Lipase-mediated Epoxidation of the Cyclic Monoterpene Limonene to Limonene Oxide and Limonene Dioxide

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Dedicated to Professor Heribert Offermanns on the occasion of his 75th birthday

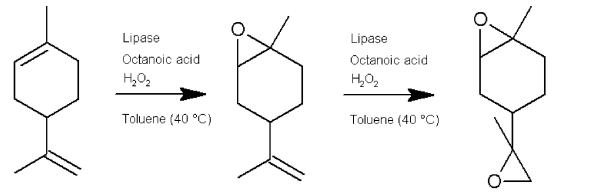
Limonene is an industrially interesting monoterpene that accumulates in bulk quantities as by-product of the fruit juice industry. The corresponding epoxides are versatile synthetic intermediates and additives for the chemical industry. Due to a number of disadvantages of classical chemical epoxidation including serious safety issues and unwanted side-reactions, we here used a mild lipase-catalyzed chemo-enzymatic epoxidation system, with either free or different immobilized forms of *Candida antarctica* lipase B. Full limonene conversion ($> 98\,\%$) was easily achieved at 40 °C within less than 24 h. The enzymatic activities in the formation of limonene monoxide significantly varied from either $1-3.4\,\mathrm{U/mg_{biocatalyst}}$ at r. t. or from $1.7-4.9\,\mathrm{U/mg_{biocatalyst}}$ at 40 °C. For the first time we showed that it is possible to generate high amounts of limonene dioxide ($\sim 30\,\%$ with CalB on carrier 350 at 40 °C) using this mild lipase-mediated epoxidation method. Enzyme activities and limonene dioxide yields strongly depend on the nature of the selected enzyme carrier, the immobilization method and the reaction temperature.

Key words: Terpene, Biocatalysis, Chemo-enzymatic Epoxidation, Enzyme Immobilization, Renewable Resources

Introduction

The ongoing "greening" of chemical production processes currently enhances research activities that try to open novel biocatalytic or combined chemo-enzymatic routes for the conversion of sustainable resources. In this context plant-derived monoterpenes are industrially interesting compounds as they accumulate in bulk amounts as extracts from biogenic waste streams. Common examples are pinene from technical forestry resins and wood pulp [1] and limonene as an inexpensive by-product from the citrus fruit juice industry [2, 3]. The value of these bio-based compounds can be increased *via* addition of further functionalities, *e. g.* by producing the corresponding epoxides as versatile synthetic intermediates and chiral building blocks for the chemical industry [4, 5]. Moreover, limonene oxide and limonene dioxide, as well as mixtures of both, are already in use in the polymer industry as reactive diluents in cationic and UV-cure applications [6].

In classical chemical synthesis organic peroxides and metal catalysts or peroxy acids such as *m*-chloroperbenzoic acid (*m*-CPBA) are added to gain the corresponding epoxides from alkenes [7]. *m*-CPBA still is considered to be the work horse in synthetic chemistry, although it is expensive and difficult to handle, but most other peroxy acids are less stable and more dangerous [8]. Moreover these techniques exhibit a high potential to catalyze unwanted hydrolytic side reactions. If possible the handling of the hazardous peroxy acids on an industrial scale generally is to be avoided [7, 9, 10]. Therefore, on an industrial level, epoxides are typically synthesized either by direct oxidation, by the chlorohydrin method or by the *in situ*-generation of peroxy acids from formic or acetic acid and hydrogen peroxide, and often with addition of min-



Scheme 1. Lipase-mediated epoxidation of limonene to limonene oxide and limonene dioxide.

eral acids [7]. In consequence this leads to the accumulation of large amounts of acidic and chlorinated waste streams, not to mention the serious safety issues and the problems with the formation of unwanted side products.

Already two decades ago Björkling *et al.* [11, 12] showed that some lipases (EC 3.1.1.3) are able to form peroxy acids from carboxylic acids in organic solvents if H_2O_2 is present. Thus, hazardous peroxy acids can be produced *in situ* in organic solvents under mild conditions and used directly in the catalyzed epoxidation of organic substrates such as alkenes *via* the Prileschajew reaction. The first industrially important products such as environment-friendly PVC stabilizers and solubilizers on the basis of epoxidized soybean oil have successfully been produced *via* this chemo-enzymatic method [13]. Further work has also proven the general feasibility of this approach for the epoxidation of cyclic monoterpenes [4, 5, 8, 14].

In this work, we investigated different commercially available carrier systems for the immobilization of *Candida antarctica* lipase B and compared the efficiency of the corresponding enzyme carriers in the epoxidation of the cyclic monoterpene limonene. We report for the first time on successfully shifting the product range to reach not only full conversion of limonene to limonene monoxide, but also to gain significant amounts of limonene dioxide (Scheme 1).

Results and Discussion

Chemo-enzymatic epoxidation of limonene

In our previous studies (unpublished data) we found that the best results for the lipase-catalyzed epoxidation of limonene (*e. g.* 25 mM) were obtained with *Candida antarctica* lipase B (CalB) in reaction systems that use toluene as solvent with a double excess of caprylic acid (*e. g.* 50 mM) and H_2O_2 (*e. g.* 50 mM, from a 30 % aqueous solution) as co-substrates for the lipase-catalyzed *in situ*-generation of peroxy acids, that in turn catalyze the Prileschajew epoxidation of limonene to limonene monoxide. Thereby we also observed a significant difference between the free lyophilized form of *Candida antarctica* lipase B (CalB) and the carrier-bound lipase-preparation Novozyme435, where CalB is adoptively bound to a PMMA-carrier. Based on these results we now used several different carrier systems exhibiting varying properties for the immobilization of CalB. These enzyme carriers were used for the epoxidation of limonene in the reaction system described above.

Enzyme immobilization

CalB was immobilized on 3 different carriers either by adsorption (carrier C435 and carrier EC1) or by covalent binding (carrier 350). The immobilization procedure is described in the Experimental Section, and details on the applied carrier types and properties are shown in Table 1. All enzyme carriers were of spherical shape with average sizes of 300 – 1000 μm for facilitated handling in technical applications and were made out of solid polyacrylic materials.

Name	Binding type	Carrier material	Functional group	Particle size (μm)	Manufacturer
CalB	–	–	–	lyophilized enzyme powder	c-LEcta (Germany)
NZ435	adsorption	PMMA	–	300 – 1000	Novozymes (Denmark)
EC1	adsorption (non-ionic)	polyacrylic	carboxylic ester	350 – 700	Chiral Vision (The Netherlands)
C435	adsorption (cationic)	polyacrylic	carboxylic acid	350 – 700	Chiral Vision (The Netherlands)
350	covalent	polyacrylic	epoxide (apolar)	300 – 700	Chiral Vision (The Netherlands)

Table 1. Properties of enzyme carriers.

Biocatalyst	Total activity per assay at r. t. (U)	Specific activity at r. t. ($U/mg_{carrier}$)	Total activity per assay at 40 °C	Specific activity at 40 °C ($U/mg_{carrier}$)
CalB lyo	51.4	1.0	84.9	1.7
NZ435	167.1	3.3	208.2	4.2
EC1	109.2	2.2	214.0	4.3
C435	170.6	3.4	244.7	4.9
350	118.9	2.4	172.5	3.5

Table 2. Biocatalytic activities for the epoxidation of limonene to limonene monoxide calculated from the initial product formation rates.

A determination of enzyme loadings *via* Bradford [15] was not feasible, probably due to the disturbing influence of undefined additives and stabilizing agent in the lyophilized enzyme powder from c-LEcta (Leipzig/Germany). The protein content given by the manufacturer is 10 – 20 %.

Chemo-enzymatic limonene epoxidation (limonene monoxide)

The lyophilized CalB powder showed the lowest specific activity ($1\,U/mg_{enzyme}$) in the synthesis of limonene monoxide at r. t. All biocatalytic systems where CalB was immobilized on a carrier (NZ435, EC1, C435 and 350) showed significantly higher specific activities, ranging from 2.2 to $3.4\,U/mg_{carrier}$ (Table 2). Assuming maximum enzyme loadings of 1 – 10 % on the carriers, the real specific activities in $U/mg_{protein}$ should be by far higher. Obviously the surface properties of the acrylic carriers have a positive impact on the micro environment of the biocatalyst that significantly enhances the initial product formation rates. Moreover we observed another considerable increase in the specific activity by simply elevating the reaction temperatures to 40 °C. Thus we reached the high specific activity of $4.9\,U/mg_{carrier}$ in case of the C435 carrier (Table 2). No conversion of limonene was observed in the absence of *Candida antarctica* lipase B as a biocatalyst.

Yields of limonene conversion to limonene monoxide and limonene dioxide

Nearly quantitative conversion (> 98 %) of limonene was achieved under the chosen reaction conditions after 24 h with free CalB powder as well as CalB-containing enzyme carriers as biocatalysts. However, the conversion products were different mixtures of limonene monoxide and limonene dioxide. To the best of our knowledge, this is the first report about lipase-mediated epoxidation of limonene where, next to limonene monoxide, significant amounts of limonene dioxide were also generated. Figure 1 shows the 24 h kinetics of the chemo-enzymatic limonene conversion using biocatalyst 350 at 40 °C as an example. The dashed line indicates total limonene conversion by summing up the amount of both products (monoxide and dioxide). Thus full limonene conversion was reached after approximately 8 h in this case. In the first 1 to 1.5 h of the reaction more than 60 % of limonene monoxide is generated, but no limonene dioxide yet. After 2 h, when about 70 % of the limonene was converted to limonene monoxide, the concentration of limonene dioxide slowly started to increase until it finally reached its maximum of 27.4 % after 24 h. On the other hand, limonene monoxide already reached its maximum of 85 % after 6 – 7 h and then started to decrease to 72.6 % as it is converted to the dioxide. Although notable variations in the ratios of limonene monoxide/dioxide after 24 h were observed for the different biocatalysts (Figs. 2 and 3), the basic kinetics were rather similar to the one shown in Fig. 1.

Fig. 2 shows the results with the different biocatalysts for limonene conversion after 24 h at r. t. The highest amounts of limonene dioxide of 14.6 and 13.6 % were detected for CalB lyo and 350, respectively, whereas C435 only reached 7.1 %, but interestingly C435 showed the highest activity in the initial

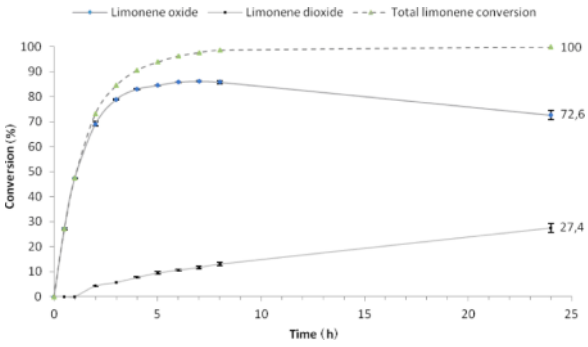


Fig. 1 (color online). Kinetics of the chemo-enzymatic limonene conversion to limonen monoxide and limonene dioxide with biocatalyst 350 at 40 °C.

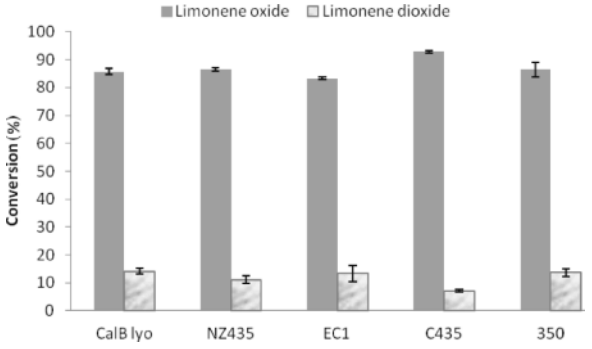


Fig. 2. Degree of limonene conversion into limonene monoxide and dioxide at r. t. after 24 h of reaction time.

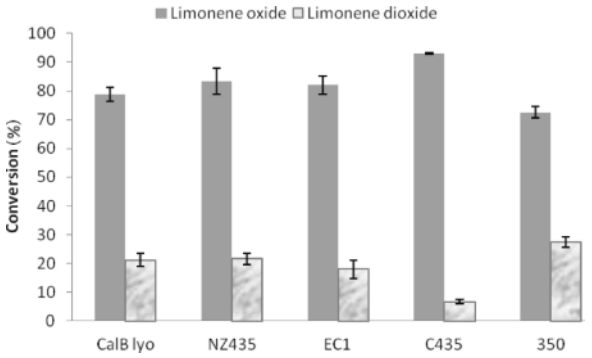


Fig. 3. Degree of limonene conversion into limonene monoxide and dioxide at 40 °C after 24 h of reaction time.

formation of limonene monoxide. Increasing the reaction temperature to 40 °C led to significantly increased limonene dioxide yields (Fig. 3), except for biocatalyst C435. The highest limonene dioxide yield of nearly 30 % was observed for the biocatalyst 350. No side reactions or rearrangement of educts or products were detected in any of the described reactions. In future studies it will be tried to elucidate the effects of immobilization and the carrier properties on the intrinsic kinetics of lipase-mediated epoxidation in more detail in order to develop even more efficient epoxidation systems.

Conclusion

We have shown for the first time that the lipase-mediated chemo-enzymatic conversion of limonene to limonene dioxide with maximum yields of 30 % is feasible using immobilized *Candida antarctica* lipase B under moderate reaction conditions (≤ 40 °C). We observed a strong dependency of the lipase activity in the synthesis of both limonene monoxide and dioxide with respect to the properties of the selected carrier materials and the reaction temperature. Thus it seems possible to predetermine certain desired product ratios of limonene monoxide/dioxide mixtures by simply adopting the reaction parameters in terms of temperature, enzyme carrier properties and immobilization method. Currently, crucial parameters such as biocatalyst stability (repetitive recycling), epoxidation yields at higher substrate concentrations, as well as the possibility to transfer this efficient, mild and bio-based epoxidation system to other valuable terpenes are under investigation in our laboratories.

Experimental Section

Materials

Novozyme 435 was obtained from Novozymes A/S (Bagsvaerd/Denmark), lyophilized CalB (*Candida Antarctica* lipase B) was a gift from c-LEcta (Leipzig/Germany). Enzyme carriers (IB350, IB-C435 and IB-EC1) were received from ChiralVision (Leiden/The Netherlands), D-Limonene (96 %) from Acros Organics (Geelen/Belgium). Solvents and all other reagents were purchased from Sigma-Aldrich (Steinheim/Germany) or Carl Roth (Karlsruhe/Germany) and used as obtained.

Enzyme immobilization

1 g of each carrier (Table 1) was mixed with 4 mL of pure ethanol (Merck) and incubated for 10 min. The samples were then filtered, thoroughly rinsed with 10 mL of aqua dest. and dried at r. t. 0.25 g of a pre-treated carrier was mixed with 1 mL of cooled enzyme solution containing 30 mg CalB (c-LEcta; Leipzig/Germany) in 100 mM acetate buffer (pH = 6.9) and left standing on ice for 16 h. Finally the samples were filtered, rinsed three times with 100 mM ice-cold acetate buffer (pH = 5), dried at r. t., and stored at 4 °C.

Lipase activity for the chemo-enzymatic epoxidation of limonene

Reactions of 50 mM caprylic acid, 25 mM limonene, 50 mM H_2O_2 (30 % aqueous solution), and 50 mg of lipase-immobilisates in toluene were carried out under permanent magnetic stirring in sealed GC-glass vials at either r. t. ($\sim$ 23 °C) or 40 °C, all in triplicate. Samples of 10 μL were withdrawn after 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h, mixed with 990 μL of ethyl acetate and then analyzed *via* GC-MS. One enzyme unit (U) corresponds to the amount of immobilized lipase that catalyzes the formation of 1 μmol limonene

oxide per minute and was calculated from the linear array of the initial product formation rates.

GC-MS

Concentrations of limonene, limonene monoxide and limonene dioxide were determined using a BPX5 column (SGE, 30 m, I. D. 0.25 mm, film 0.25 μm) connected to a QP2010 Plus gas chromatograph with a Single Quad MS-detector (both Shimadzu, Japan) with helium as carrier gas. 1 μL of the diluted sample was injected *via* SSL-injector (290 °C) starting at 60 °C for 1 min, then heated to 170 °C at 11 °C min^{-1} and finally to 270 °C at 70 °C min^{-1} and held for 3 min. Retention times, limonene: 5.72 – 5.78 min, limonene monoxide: 7.21 – 7.32 min and limonene dioxide: 9.52 – 9.65 min.

Supporting information

Mass spectra of limonene oxide and dioxide after chemo-enzymatic synthesis are given as Supporting Information available online (DOI: 10.5560/ZNB.2012-1067).

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