COMMUNICATION TO THE EDITOR

Influence of Water Activity on the Enantioselective Esterification of (*R,S*)-Ibuprofen by *Candida antarctica* Lipase B in Solventless Media

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Abstract: The lipase-catalyzed enantioselective esterification of ibuprofen has been studied in a media, composed only of substrates. When racemic ibuprofen is used, the alcohol-chain length affects the esterification rates of individual enantiomers, but it does not affect the enantioselectivity. Water activity affects the esterification rates of (*R*)- and (*S*)-ibuprofen differently, leading to higher enantioselectivity at lower water activities. Experiments were also conducted at various (*R*)- to (*S*)-ibuprofen ratios. It appears that the esterification rate of (*R*)-ibuprofen is always proportional to its concentration, whereas at low water activity the esterification rate of (*S*)-ibuprofen shows a saturation at higher concentrations. Other 2-phenyl carboxylic acids were studied, and the increase in apparent enantioselectivity at low-water activity was not observed for the molecules tested. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **63**: 502–505, 1999.
Keywords: lipase; *Candida antarctica;* ibuprofen; water activity; resolution; enantioselectivity; esterification

INTRODUCTION

Enzyme-catalyzed reactions in solvent-free media have been described in the recent past (Ergan et al., 1991). The major advantages of these systems are that no solvent is used, minimizing the environmental impact and the reaction volume, lowering the size of the equipment required, and the associated capital immobilization costs. Some examples of the use of these systems are the synthesis of glycerides from fatty acids and glycerol (Ergan et al., 1990), the production of wax esters (Trani et al., 1991), the modification of fats and oils through interesterification (Foglia et al., 1993; Marangoni et al., 1993), the synthesis of acylglucosides (Adelhorst et al., 1990), and the kinetic resolution of racemic compounds through esterification (Ducret et al., 1995; Ergan et al., 1995). It has been shown that water activity influences the activity of the enzyme, and the equilibrium and yield of the reaction (Dudal and Lortie, 1995), just as in organic solvents. Furthermore, in the case of the synthesis of dodecyl decanoate catalyzed by *Rhizomucor miehei* lipase, the enzyme activity vs. water activity relationship has the same general profile in the solventless system as in organic solvents (Valivety et al., 1992).

During the study of the enantioselective esterification of ibuprofen (2-(4-iso-butylphenyl)propionic acid) with fatty alcohols, catalyzed by the type B lipase from *Candida antarctica* (CALB) under reduced pressure (Ducret et al., 1995), it was observed that the enantiomeric excess of the remaining (*S*)-ibuprofen could depend on the pressure in the reactor, possibly because of the impeded water removal. To better understand this kinetic resolution, we decided to study the influence of factors such as water activity, fatty alcohol-chain length, and substitution around the chiral center on the kinetics of this reaction.

MATERIALS AND METHODS

Materials

The *Candida antarctica* type B lipase, immobilized on acrylic resin (Novozym 435), was a gracious gift from Novo Industries (Denmark). (*R,S*)-2-(4-iso-butylphenyl)propionic acid (ibuprofen) was purchased from Ethyl Corporation (Baton Rouge, LA). (*R,S*)-2-phenylpropionic acid, (*R,S*)-2-phenylbutyric acid, decanol, 1-dodecanol, 1-tetradecanol, 1-hexadecanol, and trifluoroacetic acid were obtained from Aldrich Chemical Company (Milwaukee, WI). Hexane, molecular sieves 3Å, magnesium nitrate, acetone, acetonitrile, lithium bromide, and 2-propanol were purchased from Fisher Scientific (Montreal, Qc., Canada). Acetic acid, disodium hydrogen orthophosphate heptahydrate, potassium acetate, and magnesium chloride were obtained from BDH

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Inc. (Ville St-Laurent, Qc., Canada). Sodium chloride and monobasic sodium phosphate were purchased from Anachemia Science (Lachine, Qc., Canada). Caprylic acid was purchased from Sigma Chemical Company (St. Louis, MO).

Reactions

All reactions were performed in open Eppendorf tubes enclosed in a 230-L plexiglass chamber. The water activity (a_W) was adjusted with saturated aqueous solutions of various salts, lithium bromide ($a_W = 0.15 \pm 0.02$), potassium acetate ($a_W = 0.32 \pm 0.02$), magnesium chloride ($a_W = 0.40 \pm 0.02$), magnesium nitrate ($a_W = 0.50 \pm 0.02$), and sodium chloride ($a_W = 0.74 \pm 0.02$) or molecular sieves 3Å ($a_W = 0.05 \pm 0.02$). A Hanna Instruments (Padova, Italy) HI 9065 hygrometer was used to measure a_W. The temperature in the chamber was maintained at 55°C by circulating hot water in a copper tubing inside the chamber. To ensure uniform temperature and water activity in the chamber, air was circulated with a fan. The chamber was insulated with cotton wool to prevent temperature variations and condensation on the plexiglass at high water activities. Sample transfer from the chamber was made through an airlock.

Reactions With Ibuprofen

Ibuprofen (0.7271 mmol) and alcohol (0.5452 mmol) were mixed and the ibuprofen was dissolved by heating to 70°C. The tubes were then transferred into the chamber, along with tubes containing 25 mg of enzyme for equilibration at the selected water activity. Reactions were started by pouring the enzyme into the tubes containing the substrates, and the enzyme was kept in suspension by placing the tubes in an Eppendorf mixer. Samples were taken at 1-h intervals and analyzed by HPLC, using methods described previously (Ducret et al., 1995). Samples were diluted in the mobile phase and filtered on a 0.45 mm filter prior to injection. The enantiomer concentration vs. time data was analyzed by linear regression to calculate the initial rates.

Reactions With Other 2-Phenyl Carboxylic Acids

The reactions were performed as above, with the exception that 0.6659 mmol 2-phenylpropionic acid were mixed with 0.4994 mmol decanol, and 0.6090 mmol 2-phenylbutyric acid were mixed with 0.4568 mmol decanol. Because of the smaller reaction volume, only 16.7 mg of enzyme were used. For easier comparison, the reactions with ibuprofen were repeated using this amount of enzyme. The progress of the reactions was monitored by HPLC, as above. All reactions were run in triplicate. The reaction volumes were evaluated by mixing larger amounts of the substrates (2 g of acid) in the same molar proportions, and melting them at 60°C in a graduated cylinder. It was then possible to compare one acid to the other, by reporting the reaction rates per gram of enzyme.

RESULTS AND DISCUSSION

To evaluate the combined effect of water activity and chain length of the alcohol, the initial esterification rates of both ibuprofen enantiomers were measured at various values of a_W and with four different fatty alcohols. The results are shown on Figure 1. Because it is difficult to determine the exact reaction volume with these small amounts of pure substrates, the reaction rates are reported in terms of concentration change rather than variation of quantity of product. Stepwise forward regression showed that the rate of esterification of (*R*)-ibuprofen is dependent on the chain length of the alcohol and on the water activity. In the case of (*S*)-ibuprofen, only the chain length has an influence, as summarized in Table I. When the ratio of the two reaction rates is examined, it can be seen that the chain length has no effect on the observed enantioselectivity whereas lower water activities foster higher enantioselectivity.

In the case of pure substrates in solventless media it is difficult to perform a kinetic study of the reaction, Because concentrations cannot be changed easily. To gain some understanding on this system despite this limitation, initial reaction rates were determined at various ratio (*S*)- to (*R*)-ibuprofen (total ibuprofen concentration being constant) and at different values of water activity. The results are shown on Figure 2. It is interesting to note that the esterification rate of the (*R*)-enantiomer is always proportional to the concentration of this enantiomer, and that the first-order rate constant for the esterification of (*R*)-ibuprofen increases as a_W decreases. In contrast, the rate of esterification of (*S*)-ibuprofen is linear at $a_W = 0.74$ and $a_W = 0.41$ and saturation kinetics appear for the lower water activities. This may explain why it is possible to obtain a 97.5% enantiomeric excess of (*S*)-ibuprofen (Ducret et al., 1995) despite a low enantioselectivity, because the rate of esterification of the (*S*)-enantiomer remains quite low, even when the rela-

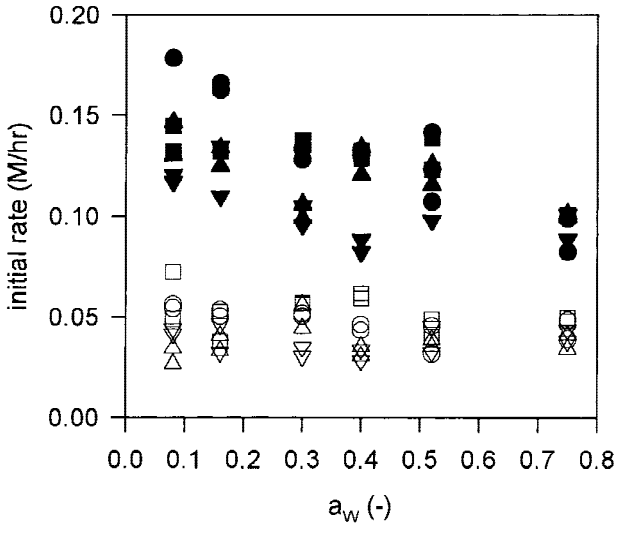

Figure 1. Initial rate of esterification of (*R*)-ibuprofen (filled symbols) and (*S*)-ibuprofen (open symbols) with decanol (●), dodecanol (■), tetradecanol (▲), and hexadecanol (▼) as a function of water activity.

Table I. *P* and *F* statistics for the forward stepwise regression analysis of the dependence of esterification rates and observed enantioselectivity on the water activity and carbon chain length of the alcohol.[a]

Dependent variable	Independent variable	*P*	*F*
ν_{iR}	a_w	< 0.0001	30.5
	chain length	0.0002	15.8
ν_{iS}	a_w	0.2411	1.41
	chain length	0.0002	16.1
ν_{iR}/ν_{iS}	a_W	0.0042	9.07
	chain length	0.3597	0.856

[a]*P* and *F* statistics are calculated at the first step of a stepwise forward regression for the inclusion of the independent variables in a simple linear model of the form $y = b_0 + b_1 a_W + b_2 C$ where *y* is the dependent variables, a_W the water activity, and *C* the number of carbon atoms of the alcohol.

tive concentration of the competing (*R*)-enantiomer is low. It also explains why poor water removal results in poor resolution. When the water activity is high, the saturation kinetics do not exist anymore, and the rate of esterification of (*S*)-ibuprofen increases when its relative concentration rises, while at the same time the rate of esterification of (*R*)-ibuprofen remains low. It is clear that the conditions in these initial rates experiments are different from those prevailing in a resolution reaction, where the polarity of the media diminishes as the esterification progresses. Nevertheless, the information obtained here parallels what has been observed for resolution reactions under reduced pressure (Ducret et al., 1995).

The fact that the esterification rate of (*R*)-ibuprofen increases when the enzyme is less hydrated is interesting. Low hydration of the enzyme is believed to decrease the flexibility of enzymes, impeding their catalytic activity, because of interactions between polar residues, no longer screened by bound water molecules (Affleck et al., 1992; Zaks and Klibanov,1988). This has been observed in the case of γ-chymotrypsin crystals suspended in anhydrous hexane for which the thermal factors are lower than those for the native structure in water (Yennawar et al., 1994). This increased rigidity of γ-chymotrypsin is accompanied by a reorientation of side chains, whereas it has been shown that the three-dimensional structures of cross-linked subtilisin crystals in anhydrous acetonitrile (Fitzpatrick et al., 1993) and of lyophilized α-lytic protease in acetone and octane (Burke et al., 1989) remain unchanged. It is possible that immobilized CALB behaves like γ-chymotrypsin, and its structure changes in a nonaqueous environment, or that the removal of water modifies the nature of the interactions in the active site.

To obtain some information about these interactions, the influence of substitution around the chiral center was evaluated. Two α-phenyl carboxylic acids were esterified: 2-phenylpropionic and 2-phenylbutyric acids. As seen in Table II, the presence of the additional carbon atom in 2-phenylbutyric acid impedes the esterification of both enantiomers, compared to 2-phenylpropionic acid. This is true

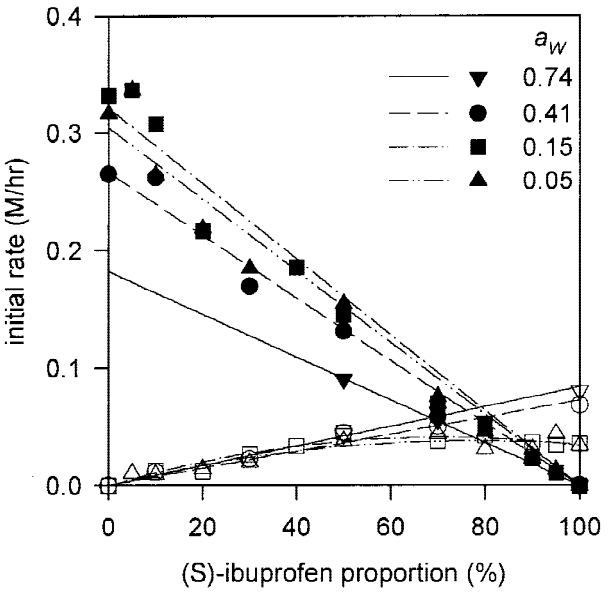

Figure 2. Initial rate of esterification of (*R*)-ibuprofen (filled symbols) and (*S*)-ibuprofen (open symbols) as a function of the enantiomeric composition of the substrate for different water activities. All the points are the mean of two to four experiments. The lines were obtained by linear regression on the complete data set, using a first-order equation, except for (*S*)-ibuprofen at $a_W = 0.15$ and $a_W = 0.05$, where a second-order equation was used.

at both water activities tested. The addition of the isobutyl group on the phenyl ring leads to comparable esterification rates between ibuprofen and 2-phenylpropionic acid at $a_W = 0.10$. At the lower water activity, the esterification rate of (*R*)-ibuprofen decreases slightly, as it was the case between $a_W = 0.15$ and $a_W = 0.05$ (Fig. 2). For (*S*)-ibuprofen, the rate falls more noticeably than for the other acids. This leads to a marked increase in the measured enantioselectivity. Because less enzyme was used in these reactions, the molecular sieve maintained the water activity at a lower level, because of the slower production of water. This lower a_W may explain why the values of the enantioselectivity are higher than those reported in Figures 1 and 2.

Lipases are known to have a mode of action similar to that of serine proteases, described by a bi–bi ping–pong mechanism. This involves the formation of two tetrahedral

Table II. Initial rates and enantioselectivites at two different water activities for the esterification of 2-phenylcarboxylic acids catalyzed by *Candida antarctica* lipase type B.

Acid	a_W	ν_{iR} (mmol/h · g)	ν_{iS} (mmol/h · g)	ν_{iR}/ν_{iS}
2-Phenylbutyric acid	0.10	0.34	0.10	3.4
	0.03	0.16	0.07	2.3
2-Phenylpropionic acid	0.10	1.4	0.42	3.4
	0.03	0.65	0.24	2.7
Ibuprofen	0.10	1.5	0.46	3.3
	0.03	1.1	0.12	9.2

intermediates (Kazlauskas, 1994; Zuegg et al., 1997). The first intermediate, Td1, contains the ester and leads to the formation of an acyl-enzyme in hydrolysis; the second intermediate, Td2, contains the acid and leads to the free acid in hydrolysis. The enantioselectivity towards optically active carboxylic acids can be determined by the two tetrahedral intermediates, because they both contain the acyl moiety. The enantioselectivity towards alcohols is determined by the first tetrahedral intermediate, Td1, because the alcohol leaves this intermediate in hydrolysis reactions. Because it has been shown that serine proteases and lipases under synthetic conditions follow the same bi–bi ping–pong kinetics (Chatterjee and Russell, 1993; Chulalaksananukul et al., 1990; Martinelle and Hult, 1995), it is reasonable to assume that these two tetrahedral intermediates exist in esterification reactions also, and govern the enantioselectivity of the reactions.

The observed enantioselectivities presented herein are rather low and correspond to differences in free energy changes of 0.54 to 0.80 kcal/mol for $v_{iR} / v_{iS} = 2.3$ to 3.4, respectively, according to Equation (1) (Faber et al., 1994; Fersht, 1985).

$$E = \frac{(k_{cat}/K_M)_R}{(k_{cat}/K_M)_S} = \exp\left(-\frac{\Delta\Delta G^*}{RT}\right) \quad (1)$$

These values are too small to be explained by molecular modeling, especially considering the fact that this system is atypical. The absence of a diluting solvent and the lack of information about enzyme three-dimensional structure at these low water activities would lead to large uncertainties in the calculated free-energy changes. Moreover, it has been shown in Figure 2 that at low a_W, when the observed enantioselectivity is higher, the reaction rate for the (*S*)-isomer is no longer proportional to the enantiomer concentration. This departure from first-order kinetics limits the use of Equation (1) in relating the differences in free-energy changes with enantioselectivity.

CONCLUSION

The results presented here have shown that there is still a lot to be understood about the functioning of enzymes in nonaqueous media. Small changes in variables such as water activity can have a significant impact on their activity and enantioselectivity. Molecular modeling could be of great help in the study of these phenomenon, however, it has been developed to simulate the behavior of enzymes in an aqueous environment.

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