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Enantioselective transesterification of a tertiary alcohol by lipase A from *Candida antarctica*

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Abstract—Chiral tertiary alcohols and their esters represent important flavor compounds and are useful building blocks. Unfortunately, they are accepted by only a few lipases/esterases as substrates and enantioselectivity is usually very low. We report here a highly enantioselective transesterification of the tertiary alcohol 2-phenylbut-3-yn-2-ol using lipase A from *Candida antarctica* (CAL-A). Under optimized conditions, the corresponding acetate was obtained with 94% ee at 35% conversion equivalent to an enantioselectivity factor of $E=65$. In contrast, satisfactory enantioselective hydrolysis of the racemic acetate was not feasible as this is very prone to autohydrolysis.

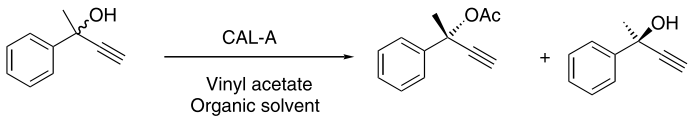
1. Introduction

Tertiary alcohols and their esters are an important class of compounds, which are found in numerous natural products (e.g. α-terpineol and linalool are important flavor compounds) and also represent a useful group of building blocks (e.g. 2-phenylbut-3-yn-2-ol and 3-methylpent-1-yn-3-ol).[1,2] Hydrolases constitute a group of versatile biocatalysts finding tremendous attention in organic synthesis[3,4] and industrial applications.[5–7] However the vast majority of chiral substrates resolved have been secondary alcohols, carboxylic acids and primary alcohols. Only a few hydrolases were shown to be active on tertiary alcohols.[1,8–12] This is basically due to the adverse steric interactions caused by the tertiary alcohols, making them challenging and tougher candidates for both chemical and biotransformations.

Recently, we discovered that a certain amino acid motif (GGGX-motif, in single-letter amino acid code where G denotes glycine and X denotes any amino acid) located in the oxyanion binding pocket of lipases and esterases determines activity towards tertiary alcohols.[13] All enzymes bearing this motif (e.g. CAL-A, lipase from *Candida rugosa*, pig liver esterase, acetyl choline esterases and an esterase from *Bacillus subtilis*) were active towards several acetates of tertiary alcohols, while enzymes bearing the more common GX-motif did not hydrolyze the model compounds.

For 2-phenylbut-3-yn-2-ol, which is an important building block,[1,2] we observed strong autohydrolysis during enzymatic hydrolysis, which circumvented high enantioselectivity in accordance with a previous study.[1]

In this report, we demonstrate that transesterification of 2-phenylbut-3-yn-2-ol (Scheme 1) avoids this autohydrolysis and thus it is possible to obtain enantiomerically enriched ester using CAL-A as biocatalyst. This is the first example for a highly enantioselective enzyme-catalyzed resolution of a tertiary alcohol.



Scheme 1.

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2. Results and discussion

Initially eight different lipases and esterases were investigated for transesterification activity of 2-phenylbut-3-yn-2-ol with vinyl acetate in hexane (Scheme 1). As expected, one esterase (BsteE) and three lipases (RML, CAL-B and TLL), which do not contain the GGGX-motif, did not show any transesterification activity. Of the GGGX-containing lipases and esterases, CAL-A from *C. antarctica* immobilized on carrier 1 (CAL-A-C1) and a thermophilic esterase (PestE) from *Pyrobaculum calidifontis*[12] immobilized on Celite, exhibited promising activity and selectivity (Table 1), while lipase from *C. rugosa* (CRL-AY) and the *p*-nitrobenzyl esterase from *B. subtilis* (BsubpNBE) showed considerably lower or no activity, albeit they are highly active in hydrolysis reactions.[13] CRL is known to be sensitive to the acetaldehyde released in the transesterification reaction using vinyl acetate as acyl donor. To exclude that the low activity of CRL is due to inactivation by acetaldehyde, the reaction was performed in isopropenyl acetate instead. However again, low enantioselectivity and conversion below 2% were found. Thus CAL-A was used for further optimization, since this enzyme exhibited higher enantioselectivity as compared to the esterase from *P. calidifontis*.

Different solvents exhibit different effects on enzyme activity and enantioselectivity.[14–16] We investigated various commonly employed organic solvents and an ionic liquid ([BMIM][BTA]) for the transesterification reaction using CAL-A-C1 as catalyst. The reaction was also performed with a large excess of the acyl donor vinyl acetate making the reaction system 'solvent-free'. Highest enantioselectivity and conversion were obtained in isooctane ($E=49$, $C=21\%$) and in vinyl acetate ($E=48$, $C=17\%$), while the other solvents were found less useful (Table 2). Isooctane was selected for all further experiments.

The effect of water activity (a_w) was also investigated. Highest conversion ($C=21\%$ after 48 h reaction) was obtained in isooctane at a water activity of 0.11. Higher water activities resulted in a decrease in the conversion, most likely due to the action of water as a competitive nucleophile in the reaction.[17,18] When the solvent (isooctane) and substrates were dried over molecular sieves, very low conversion ($C=3\%$, after 48 h) was obtained, hence a small amount of water is essential to maintain the enzyme catalytically active. The enantioselectivity ($E=50$) was however not influenced by the water activity.

Enantioselectivity and conversion were not affected when different acyl donor concentrations (0.025 M, 0.2 M and 1.0 M) were employed. A conversion of 20% (after 48 h) and an enantioselectivity factor of $E=50$ was obtained in all cases. Isopropenyl acetate (200 mM) was used as an alternative acyl donor to vinyl acetate to investigate if conversion and (or) enantioselectivity could be increased in the transesterification reaction. The use of isopropenyl acetate (200 mM) resulted, however, in a decreased conversion ($C=9\%$, after 48 h) as compared to vinyl acetate ($C=22\%$). Enantioselectivity ($E=50$) was, however, not influenced by the choice of acyl donor.

Immobilization support has been shown to influence conversions and selectivities of enzyme-catalyzed reactions.[19–21] In this study, we immobilized the lyophilized powder of lipase A from *C. antarctica* (Chirazyme L5) on a porous polypropylene support, EP-100, which has proved to be a suitable support for different lipases.[22–24] CAL-A immobilized onto EP-100 was then compared with the commercial preparation CAL-A-C1. The reaction proceeded up to 35% conversion and high enantiomeric excess of product ($ee_p=94\%$) and substrate ($ee_s=50\%$) with CAL-A-EP-100 as catalyst, while the reaction tend to stop at about 20–22% after 24 h for CAL-A-C1 (Fig. 1). The enantioselectivity was, however, not affected by the enzyme preparation applied.

Temperature has also been shown to be an important parameter for enzyme activity and selectivity.[25–27] Reactions at 20 and 40°C revealed that the conversions were reduced at 40°C with both enzyme preparations (Table 3). A slightly higher *E*-value ($E=87$) was obtained with CAL-A-EP-100 at 40°C.

3. Conclusion

In conclusion, it was shown that a CAL-A-catalyzed resolution of a tertiary alcohol is feasible by performing a transesterification reaction in an organic solvent.

Table 1. Conversion, ee_p, ee_s and *E*-value in the transesterification of 2-phenylbut-3-yn-2-ol (5 mM) with vinyl acetate (200 mM) in hexane using different GGGX-motif containing lipases and esterases at $a_w=0.11$, $T=20$°C, enzyme = 100 mg; reaction time = 48 h. The esterases were immobilized onto Celite, while the lipases were used as received from the manufacturer

Enzyme	Conv. (%)	ee_p (%)	ee_s (%)	E[b]
CAL-A-C1	9.3	91	9.3	22
PestE[a]	7.6	73	6.0	8
BsubpNBE	0	–	–	–
CRL-AY	1.3	8.7	0.1	1.2

[a] Reaction at 60°C, since the hyperthermophilic PestE exhibited considerably lower activity at 20°C.
[b] The (*R*)-enantiomer was preferred in all cases.

Table 2. Conversion, ee_p, ee_s and *E*-value in the transesterification of 2-phenylbut-3-yn-2-ol (5 mM) with vinyl acetate (200 mM) in different solvents using lipase A from *C. antarctica* on carrier-1 (CAL-A-C1) at $a_w=0.11$, $T=20$°C, enzyme = 100 mg; reaction time = 48 h

Solvent	Conv. (%)	ee_p (%)	ee_s (%)	E
Isooctane	21	95	26	49
Vinyl acetate	17	97	20	48
Hexane	9.3	91	9.3	22
MTBE	2.2	100	n.d.	n.d.
[BMIM][BTA]	3.6	100	n.d.	n.d.

n.d., not determined since only one enantiomer of the ester could be detected due to the low conversions.

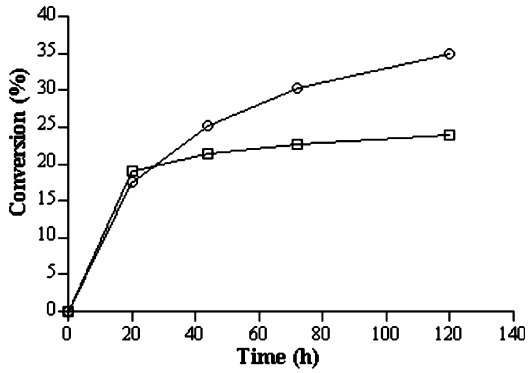

Figure 1. Influence of immobilization support on conversion in the transesterification of 2-phenylbut-3-yn-2-ol (5 mM) with vinyl acetate (200 mM) in isooctane at $a_w = 0.11$, $T = 20°C$, enzyme = 100 mg, reaction time = 48 h. CAL-A-EP-100 (○) and CAL-A-C1 (□).

Under optimized conditions, 35% conversion and an enantioselectivity of $E = 65$ can be achieved.

4. Experimental

4.1. Enzymes and chemicals

Vinyl acetate (>99%), isopropenyl acetate (>99%), (*R*,*S*)-2-phenylbut-3-yn-2-ol (>98%), 2,2,4-trimethylpentane (isooctane) (99%), *tert*-butyl-methylether (MTBE) (>99.8%) and *n*-hexane (>99%) were purchased from Fluka. The ionic liquid 1-butyl-3-methyl imidazolium bis[(triflouromethyl)sulfonyl]amide, [BMIM][BTA] (>97%) was a gift from Solvent Innovation (Cologne, Germany).

Polypropylene powder Accurel EP-100 (200–1000 μm; Akzo, Obernburg, Germany) was kindly donated by Professor Patrick Adlercreutz (Department of Biotechnology, Lund University, Sweden). Immobilized lipase from *Rhizomucor miehei* (Chirazyme L9 on carrier 2, designated as RML), lipase B from *C. antarctica* (Chirazyme L2 on carrier 2, designated as CALB), immobilized lipase A from *C. antarctica* (on carrier 1, CAL-A-C1) and lyophilized lipase A from *C. antarctica* (Chirazyme L5) were donated by Roche Diagnostics (Penzberg, Germany). Lipase from *C. rugosa* (designated as CRL-AY) came from Amano Pharmaceutical Co. (Nagoya, Japan) and immobilized lipase from *Thermomyces lanuginosa* (Lipozyme TL IM, designated as TLL) was a gift from Novozymes A/S (Bagsvaerd, Denmark). The *p*-nitrobenzyl esterase from *B. subtilis* (BsubpNBE), the thermophilic esterases from *Bacillus stearothermophilus* (BsteE) and from *P. calidifontis* (PestE) have previously been cloned and overexpressed in *Escherichia coli* and were produced according to published protocols.[12,28] Other chemicals used were of analytical reagent grade.

4.2. Immobilization of esterases by deposition onto Celite and lipase by adsorption onto Accurel EP-100

The esterases (15 mg lyophilized powder) were dissolved in sodium phosphate buffer (50 mM, pH 7.5). The resulting enzyme solution (2 ml) was then added to Celite (1 g) and the preparation was thereafter dried for 12 h under vacuum.

Lipase A from *C. antarctica* was immobilized by adsorption on the hydrophobic support Accurel EP-100. Lyophilized lipase powder (60 mg) was dissolved in 20 ml sodium phosphate buffer (pH 6.0, 20 mM) and centrifuged. The supernatant was added to Accurel EP-100 (0.4 g), which was prewetted with ethanol (3 ml/g support). The support and the enzyme solution were incubated overnight at room temperature and thereafter filtered. Finally 1 ml sodium phosphate buffer (50 mM, pH 7.5) was added and the preparation was dried overnight under reduced pressure. The resultant preparation was designated as CAL-A-EP-100.

4.3. Transesterification reaction

Substrate solution (5 mM alcohol and 200 mM vinyl acetate in solvent) and enzyme preparation were incubated over a saturated salt solution to obtain a defined initial water activity. The salts used for equilibration were LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $MgNO_3$ ($a_w = 0.54$) and KCl ($a_w = 0.84$). The equilibrations were performed overnight. Reactions were performed in 2 ml screw-capped vials with Teflon-lined septa and started by adding enzyme preparation (100 mg) to the substrate solution (1 ml). Reaction vials were shaken (1300 rpm) on a thermoshaker. Samples (50 μl) were withdrawn at different intervals of time and analyzed by gas chromatography.

4.4. GC analysis

Alcohol substrate and ester product were analyzed by a Shimadzu Gas Chromatograph (GC-14 A) equipped with a flame ionization detector. The chiral column used was a Hydrodex-β-3P (25 m×0.25 mm, Macherey-

Table 3. Effect of temperature and carrier in the transesterification of 2-phenylbut-3-yn-2-ol (5 mM) with vinyl acetate (200 mM) in isooctane using CAL-A as catalyst at $a_w = 0.11$, enzyme = 100 mg, reaction time = 48 h

Temp. (°C)	Preparation	Conv. (%)	ee_p (%)	ee_s (%)	*E*
20	EP-100	25	96	32	65
40	EP-100	18	97	21	87
20	C1	21	95	26	49
40	C1	7.6	89	7.3	18

Nagel, Düren, Germany). Hydrogen was used as a carrier gas and the temperature of the injector and detector was 200°C. The temperature of the column was 110°C. Absolute configurations were assigned based on published data.[1,13]

4.5. Calculation of enantioselectivity

The enantiomers of the product ester were baseline separated in the GC analysis giving ee_p, while the enantiomers of the alcohol were not. The conversion (C) was therefore calculated from the relative amounts of ester and alcohol. The ee_s values were then obtained from the equation $C = ee_s/(ee_s+ee_p)$. Enantioselectivity was calculated using the equation $E = \ln[(1-C(1+ee_p)]/\ln[(1-C(1-ee_p)]$ described by Chen et al.[29]

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