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Improvement of Enantioselective Enzymatic Ester Hydrolysis in Organic Solvents

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Abstract: A method is presented to improve the enzymatic enantioselective hydrolysis of chiral esters in water-saturated organic solvents. It is shown that addition of a non-reactive amine to the reaction mixture leads to the formation of an ion-pair with the produced carboxylic acid. Yield and enantiomeric excess are increased in lipase catalyzed kinetic resolutions of methyl 2-chloropropionate and glycidyl butyrate in various solvents. Chemical background hydrolysis was reduced with respect to aqueous solvents. A second phase was formed during reaction in apolar solvents, due to ion-pair formation, which may facilitate the isolation of the desired compound.

Introduction

The enzymatic kinetic resolution of enantiomers has become a common tool in organic chemistry to prepare enantiomerically pure compounds. In order to prepare a pure product one needs a very high enzyme enantioselectivity. The remaining substrate might be obtained in highly pure form with moderately selective enzymes, provided that the reaction equilibrium is favorable[1]. If the target compound is a chiral ester, the most suitable strategy thus is either hydrolysis or transesterification (or aminolysis) of the target ester, rather than esterification of the chiral alcohol or carboxylic acid.

The conventional method for kinetic resolution of an ester is through hydrolysis in an aqueous system. Although the reactions mostly run to completion because of dissociation of the produced carboxylic acid, this approach has several drawbacks: Firstly, substrate solubilities are usually low, resulting in low enzymatic reaction rates[2]. Secondly, non-selective chemical background hydrolysis of the ester may occur in water, leading to a lower enantiomeric purity of the reaction product[3,4,5]. Although the enantiomeric purity of the remaining substrate will not be affected, its yield will be lower. Table 1 shows the quantitative effect of the relative chemical background hydrolysis rate of the overall reaction on the maximum attainable, i.e. the optimum, substrate yield, when the purity requirement for the remaining substrate $ee_S = 0.98$. It is also shown that the maximal (= initial) product enantiomeric excess, ee_P, may drop below 98% because of background hydrolysis, despite the use of an enzyme with a very high enantioselectivity. A third

disadvantage of water as solvent may in some cases be the difficulty to work-up the reaction product from an aqueous system[6].

Table 1. Calculated effect of the relative rate of non-selective chemical background hydrolysis (calculated as the ratio of initial chemical hydrolysis rate and the total (= chemical + enzymatic) hydrolysis rate) on the optimum substrate yield, y_S, (when $ee_S = 0.98$) and the maximal ee_P (at zero conversion, $\xi = 0$) for various enzyme enantioselectivities, E. All rates are assumed to be first order in substrate.

	$E = 10$		$E = 100$		$E = \infty$	
relative chemical hydrolysis rate	max ee_P (ξ=0)	opt y_S (ee_S=0.98)	max ee_P (ξ=0)	opt y_S (ee_S=0.98)	max ee_P (ξ=0)	opt y_S (ee_S=0.98)
0	0.82	0.30	0.98	0.48	1.00	0.51
0.01	0.81	0.29	0.97	0.47	0.99	0.49
0.05	0.78	0.26	0.93	0.43	0.95	0.45
0.10	0.70	0.22	0.88	0.37	0.90	0.40

An alternative to a homogeneous aqueous medium for ester hydrolysis is an aqueous/organic two-phase system[1,7]. Such a system overcomes the problem of ester solubility, and remains irreversible because of dissociation of the carboxylic acid in the aqueous phase. However, it has been shown that it may be not an appropriate system to perform a kinetic resolution: As a result of partitioning of the ester enantiomers between both phases, pseudo-equilibria may establish, causing a decrease in the enantiomeric excess[8]. Additionally, the formation of an emulsion in an aqueous/organic two-phase system may complicate work-up.

Another possibility is to perform the hydrolysis reaction in a water-saturated organic solvent[2,4,9], provided that the enzyme remains active and enantioselective. Substrate solubility may be high, and enzyme and products might be easily recovered and recycled. Since non-enzymatic hydrolysis is repressed, negative effects on yield and enantiomeric purity as presented in Table 1 are expected to be absent. Furthermore the enzyme enantioselectivity may be favorably affected by an organic (co)solvent[4,10]. However, two problems may hamper the achievement of the desired extent of conversion and enantiomeric excess: The thermodynamic reaction equilibrium may be unfavorable and the water capacity of some solvents may be too low.

The latter problem might be overcome by replacing water by another, more soluble nucleophile, for example an alcohol[11] or amine[12], carrying out a transesterification or aminolysis reaction as an alternative to hydrolysis. If one prefers transesterification or aminolysis in an organic solvent, the equilibrium position

may still be unfavorable. High yields are out of reach, unless one of the reaction products is removed from the medium during reaction, e.g. by using vinyl ester as substrate.

Palomer et al.[9] preferred to perform the kinetic resolutions of chiral acid esters by hydrolysis in organic medium rather than by transesterification or aminolysis, because of much easier work-up and isolation of the desired compound: No additional reagents were required, and separation of the produced carboxylic acid from the unreacted ester was easy by washing with alkali. Another reason *not* to choose the transesterification strategy may be the need to work in an anhydrous medium, considering the possibility of undesired enzymatic hydrolysis as a background reaction.

In this paper we will present a method to overcome the problem of the unfavorable equilibrium of hydrolysis reactions in water-saturated water-immiscible organic medium, in order to enhance the enantiomeric excess and yield in enzymatic kinetic resolutions of chiral esters. To this end we will perform the reaction in the presence of a non-reactive amine. An insoluble ion-pair is now expected to be formed by the chiral product carboxylic acid and this amine:

$$R_1^*COOR_2 + H_2O \rightleftarrows R_1^*COOH + R_2OH \quad \text{and} \quad R_1^*COOH + RNH_2 \rightarrow RNH_3^+R_1^*COO^-$$

Consequently the equilibrium yield and the maximum obtainable enantiomeric excess may be enhanced. Work-up may be facilitated by precipitation of the ion-pair produced. The same method applies to the resolution of chiral alcohols, but in this case the chiral compounds will stay in solution while the produced non-chiral acid may precipitate.

According to García et al.[13] a salt was formed in the lipase-catalyzed hydrolysis of activated racemic 2-chloro-2-phenylacetate ester in organic solvent, when aniline was added. This gave improvements in conversion and enantiomeric excess values, due to loss of reversibility of the reaction, but was not studied in any detail. Berger et al.[14] and Theil et al.[15] performed alcohol resolution using activated carboxylic acids in the presence of amines or $KHCO_3$. The reported[14] increase in E in the presence of base, however, is equally well described by a *fixed* E-value, taking reversibility into account, and improvements may be subscribed to a shift in the equilibrium position.

In this study we will investigate the effect of ion-pair formation on a kinetic resolution of chiral esters. Various solvent systems will be tested and the occurrence of chemical background hydrolysis will be examined. Model reactions are the *C. cylindracea* lipase (CCL) catalyzed hydrolysis of racemic methyl 2-chloropropionate (chirality in acid) and the porcine pancreas lipase (PPL) catalyzed hydrolysis of glycidyl butyrate (chirality in alcohol), in absence and presence of pyrrolidine.

Results and Discussion

Ion-pair formation: In order to investigate the effect of the addition of an amine on ion-pair formation during the kinetic resolution of an ester, it is required that the amine does not interfere with the

enantioselective enzymatic hydrolysis. Various amines are inadequate in this respect, since their application would lead to aminolysis of the ester[12]. Therefore we chose pyrrolidine, which not only appeared not to react with the substrates we used, but also is a rather strong base (pK_A = 11.4), and was thus likely to form an ion-pair with the carboxylic acids. When pyrrolidine (0.1 M) was added to a solution of 2-chloropropionic acid (0.1 M) in heptane or cyclohexane exothermic formation of a light yellow viscous second phase was observed. This heavy syrup most likely contained the ion-pair. In other solvents such as acetonitrile, cyclohexanone, 2-pentanone, diethyl ether, 1-octanol, DMF, dichloromethane or toluene no second liquid phase was noticed, although a light yellow tint again appeared. Probably the ion-pair was completely soluble in these more polar solvents at the applied concentrations. Ion-pair formation will be more favorable when the solvent hydrophobicity increases (as in heptane). Furthermore, phase separation in apolar solvents should facilitate work-up of the desired product. However, the water capacity of the apolar solvent may cause a problem, as will be pointed out below.

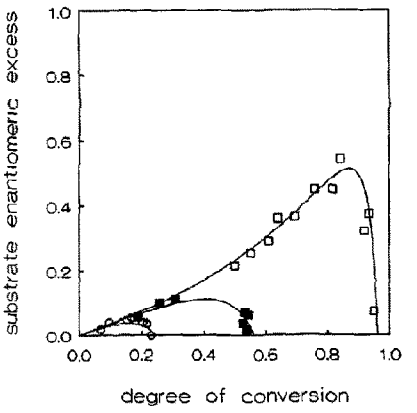

Figure 1. CCL catalyzed hydrolysis of racemic methyl 2-chloropropionate (20 mM) in water saturated dichloromethane. The enantiomeric excess of the remaining (S)-ester is shown as a function of the reaction progress. ○ = without pyrrolidine, ■ = 20 mM pyrrolidine, □ = 50 mM pyrrolidine. The curves show the best fit (E = 2.0 for all experiments) of the model (reversible uni-uni kinetics[1]) to the data.

Hydrolysis of chiral acid ester: The CCL catalyzed enantioselective hydrolysis of racemic methyl 2-chloropropionate in buffer saturated dichloromethane leads to low values of the ester enantiomeric excess, because of the unfavorable reaction equilibrium (Figure 1, Table 2). In the presence of an equimolar (20 mM) amount of pyrrolidine, however, the equilibrium conversion, ξ_{EQ}, increased from 24% to 56%, and to 94% in presence of 50 mM pyrrolidine (150% molar excess), as is depicted in Figure 1. From the same figure it is clear that as a consequence of the improved equilibrium also higher enantiomeric excess values were measured. In heptane an even more dramatic result was obtained, shifting the equilibrium from 9% to approximately 94% conversion. In 2-pentanone, which is more polar, the equilibrium conversion is high, and by addition of pyrrolidine only a small improvement was obtained. All results are summarized in Table

2. From the calculated *E*-values it appeared that pyrrolidine does not affect the enantioselectivity of the enzyme.

Table 2. The effect of pyrrolidine addition on CCL activity and enantioselectivity and the equilibrium conversion in hydrolysis of methyl 2-chloropropionate by 2 g CCL in various water-saturated solvents.

solvent	pyrrolidine conc. (mM)	*E*-value	ξ (1 h)	ξ_{EQ}[a]
heptane	0	~2	0.07[b]	0.087
heptane	50	1.9	0.27	0.94
dichloromethane	0	2.0	0.15	0.24
dichloromethane	20	2.0	0.11	0.56
dichloromethane	50	2.0	0.055	0.94
dichloromethane	100	~2	0.013	n.d.[c]
2-pentanone	0	1.5	0.56[d]	0.95
2-pentanone	50	1.6	0.91	0.98
phosphate buffer	0	2.4	1[e]	1

[a] These values were estimated from the best fit of the uni-uni model reported by Chen et al.[1] to experimental data at lower ξ-values (as in figure 1 and 2). [b] Equilibrium was reached in an early stage of the reaction. [c] Not determined. [d] Only 1 g CCL was used. [e] Only 50 mg CCL was used, indicating the high enzyme activity. The reaction was completed within 1 h.

Hydrolysis of chiral alcohol ester: We also investigated the PPL catalyzed kinetic resolution of racemic glycidyl butyrate by hydrolysis in organic medium. In this case the chiral product, glycidol, remains in solution, while the non-chiral product, butyric acid, forms an ion-pair with pyrrolidine. We analyzed both the remaining ester and the product alcohol. The results are presented in Figure 2. Again improvements of the resolution were established. The effect seems to be the most profound for the ester. In addition to this expected effect we found to our surprise that the observed enantioselectivity of the enzyme was higher in the presence of pyrrolidine. We have no direct explanation for this.

Reversibility: Since we found pseudo-equilibria in our experiments (Figure 1 and 2) it is clear that ion-pair formation as it occurs in our systems is reversible and therefore not appropriate to make a reaction run to completion. Apparently the ion-interactions are rather weak. From Table 2 it is read that 50 mM pyrrolidine gave an 11-fold increase in equilibrium conversion in heptane, a 4-fold increase in dichloromethane, and only a minor improvement in 2-pentanone. From these results it can be reasoned that in an apolar solvent the equilibrium for ion-pair formation will be more profound than in polar solvents.

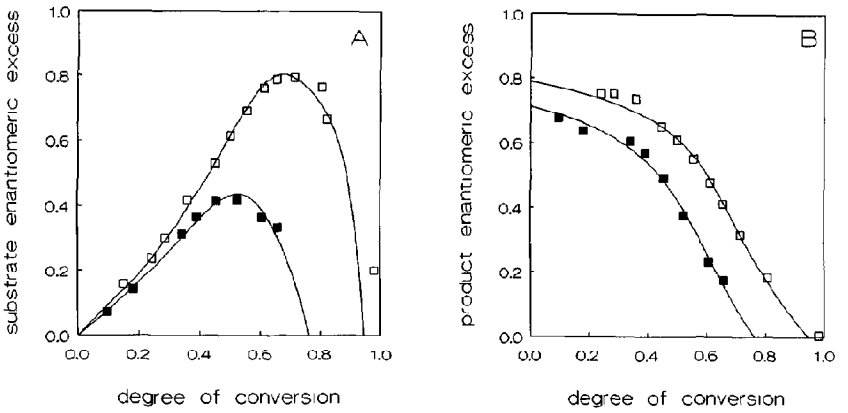

Figure 2. PPL catalyzed hydrolysis of racemic glycidyl butyrate (20 mM) in water saturated dichloromethane in absence (■) and presence (□) of pyrrolidine (50 mM). Figure 2A shows the enantiomeric excess of the remaining (*S*)-ester, figure 2B of the product glycidol as a function of the reaction progress. The curves show the best fit of the model[1] to the data: E = 6.0 and ξ_{eQ} = 76 % without pyrrolidine, E = 8.5 and ξ_{eQ} = 94% in presence of pyrrolidine.

Catalytic activity: From Table 2 it is clear that reaction rates drop in presence of pyrrolidine. Considering the dichloromethane experiments, the conversion after 1 h reaction time in presence of 100 mM pyrrolidine is significantly lower than in presence of 50 mM pyrrolidine. From this it may be concluded that inhibition of the enzyme by pyrrolidine probably occurs. To minimize this negative effect on the enzyme activity, it is suggested to keep its concentration as low as possible, by adding it to the vessel during the progress of the reaction (fed-batch).

Chemical hydrolysis: Chemical background hydrolysis of methyl 2-chloropropionate was not detected in buffer saturated heptane, dichloromethane and 2-pentanone after 1 h, 6 h and 24 h reaction time, also after addition of pyrrolidine. In phosphate buffer (10 mM, pH 7.0) a first order chemical hydrolysis constant $k_h = 2.5 \cdot 10^{-6}$ s^{-1} was measured[4]; this corresponds to 1 % hydrolysis after 1 h. No chemical background hydrolysis of glycidyl butyrate in buffer saturated dichloromethane was measured after 1 h, 2 h, 6h, and 22 h. Apparently chemical background hydrolysis is repressed in our systems, as was expected.

Water content: The water content may be the limiting factor in hydrolysis in organic media. The water requirement is dependent on the initial substrate concentration. For the solvents we applied in this work, the maximum water solubility in heptane was 0.035 mM, in dichloromethane 143 mM and in 2-pentanone 681 mM[16]. Since heptane could in our case (initial ester concentration is 20 mM) not have

contained sufficient water to catalyze the reaction to the extent we measured, water probably originated from the enzyme powder[17]. The water content of the CCL powder was determined to be ≈3 % w/w. Therefore the 2 g CCL used in the experiments provided circa 170 mM water. At higher substrate concentrations lack of water may play a limiting role in such systems, so care should be taken to keep the solvent water-saturated by feeding water to the system or by using salt hydrates as water buffer[18]. Lump-formation, as occurring in our suspended-enzyme system, may be simply overcome by immobilization of the catalyst on a macroscopic carrier.

Experimental section

Enzymes and chemicals: *Candida cylindracea* lipase (EC 3.1.1.3, Type VII) and porcine pancreatic lipase (Type II) were purchased from Sigma Chemical Co, methyl 2-chloropropionate and glycidyl butyrate were from Aldrich Chemie. All solvents were analytical grade from J.T. Baker.

Hydrolyses: Experiments were performed in a magnetically stirred closed vessel (kept at 30 °C), containing 20 ml buffer saturated solvent (by shaking with 10 mM phosphate buffer, pH 7) 50 µl n-decane (internal standard) and 2 g lipase. The reaction was started by adding substrate (20 mM). Samples of 0.5 ml were centrifuged, and an aliquot of the supernatant was directly analyzed.

Analysis: The enantiomeric excess values of methyl 2-chloropropionate, glycidyl butyrate and glycidol were determined on GC (Varian, model 3700), using a Chiraldex column (20 m x 0.25 mm) from Astec. This is a capillary γ-cyclodextrin column with trifluoroacetyl coating. The extent of conversion of substrate was calculated by the internal standard method using the sample at zero time for calibration.

Calculations: Values for the enantiomeric ratio, E, and the equilibrium conversion, ξ_{EQ}, were obtained using the equation reported by Chen[1] for reversible enantioselective reactions obeying uni-uni kinetics. Model parameters were obtained from the experimental data using the non-linear regression program RRGraph (Stichting Reactor Research, Delft, The Netherlands) or Simfit (J.B.A. van Tol, Department of Microbiology and Enzymology, Delft University of Technology, freely available).

Enzyme water content: A weighed amount of CCL powder (4 g) was dried over molecular sieve 3 Å in a vacuum exsiccator, and weighed again after 1 and 2 days. The difference was assumed to be free water in the enzyme powder.

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