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Novel Carboxyl Esterase Preparations for the Resolution of Linalyl Acetate

Mateja Pogorevc[1], **Ulrike T. Strauss**[1], **Marianne Hayn**[2], and **Kurt Faber**[1,*]

[1] Institute of Organic Chemistry, University of Graz, A-8010 Graz, Austria
[2] Institute of Biochemistry, University of Graz, A-8010 Graz, Austria

Summary. Biocatalytic resolution of the tertiary terpene alcohol (±)-linalool was accomplished *via* hydrolysis of its corresponding acetate ester using two highly enantiospecific enzymes ($E > 100$). The latter were identified in a crude cell-free extract of *Rhodococcus ruber* DSM 43338 and could be separated by (partial) protein purification. Since they showed opposite enantiopreference, they were termed (*R*)- and (*S*)-linalyl acetate hydrolase (LAH). The activity and selectivity of the enzyme preparations was markedly dependent on the fermentation conditions.

Keywords. Linalool; Enzymatic resolution; *tert*-Alcohol; Linalyl acetate hydrolase.

Introduction

Linalool is an important chiral terpene alcohol used in flavor and fragrance industry. Since its enantiomers differ in odor [1], their availability in optically pure form is desirable for flavor and fragrance composition. The (*R*)-(−)-enantiomer – also denoted as licareol – is a major constituent of *Cinnamonium camphora* and *Cayenne linaloe* extract, whereas the (*S*)-(+)-counterpart primarily occurs in coriander oil (coriandrol).

Several attempts to resolve (±)-linalool (**1a**) by means of biocatalytic methods have been reported to date. For instance, (±)-**1a** was shown to be a non-substrate for several microbial lipases in esterification reactions [2, 3]. In an analogous fashion, a broad range of esterases, proteases, and lipases of various origins such as microbial, plant, and mammalian sources were found to be inactive in the hydrolysis of the corresponding acetate (±)-**1** [4]. This is not surprising, since tertiary alcohols and esters thereof are generally not accepted as substrates by the most commonly employed carboxyl ester hydrolases [5, 6]. As a consequence, several studies in search for novel microbial enzymes being capable to hydrolyze linalyl acetate in a selective fashion have been initiated. When using whole cells of *Aspergillus niger*, the hydrolysis succeeded without noticeable enantioselectivity ($E < 2$) [7]. In addition, several by-products arising from hydroxylation and rearrangement of the carbon skeleton were formed in significant amounts. Similar activities have been reported using whole resting cells of *Bacillus subtilis* [8]. Although (*S*)-linalool was

* Corresponding author

(+/-)-**1**

(*R*)- or (*S*)-
Linalyl Acetate
Hydrolase

Buffer, pH 7.5

OH

+

O

(*S*)-Linalool or (*R*)-Linalyl Acetate

(*R*)-Linalool (*S*)-Linalyl Acetate

1a **1**

Scheme 1. Biocatalytic resolution of (±)-linalyl acetate

formed from racemic linalyl acetate, its optical purity was exceedingly low (~8%). In addition, significant amounts of α-terpineol and the (non-chiral) terpene alcohols geraniol and nerol were isolated due to undesired allylic rearrangement. On the contrary, various bacterial cells of the genus *Actinomyces* have shown clean hydrolysis without formation of by-products, but the enantioselectivities remained too low to be of practical value ($E < 5$) [4]. Most recently, an interesting study has proposed the use of a spacer moiety which is introduced between the sterically offending chiral center and the ester moiety to be hydrolyzed [9]. As a result, the carbonyl moiety of pivaloyloxymethyl- and *n*-butanoyloxymethyl derivatives of linalool became more accessible, making these substrates acceptable for several standard lipases, esterases, and proteases. Although reasonable reaction rates were achieved, the selectivities were not optimal. Best results were obtained with *Candida rugosa* lipase for the corresponding butanoyloxymethyl derivative of linalool ($E = 9.7$–9.9).

The reasons for the low enantioselectivities obtained so far may be inherent to the substrate, *i.e.* due to the steric similarity of the methyl and the ethenyl group of linalool, which impedes the chiral recognition process. Alternatively, low selectivities may result from the presence of several competing carboxyl esterases of opposite enantiopreference. This phenomenon is not uncommon in biotransformations; it has been observed during the microbial asymmetric reduction of ketones [10] and β-ketoesters [11], in the enzymatic oxidation of *sec*-alcohols [12], and in *Baeyer-Villiger* oxidations of ketones [13]. This latter option encouraged us to study the enzymes involved in more detail.

Results and Discussion

By extensive screening [4], the best activity on (±)-linalyl acetate was found in cells of *Rhodococcus ruber* DSM 43338; however, the selectivity displayed by whole cells was disappointingly low ($E = 4.2$). Two strategies were chosen:

(*i*) Protein purification employing FPLC on hydrophobic interaction material in the first step, following by anion exchange chromatography, revealed that the low selectivities displayed by whole cells are indeed due to the presence of several competing carboxyl esterases. Thus, two active (partially purified) fractions

were obtained which showed opposite enantiopreference. However, the low overall yield and the instability of the semipurified enzyme preparations prevented their purification to homogeneity and characterization. However, all of the active enzymes were found to be located in the cytosol, and from SDS-PAGE gels using partially purified preparations it was concluded that the molecular weight was in the range of approximately 30 kDa.

(*ii*) As an alternative approach to protein purification, selective protein extraction experiments based on *PEG*-dextran systems were carried out. This technique allows the extraction of proteins with concomitant (partial) purification in a single operation [14]. Undesired by-products, such as nucleic acids, polysaccharides, and colored dyes (*e.g.* carotenoids) arising from the fermentation are removed in a single step [15]. The system was optimized by variation of three parameters, *i.e.* *pH* (6–8), *PEG*-OMe concentration (5–20% (w/w)), and dextran content (6–20% (w/w)). For the analysis of the data, a response surface central composite design was employed [16]. Based on recommendations given in the literature [17], polyethylene glycol methyl ether (*PEG*-OMe) and dextran from *Leuconostoc mesenteroides* showing molecular weights of 5000 and 260.000, respectively, were chosen.

Aiming at the separation of competing enzymes, the system was first optimized with respect to the phase distribution, *i.e.* enzymes extracted into the (upper) *PEG*-OMe and (lower) dextran phase should display opposite enantiopreference. This approach proved to be successful. Modelling of the response surface for both criteria showed that a relative maximum was found for the following conditions: 11% *PEG*-OMe, 12% dextran, $pH = 8.0$. When protein extracts from both phases were used as catalysts, the desired opposite enantiopreference was observed. The extract from the *PEG*-OMe and the dextran phase gave (*S*)-**1a** ($ee = 20\%$) and (*R*)-**1a** (*ee* 48%) from (±)-**1** at 10% conversion. Next, the system was optimized towards a maximum of activity and a minimum of protein concentration in the *PEG*-OMe-phase. Response surface modelling and calculation of the relative optima for both criteria gave the following data set: using a system consisting of 15% *PEG*-OMe and 6% dextran at *pH* 6.0, 18% of the total protein containing 88% of the activity were located in the *PEG*-OMe-phase. The latter was then subjected to protein purification as described above. The properties of the enzyme preparations thus obtained were very similar to those from the crude cell-free extract by protein chromatography, *i.e.* the selectivity of the enzyme(s) remained intact.

Table 1. Selectivities of linalyl acetate hydrolases from *Rhodococcus ruber* DSM 43338

	(*S*)-enzyme[a] ee_p %[b]		(*R*)-enzyme[a] ee_p %[b]	
	HIC[c]	IEC[c]	HIC[c]	IEC[c]
Crude cell-free extract	< 99	n.d.[d]	< 99	< 99
PEG-OMe/dextran extract	n.d.[d]	n.d.[d]	< 99	< 99[e]

[a] (*S*)- or (*R*)-linalool were formed from (±)-linalyl acetate, respectively; [b]enantiomeric excess of linalool formed; [c]HIC = hydrophobic interaction chromatography, IEC = ion exchange chromatography; [d]not determined due to loss of activity; [e]the selectivity corresponds to $E = 109$

In order to overcome the limited hydrolytic activity, several attempts towards enzyme induction were carried out. It was found that the presence of competing enzymes possessing different or (almost) no stereoselectivity varied depending on the fermentation conditions. In the absence of any inducer, the cells had hardly any activity at all on (±)-**1**. Thus, *Rhodococcus ruber* DSM 43338 was first grown on a medium composed of glucose and peptone. After reaching the stationary phase, an inducer was added to the culture. Quite surprisingly, the addition of either racemic or (*R*)-(−)-linalyl acetate caused no induction of an enzyme. On the contrary, several carboxyl ester hydrolases capable of accepting linalyl acetate were obtained by using *tert*-butyl acetate as inducer:

(*i*) A highly active enzyme was strongly expressed towards the end of the logarithmic phase of the growth. Unfortunately, it was shown to be non-selective towards (±)-linalyl acetate.

(*ii*) In addition, two enzymes possessing opposite stereopreference were formed when the cells were harvested during the stationary phase, *i.e*. an (*R*)- and an (*S*)-linalyl acetate hydrolase.

Both of these proteins differ significantly in their stability. The (*R*)-selective enzyme proved to be reasonably stable, as only a minor loss of activity was observed upon chromatography, lyophilisation, and freezing at −25°C. Since the addition of *EDTA* had no effect on the activity, the protein was assumed to be devoid of metal ions. On the contrary, (*S*)-linalyl acetate hydrolase was found to be highly sensitive and easily deactivated during purification. Furthermore, a certain degree of deactivation was also observed during protein-selective extraction using the *PEG*-OMe/dextran system.

Experimental

Materials

(±)-Linalyl acetate (Fluka) was purified by silica gel chromatography using petroleum ether (b.p. 60–90°C)/ethyl acetate (20/1) as eluent, followed by *Kugelrohr* distillation (b.p.: 115°C/25 mm). (*R*)-(−)-Linalool (Fluka) was used as received. Polyethylene glycol methyl ether (*MW* = 5000) and dextran from *Leucomostoc mesenteroides* strain no. B-512 (MW 260.000, industrial grade) were obtained from Sigma.

TLC analysis

Samples were analyzed by TLC using Merck silica gel 60 F_{254} and cyclohexane: ethyl acetate = 3:1 as eluent. Compounds were visualized by spraying with vanillin/H_2SO_4 conc. and heat treatment.

GLC analysis

The extent of conversion of the reactions and the enantiomeric excess of linalool was determined *via* GLC analysis using a Varian gas chromatograph 3800 equipped with FID and split/splitless injector using a Chirasil-DEX CB column (25 m×0.32 mm×0.25 μm film, Chrompack) and H_2 as carrier gas at 87°C (isothermal). Retention times: (*R*)-linalool, 11.6 min; (*S*)-linalool, 12.0 min; (±)-linalyl acetate, 12.6 min.

Medium and growth conditions

Cells of *Rhodococcus ruber* DSM 43338 were grown in baffled *Erlenmeyer* flasks at 30°C and 130 rpm using the following medium: 10 g/dm^3 glucose, 10 g/dm^3 peptone, 10 g/dm^3 yeast extract, 2 g/dm^3 NaCl, 0.15 g/dm^3 $MgSO_4 \cdot 7H_2O$, 1.3 g/dm^3 NaH_2PO_4 and 4.4 g/dm^3 K_2HPO_4. Enzyme induction was achieved by addition of 4 ml/dm^3 *tert*-butyl acetate or 0.4 ml/dm^3 (*R*)-(−)-linalyl acetate, respectively. The growth of cells was monitored by measurement of the optical density *via* the absorption at 600 nm.

Disruption of cells

To a suspension of *R. ruber* cells (*ca.* 26 g wet weight) in 120 cm^3 of tris-buffer (10 m*M*, *pH* = 7.5), 120 cm^3 of glass beads (diameter 0.35 mm) were added. The cells were disrupted using a Vibrogen cell mill with external cooling (*ca.*+4°C) and 3 shaking cycles of 2 min agitation/5 min cooling each. After removal of the beads by filtration, the crude cell lysate was centrifuged at +4°C and 38000 g.

Protein extraction

To 50 cm^3 of cell-free extract prepared as described above (*pH* adjusted to 6.0), 9.5 g of *PEG*-OMe and 3.8 g dextran were added. The mixture was shaken at 150 rpm at 20°C for 4 h. The phases were then separated by centrifugation (1000 g, 10 min) and directly used for further experiments.

Protein purification

The cell-free extract was loaded directly onto a phenyl sepharose high performance column (d = 16 mm, h = 20 cm, total volume 8 cm^3, Pharmacia). The column was pre-equilibrated with 10 m*M* tris containing 1 *M* of $(NH_4)_2SO_4$ (*pH* = 7.5) at a flow rate of 3 cm^3/min. Elution was effected using a stepwise gradient with 10 m*M* tris (*pH* = 7.5) at intervals of 100/75/50/25/0%. The (*S*)- and the non-selective enzyme were desorbed with 100% 10 m*M* tris (*pH* = 7.5), whereas the (*R*)-enzyme proved to be more lipophilic as it eluted with 100% destilled H_2O. Active fractions were subjected to further purification by ion exchange chromatography using a Q6 column (total volume 6 cm^3, BioRad), pre-equilibrated with 10 m*M* tris-buffer (*pH* = 7.5). Elution was performed with 10 m*M* tris-buffer containing 1 *M* NaCl (*pH* = 7.5, buffer B) with a linear gradient from 0–1 *M* NaCl at a flow rate of 6 cm^3/min. Desorption of (*R*)- and (*S*)-enzyme took place in the range of 13.4–19.4% and 25.3–27.2% of buffer B, respectively. For the SDS-PAGE experiments the Pharmacia Phastsystem equipment was used.

Enzyme assay

Samples of the eluent (6 mm^3 total volume) were mixed with 10 mm^3 (±)-linalyl acetate on a vortex mixer and incubated for 16 h at room temperature under gentle rolling. The reaction was quenched by addition of 1 cm^3 of ethyl acetate. The samples were extracted four times with 4 cm^3 ethyl acetate each. The organic phase was dried over Na_2SO_2 and concentrated by evaporation. The samples were analyzed by GLC as described above. The absolute configurations of **1** and **1a** was determined by chiral GLC *via* co-injection with commerically available samples.

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