mL medium) were kept on a rotary shaker (25 mm shaking diameter; Multitron, Infors, Einsbach, Germany) at 150 rpm and 24 °C in Erlenmeyer flasks (500 mL) for 4 days in darkness. Afterwards, the cultures were homogenised and transferred into a 3.5 L bioreactor (Meredos, Bovenden, Germany) containing 2.3 L of fresh standard nutrition solution. After 3–5 days of growth (24 °C, 200–400 rpm, 10 L h^{-1} aeration), the culture was harvested by filtration through a cotton cloth, and the fungal mycelium was washed twice with distilled water. The obtained biomass was freeze-dried (Vaco2, Zirbus Technology, Bad Grund, Germany) and stored at −70 °C until usage.

2.5. Enzyme purification by fast protein liquid chromatography (FPLC)

30 mL Tris–HCl buffer (20 mM, pH 7.5) were added to 1 g lyophilisate of *P. sapidus*, and the suspension was agitated for 1 h at 150 rpm and 24 °C in horizontal position in an orbital shaker (25 mm shaking diameter, Multitron, Infors). After centrifugation (60 min, 100,000 × *g*, 4 °C), the supernatant was filtered through a membrane (0.45 μm, 25 mm, PET, Carl Roth GmbH), and was subjected to anion exchange chromatography.

The protein purification was performed in a cooling chamber at 4 °C using a Biologic Duo Flow Chromatography System (Bio-Rad, Hercules, CA, USA), equipped with a Fraction Collector 2128 (Bio-Rad). The samples were centrifuged (10 min, 16,060 × *g*, 4 °C) before injection. The elution of the proteins was monitored at λ = 280 nm, and protein containing fractions were tested for their capability to transform (+)-valencene (*cf.* Section 2.7).

2.5.1. Anion exchange chromatography

A HiPrep DEAE 16/10 fast flow column (20 mL, Amersham Pharmacia Biotech, Uppsala, Sweden) was employed for anion exchange chromatography. Tris–HCl (20 mM, pH 7.5) served as a start buffer (buffer A), and the proteins were eluted with a step gradient of Tris–HCl containing 1.0 M sodium chloride (buffer B) over three steps: 7 CV 100% buffer A, 6 CV 65% buffer A, 5 CV 0% buffer A. The flow rate was adjusted to 3.0 mL min^{-1}, and the fraction size was 2.0 mL. The fractions showing transformation activity were pooled. Afterwards, the solvent was exchanged by sodium citrate (20 mM, pH 3.0) by ultrafiltration (Centricon Plus-70, 10,000 Da MWCO, Millipore, Billerica, MA, USA), and the retentate was subjected to cation exchange chromatography.

2.5.2. Cation exchange chromatography

A HiTrap SP Sepharose fast flow column (1 mL, Amersham Pharmacia Biotech) was used, and sodium citrate (20 mM, pH 3.0) served as a start buffer. The proteins were eluted with a linear gradient over 15 CV of 0–100% sodium citrate containing 1.0 M sodium chloride. The flow rate was 1.0 mL min^{-1}, and the fraction size was 2.0 mL. Fractions showing transformation activity were pooled, and the solvent was exchanged to Tris–HCl (200 mM, pH 7.5) by means of ultrafiltration (Amicon Ultra-15, 10,000 Da MWCO, Millipore). The retentate was subjected to size exclusion chromatography.

2.5.3. Size exclusion chromatography

A Superdex 200 10/300 GL (Amersham Pharmacia Biotech) with a bed volume of approximately 24 mL and an optimum separation range of 10–600 kDa was used. The elution buffer contained Tris–HCl (200 mM, pH 7.5), and the flow rate was 0.5 mL min^{-1}. Fractions were collected every 2nd minute. Gel filtration calibration kits (High Molecular Weight and Low Molecular Weight, Amersham Pharmacia Biotech) were used to obtain a calibration curve.

2.6. Transformation of (+)-valencene with lyophilisates of P. sapidus

The transformation of (+)-valencene was carried out in glass vials (4 mL) in horizontal position in an orbital shaker (150 rpm, 25 mm shaking diameter; Multitron, Infors) at 24 °C for 24 h. 1, 5, or 10 μL (+)-valencene (Fluka, ≥90%) were added to 50 mg *P. sapidus* lyophilisate and 1.5 mL Tris–HCl buffer (20 mM, pH 7.5).

2.7. Transformation of (+)-valencene with purified enzyme fractions

0.5 mL of purified enzyme fraction was added to 1.0 mL Tris–HCl (20 mM, pH 7.5). The suspension was thoroughly mixed, and the biotransformation was initiated by addition of 1 μL (+)-valencene (Fluka, ≥90%). The transformation was performed in glass vials (4 mL) in horizontal position in an orbital shaker (150 rpm, 25 mm shaking diameter; Multitron, Infors) at 24 °C for 20 h.

2.8. Identification of transformation products

The transformation products were identified by means of high resolution GC–MS, LC–MS, and NMR spectroscopy as reported by Kaspera et al. [7].

2.9. Quantification of α-, β-nootkatol, and (+)-nootkatone

After transformation, 100 μL of internal standard solution (500–1500 mg L^{-1} thymol in pentane/diethyl ether, 1:1.12 v/v) and 2 mL pentane/diethyl ether (1:1.12 v/v) were added. The suspension was thoroughly mixed, and phase separation was achieved by centrifugation (10 min, 3313 × *g*, 4 °C). The organic phase was

dried over anhydrous sodium sulfate, and 1 μL was injected into a Hewlett Packard HP 6890 GC system (Hewlett Packard, Santa Clara, CA, USA) equipped with an CIS 4 injector with controller (0.5 min splitless; 60 °C (0 min), 12 °C s^{-1} – 240 °C (3 min); Gerstel, Mülheim an der Ruhr, Germany), a Varian WCOT Fused Silica CP Wax 52CB capillary column (30 m × 0.25 mm id, 0.25 μm film thickness; Varian, Palo Alto, CA, USA), and a flame ionisation detector (250 °C, 40 mL min^{-1} H_2, 450 mL min^{-1} air flow, 45 mL min^{-1} make up gas) using the following temperature program: 40 °C (2 min), 5 °C min^{-1} – 150 °C (0 min), 3 °C min^{-1} – 222 °C (0 min), 10 °C min^{-1} – 240 °C (10 min). Hydrogen was used as carrier gas (58.4 kPa head pressure, 1.5 mL min^{-1} constant flow).

2.10. UV/VIS-spectroscopy

Absorption spectra were recorded with a UV/VIS Spectrometer Lambda 12 (Perkin Elmer, RodgauJügesheim, Germany), equipped with a tempered cell holder.

2.11. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a modified method of Laemmli [8] with 6% (w/v) polyacrylamide in the stacking gels and 12% (w/v) polyacrylamide in the resolving gels. After electrophoresis, the proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R 250 (Serva, Heidelberg, Germany) or with silver as described by Blum et al. [9]. For calibration, protein standards (Precision Plus Protein Unstained Standard, Bio-Rad, Munich, Germany) with molecular weights of 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa were used.

Metalloprotein staining was performed with 6.3 mM 3,3′,5,5′-tetramethylbenzidine (3,3′,5,5′-TMBZ) in methanol according to Thomas et al. [10] and Henne et al. [11]. The gels were run at 4 °C under non-denaturing conditions.

2.12. Electrospray–ionisation tandem mass spectrometry

The ESI–MS/MS analyses for the *de novo* sequencing of proteins after digestion with trypsin were performed as reported previously [12].

2.13. cDNA synthesis and PCR-screening

For the cloning of the oxygenase encoding cDNA sequence, a cDNA library of *P. sapidus* was synthesised and screened by means of polymerase chain reaction (PCR). Mycelium of *P. sapidus* was harvested on the fourth culture day, and total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quality of the RNA was checked with a denaturing formaldehyde agarose gel electrophoresis and ethidium bromide staining. The cDNA was synthesised with the SMART™ PCR cDNA Synthesis Kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), and the first-strand was synthesised with SuperScript™ II RNase H^- (Invitrogen, Karlsruhe, Germany). PCR primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). For the PCR approximately 20 ng cDNA were used as template in 50 μL reaction mixtures with 1× CoralLoad buffer (Qiagen), 0.2 mM dNTPs, 0.2 μM primers, and 1.25 U HotStarTaq DNA polymerase (Qiagen). A PCR Mastercycler personal (Eppendorf) was employed for the amplification experiments and the following primers were used: forward (5′ > AAA CCT GAT GAG GAG CTG TTT < 3′), reverse (5′ > ACA GGA TAC GGT GAT GAA TG < 3′). The entire cDNA sequence of the *P. sapidus* oxygenase was determined by primer walking.

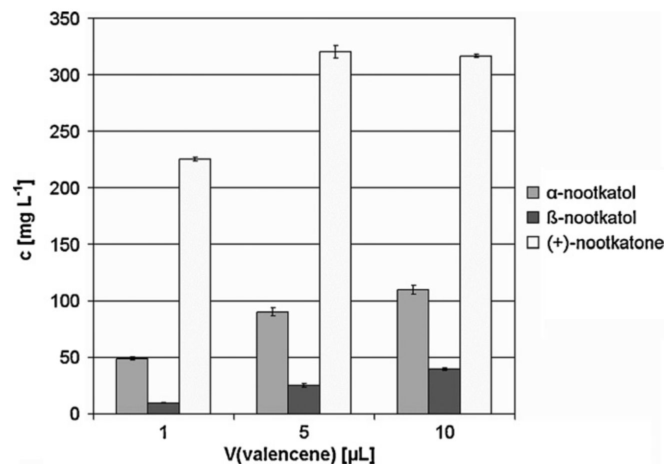

Fig. 2. Transformation of (+)-valencene with lyophilisates of *P. sapidus*; 50 mg lyophilisate in 1.5 mL Tris–HCl (20 mM, pH 7.5) for 24 h.

2.14. Cloning and sequencing of PCR products

The PCR products were cloned into the vector pCR®2.1 using the TA Cloning Kit (Invitrogen). The sequencing was done with M13 reverse and forward primers at MWG Operon.

2.15. Nucleotide sequence accession number

The *P. sapidus* mRNA for valencene oxygenase sequence was submitted to the EMBL Nucleotide Sequence Database under the accession number FM200795.

3. Results

3.1. Transformation of (+)-valencene with lyophilisates of P. sapidus

(+)-Valencene was oxidised with lyophilisate of *P. sapidus*. The transformation of 5 and 10 μL (+)-valencene yielded similar product concentrations of 436 and 466 mg L^{-1}. A product inhibition may be responsible for this observation. The dosage of the 1 μL (+)-valencene resulted in significantly lower product concentrations (284 mg L^{-1}; sum of α- , ß-nootkatol and (+)-nootkatone) (Fig. 2), but higher molar yields. Oxygen was found highly regioselectively inserted in allylic position to form the two diastereomeric nootkatols and (+)-nootkatone (Fig. 3).

3.2. Purification of P. sapidus oxygenase

A three-step chromatographic purification scheme was developed. Supernatant of rehydrated lyophilisate of *P. sapidus* was subjected to ion exchange chromatography (IEX) on a weak anion exchanger, followed by a second IEX step on a strong cation exchanger. Activity containing fractions were pooled and subjected to size exclusion chromatography. After the last purification step, the protein containing fractions 08, 09, and 12–22 were tested for enzyme activity. Significant concentrations of transformation products were detected only in fractions 15 and 16. A molecular weight of ∼54 kDa was calculated for the peak maximum (30 min). UV/VIS spectra of the active FPLC fractions did not show any distinct absorption between 300 and 800 nm, indicating the absence of a haem group. An SDS-PAGE with silver staining showed a number of protein bands in the fractions 13–19 (Fig. 4). By comparison of active and inactive fractions on a band by band basis, the protein

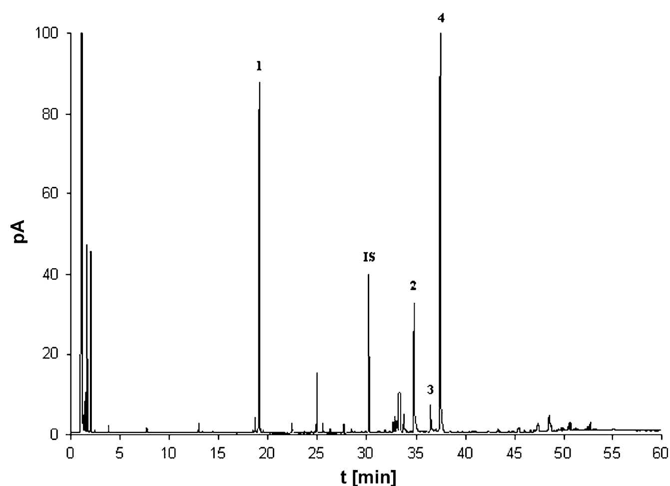

Fig. 3. GC chromatogram of a biotransformation of (+)-valencene with lyophilisate of *P. sapidus*; (+)-valencene (1), thymol (IS) (internal standard), α-nootkatol (2), β-nootkatol (3), and (+)-nootkatone (4).

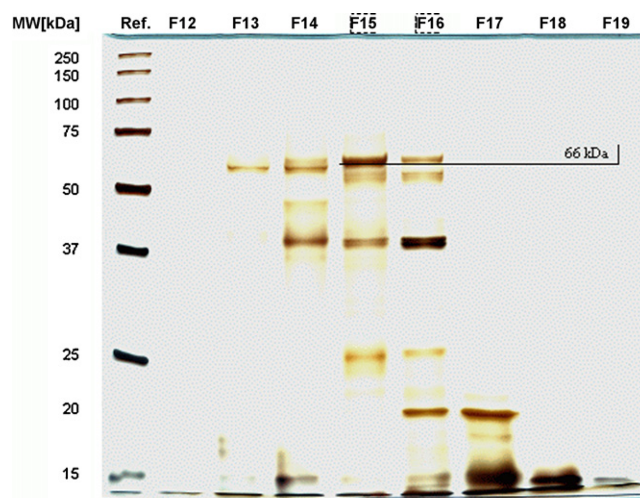

Fig. 4. Silver stained SDS-PAGE gel (12%) after three-step protein purification by means of FPLC; MW = molecular weights of reference proteins, Ref. = reference (1 μL), F = fraction (15 μL), F15 and F16 = active fractions.

with a molecular weight of 66 kDa was unambiguously identified as the target enzyme. Additionally, an SDS-PAGE analysis was performed under non-denaturing conditions using a staining protocol specific for metalloproteins. Horseradish peroxidase and a lipoxygenase from soy bean served as positive controls. Two bands were activity stained in the supernatant of rehydrated lyophilisates as well as in the active FPLC fractions. Heating of the samples prior to SDS-PAGE analysis led to a complete loss of activity (data not shown).

3.3. Peptide and protein sequences

Analogous to the SDS-PAGE with silver staining, an additional PAGE was performed with Coomassie® R staining. The protein band with enzyme activity was excised, digested with trypsin, and subjected to ESI–MS/MS analysis (Fig. 5). Database homology searches (NCBI Blast, programme *blastp* [13]) revealed homologies to hypothetical lipoxygenases from ascomycetes. Degenerated PCR primers were derived from the translated sequences based on the alignment

```
P. sapidus     --------------------------------------------------MRYG--- 4
G. max         GLEEFPPKSNLDPAIYGDQSSKITADSLDLDGYTMDEALGSRRLFMLDYHDIFMPYVRQI 420
                                                                    * *

P. sapidus     --------CAAVALFYLTAMGKLHPLAIIPDYKGSMAASVTIFNKRTNPLDISVNQANDW 56
G. max         NQLNSAKTYATRTILFLREDGTLKPVAIELSLPHSAGDLSAAVSQVVLPAKEGVEST--- 477
                        *: ::::*   *.*:*:**  .   * .   : ..: . * . .*:.:

P. sapidus     PWRYAKTCVLSSDWALHEMIIHLNNTHLVEEAVIVAAQRKLSPSHIVFRLLEPHWVVTLS 116
G. max         IWLLAKAYVIVNDSCYHQLMSHWLNTHAAMEPFVIATHRHLSVLHPIYKLLTPHYRNNMN 537
                *  **: *: .* . *::: *  *** . *..::*::*:**  * :::** **:  .:.

P. sapidus     LNALARSVLI-PEVIVPIAGFSAPHIFQFIRESFTNFDWKSLYVPADLESRGFPVDQLNS 175
G. max         INALARQSLINANGIIETTFLPSKYSVEMSSAVYKNWVFTDQALPADLIKRGVAIKDPST 597
               :*****. ** .: *:  : :.: : .::    :.*: :..  :**** .**..:.: .:

P. sapidus     PK-----FHNYAYARDINDMWTTLKKFVSSVLQDAQYYPDDASVAGDTQIQAWCDEMRSG 230
G. max         PHGVRLLIEDYPYAADGLEIWAAIKTWVQEYVP--LYYARDDDVKNDSELQHWWKEAVEK 655
               *:     :.:*.** *  ::*:::*.:*.. :    **. * .* .*:::* * .*  .

P. sapidus     MGAGMTN--FPESITTVDDLVNMVTMCIHIAAPQHTAVNYLQQYYQTFVSNKPSALFSPL 288
G.max          GHGDLKDKPWWPKLQTLEDLVEVCLIIIWIASALHAAVNFGQYPYGGLIMNRPTASRRLL 715
                 ..:.:  :  .: *::***::  : * **:. *:***: *  *  :: *:*:*    *

P. sapidus     P-TSIAQLQKYTESDLMAALPLNAKRQWLLMAQIPYLLSMQVQEDENIVTYAANASTDKD 347
G. max         PEKGTPEYEEMINNHEKAYLRTITSKLPTLISLSVIEILSTHASDEVYLGQRDNPHWTSD 775
               * .. .: ::  :..  * *   :.:   *::     :     .**  :    *.   .*

P. sapidus     PIIASAGRQLAADLKKLAAVFLVN-------SAQLDDQNTPYDVLAP--------EQLAN 392
G. max         SKALQAFQKFGNKLKEIEEKLVRRNNDPSLQGNRLGPVQLPYTLLYPSSEEGLTFRGIPN 835
               .   .* :::. .**::   :: .       . :*.  : ** :* *        . :.*

P. sapidus     AIVI 396
G. max         SISI 839
               :* *
```

Fig. 5. Alignment of the *P. sapidus* oxygenase amino acid sequence with the sequence of the lipoxygenase-1 from soy bean (P08170) with Clustal W [38]; bold: amino acids involved in complexing of iron within the catalytic centre, underlined: amino acid sequences of tryptic peptides determined by ESI–MS/MS.

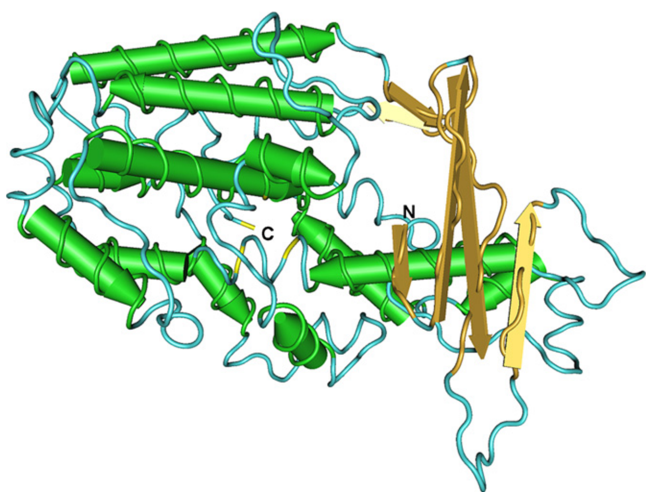

Fig. 6. Three-dimensional protein structure homology model of the oxygenase from *P. sapidus* based on lipoxygenase-1 from soy bean (PDB # 1YGE [14]) computed with SWISS-MODEL [15].

of the peptide sequences *GFPVDQLNSPK* and *YTESDLMAALPLN* with the sequence of an oxygenase from *Aspergillus fumigatus*. By primer walking, the entire coding region of the cDNA of the *P. sapidus* oxygenase was sequenced (Fig. 5). The cDNA contained an open reading frame of 1191 bp (396 amino acids). The translated protein sequence showed homologies of 51% to the sequences of potential lipoxygenases from the ascomycete *A. fumigatus* and from the basidiomycete *Laccaria bicolor* and 26% homology to the sequence of lipoxygenase-1 from soy bean (NCBI Blast, programme *blastp* [13]). Based on the crystal structure of the lipoxygenase-1 from soy bean (1YGE [14]) a three-dimensional protein structure homology model of the oxygenase from *P. sapidus* was computed with SWISS-MODEL [15] (Fig. 6). The oxygenase consisted of two β-sheets and twelve α-helices.

4. Discussion

Since the first published biotransformation of (+)-valencene by *Enterobacter* species [16], several approaches for producing the desired grapefruit flavour compound (+)-nootkatone have been proposed. Whole cell systems or crude protein extracts of bacteria, higher plants, and fungi were employed. In addition to *Enterobacter* species, a *Rhodococcus* strain (KSM-5706) was used for biotransformation [17]. The use of recombinant $P450_{BM-3}$ after single base substitutions in whole cell systems of *Escherichia coli* was proposed by Sowden et al. [18]. For the biotransformation of (+)-valencene with plant cell cultures, *Citrus* species were employed [19]. Additionally, microsomal enzyme preparations of chicory roots (*Cichorium intybus* L.) were suggested for nootkatone synthesis [20] as well as cultured cells of *Gynostemma pentaphyllum*, *Caragana chamlagu*, *Hibiscus cannabinus* [21], and some *Chlorella* species [22]. Kaspera et al. [7] described the biotransformation of (+)-valencene with submerged cultures of the ascomycete *Chaetomium globosum*, and *Mucor* and *Botryosphaeria* species were used by Furusawa et al. [22]. However, the use of these whole cell systems for the biotransformation of (+)-valencene suffered from several drawbacks. They often showed low productivities and especially plant cell cultures took extensive cultivation times or required constant illumination.

In the present investigation, numerous different fungi of the classes of asco- and basidiomycota were screened for their capability to oxidise (+)-valencene to (+)-nootkatone. *P. sapidus* was identified as the most promising biocatalyst when the mycelia were lyophilised prior to the transformation. The use of freeze-dried microorganisms for the biotransformation of terpenoid substrates

Table 1
Comparison of different transformation systems using whole cells or raw culture supernatants.

Transformation system	*t*	*c* [$mg\,L^{-1}$]	*c* [$mg\,L^{-1}\,h^{-1}$]	Literature
Plant cells (*C. paradisi*)	6 h	1	<1	[19]
Bacteria (*Rhodococcus* spec.)	5 d	50 [25]	<1	[17]
Fungi (laccases from *Botrytis cinerea*)	2 d	1 296[a]	27	[25]
Fungi (*C. globosum*)	3 d	8	<1	[7]
Plant cells (*G. pentaphyllum*)	20 d	600[a]	1	[21]
Plant cells (*C. fusca*)	18 d	252[a]	<1	[22]
Fungi, lyophilised (*P. sapidus*)	24 h	320	13	Present work

[a] Calculated based on the literature data.

to flavour compounds has been described previously for a number of bacteria [23]. The oxyfunctionalisation was highly regioselective, and α- and β-nootkatol represented the only relevant side products. As both alcohols impart pleasant, grapefruit-like flavour impressions [24], they might or might not be downstream separated from the main product (+)-nootkatone. The established transformation system showed high productivities compared to the methods published previously (*cf.* Table 1). Different from the method patented by Huang et al. [25] no subsequent degradation steps were necessary. To overcome the disadvantages associated with whole cell transformations, isolated enzymes may be employed. In recent years, the allylic oxidation of valencene by cytochrome P450 monooxygenases was discussed [7,20]. Cytochrome P450 monooxygenases (EC 1.14.14.1 and others) are widely found in bacteria, yeasts, filamentous fungi, insects, mammals, and in plants [26]. However, the direct transformation of (+)-valencene to (+)-nootkatone by means of isolated P450 monooxygenases only succeeded after point mutation of recombinant $P450_{cam}$ monooxygenases from *Pseudomonas putida* [18]. Recently, a cytochrome P450 monooxygenase from *Hyoscyamus muticus* (*Hyoscyamus* premnaspirodiene oxygenase) was characterised biochemically [27]. It catalyses the allylic oxidation of diverse sesquiterpenoid substrates. Amongst others (+)-valencene was accepted as a substrate. In contrast to $P450_{cam}$, only the hydroxylation of (+)-valencene was catalysed by *Hyoscyamus* premnaspirodiene oxygenase, and it was thus not capable of (+)-nootkatone synthesis. The overall complexity and instability of cytochrome P450 monooxygenases, their dependence of cofactors, and the difficulties in isolating them under preservation of the catalytic activity still represent major drawbacks for their application in large scale production of (+)-nootkatone [28].

The novel oxygenase from *P. sapidus* was capable of oxidising (+)-valencene in allylic position to yield (+)-nootkatone. To identify the catalytically active protein band, and to investigate the potential involvement of a cytochrome P450 monooxygenase in the biotransformation, a haem sensitive staining [10,11] was performed after SDS-PAGE. The electrophoresis was run under non-denaturing conditions, and the gels were incubated with methanolic 3,3′,5,5′-TMBZ solution. A blue coloration of a protein band upon addition of hydrogen peroxide indicated the presence of haem groups, as verified for the reference protein horseradish peroxidase. Certain non-haem metalloproteins, such as the copper containing cytochrome oxidase from *Nitrosomonas europaea* are stained by TMBZ already prior to the dosage of hydrogen peroxide [29–31]. Likewise, no hydrogen peroxide was required for the staining of the non-haem enzyme lipoxygenase from soy bean (*Glycine max*) and of the *P. sapidus* oxygenase. In accordance with this observation, no UV/VIS absorption characteristic for haem groups was observed between 380 and 420 nm (Soret band) with the purified enzyme solution. The translated amino acid sequence data confirmed these findings. Database searches returned no homologies (≤2%) of the *P. sapidus* oxygenase to *Hyoscyamus* premnaspirodiene oxygenase or other

cytochrome P450 monooxygenases. The occurrence of two activity stained bands in the SDS-PAGE gel could be addressed to the existence of isoenzymes or catalytically active subunits. Another explanation for the appearance of a second, less intense band might be due to artefactual transfer of the catalytic moiety to other proteins [31].

According to database searches, the translated *P. sapidus* oxygenase protein sequence showed homologies to lipoxygenases from *A. ochraceus* (NCBI Accession # AAZ13594; partial sequence, 205 aa, score 50), *A. fumigatus* (XP_746844, genome project [32], 744 aa, score 51), *Gibberella moniliformis* (AAW21637, 805 aa, score 44), and *L. bicolor* (EDR07700, genome project [33], 485 aa, score 51). None of them has been biochemically characterised yet. Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are iron, but not haem containing dioxygenases which naturally occur in plants [34], animals [35], bacteria [36], and fungi [37]. The iron ion of the active site of lipoxygenases is bound by four ligands. Regarding the lipoxygenase-1 from *G. max* it is complexed by three histidine residues and the C-terminal carboxyl group of isoleucine. These important amino acids occur in comparable distance in the obtained sequence of the *P. sapidus* oxygenase, and may play a major role in the chelation of the catalytic active ion (*cf.* Fig. 5). Lipoxygenases catalyse the incorporation of two atoms of oxygen into polyunsaturated fatty acids providing (*Z,Z*)-penta-1,4-dien structures. As these are not present in (+)-valencene, lipoxygenases are not capable of the direct oxidation of (+)-valencene to (+)-nootkatone. An indirect co-oxidative pathway for (+)-nootkatone production involves the lipoxygenase catalysed synthesis of fatty acid hydroperoxides, and the subsequent autoxidation of (+)-valencene to (+)-nootkatone [24]. However, co-oxidative approaches typically suffer from a low selectivity.

To date, only few sequences of lipoxygenases from filamentous fungi have been published. With one exception, all of them belong to 14 different ascomycetes. The vast majority are putative, hypothetical, or partial and emanated from genome sequence projects or shotgun approaches. Only the putative lipoxygenase of *L. bicolor* originates from a fungus of the class of basidiomycotes [33]. Although the purification of a lipoxygenase from *P. ostreatus* has been published [37], no sequence data of a purified lipoxygenase from a basidiomycete is available yet.

The formation of the ketone (+)-nootkatone from the hydrocarbon compound (+)-valencene suggests a two-step reaction via the intermediate formation of the corresponding alcohols, α- and β-nootkatol. Both nootkatols were detected in the transformation batches. When nootkatone was reduced by $LiAlH_4$ [7,39] and the resulting mixture of 95% β-nootkatoland 5% α-nootkatol was subjected to the enzymatic transformation, only β-nootkatol was further oxidised to (+)-nootkatone (data not shown). β-Nootkatol could thus represent the direct biosynthetic precursor of nootkatone. This finding is in good agreement with the low concentrations of β-nootkatol detected in the transformation experiments with (+)-valencene as substrate (Fig. 2). Considering the sequence homologies of the cloned enzyme to plant and fungal lipoxygenases, an intermediate formation of valencene hydroperoxides represents an alternative hypothesis for the reaction mechanism. Detailed mechanistic studies using wild-type and recombinant enzyme batches are subject of ongoing research.

5. Conclusion

A novel enzyme which selectively and highly efficiently oxidises the sesquiterpene (+)-valencene to the grapefruit flavour (+)-nootkatone was purified from lyophilisates of the basidiomycete *P. sapidus*. The cloned cDNA sequence showed homologies to lipoxygenases from plants and fungal genome data. As only small amounts of the enzyme could be purified by a complex purification procedure, kinetic data including K_M and K_{cat} have not become accessible yet. The oxygenase will be heterologously expressed in an appropriate host to further investigate its biochemical and structural properties as well as its substrate specificity.

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