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Water Activity Dependence of Lipases in Non-aqueous Biocatalysis

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Abstract Eleven lipases are tested and it was found that lipases can be divided into three types according to water activity dependence. The first type is lipase that has low water activity dependence and works in a low water activity, its performance changes little with the change of water activity. The optimum water activity is 0.19 and Newlase F (*Rhizopus niveus*), lipase FAP-15 (*Rhizopus oryzae*) belong to this type. The second type is lipase that has medium water activity dependence and its performance changes with the change of water activity. Most lipases belong to this type and the optimum water activity in this type is about 0.60. The third type is lipase that has a high water activity dependence and works only in a high water activity (a_w>0.75). WGL (wheat germ) belongs to this type and the optimum water activity is 0.90. The relationship between enantioselectivity and water activity is also discussed and the enantioselectivity seems to be independent of water activity. And we also compared the two control methods of water activity, it was found that the method which add solid salt hydrates to the reaction mixture (method II) is more stable and effective throughout the reaction than the method that pre-equilibrate via the vapor phase (method I). The addition concentration of salt hydrates is also investigated and the optimum concentration is 1 g/l.

Keywords Water activity dependence · Non-aqueous biocatalysis · Lipase · Kinetic resolution

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Introduction

Lipase catalyzed reaction in organic media have been widely reported and used in industry [1]. Such catalysis is affected by many factors, and it is generally appreciated that obtaining the correct water level is very important. In general, the hydration level of the enzyme greatly influences the flexibility of the protein and thereby catalytic activity [2].

First, water content was considered as the parameter to measure the water level, it was found not the best one soon. Then the thermodynamic activity of water (a_w) was considered as the convenient way of characterizing water level of these systems, because it determines the distribution of water between the various phases that can compete in binding water [3]. It also found that the optimum a_w is often unchanged as other aspects of the system are altered [4].

The relationship between mole fraction of water and enzyme activity in the case of several organic solvents has been reported [5]. A simple method of fixing water activity in organic systems, first introduced by Goderis et al., is to pre-equilibrate via the vapor phase with saturated solutions of particular salt before use [3]. This method, however, can only control the initial water activity. Although some works want to improve it, it still seems unstable throughout the reaction [6]. Another method, which is becoming increasingly popular, is the use of a pair of salt hydrates to maintain a constant water activity level throughout the reaction [7–9]. Here the lower hydrate or anhydrous form absorbs water and the higher hydrate releases it during the reaction.

Although many researchers have done a lot of work on it, there are two areas which needs more work on the effect of water activity. One is the differences of water activity dependence of various lipases. Lipases comes from different sources, most are from microorganism, some from animal and some from plant. Different lipase has different structure and different property, so is water activity dependence [10]. Some articles have discussed it, but no detailed works were published [11]. The second is the relation between enantioselectivity and water activity of the reaction. Although there are already a few reports on it, the relation between the E value and water activity remains unclear [9]. In the literature there are reports show that increasing water activity will increase the enantioselectivity [12] but in some cases the enantioselectivity decreases [13] or is unaffected by additions of water [14]. However, one should bear in mind that these investigations were performed with different types of lipases and substrates and systems, it gives very little information.

In this research, we have discussed the difference of water activity dependence of various lipases and the relation between enantioselectivity and water activity. We also have compared the stability of two control methods throughout the reaction.

Material and Methods

Materials

(R,S)-1-Phenylethanol was from Acros, USA. Vinyl acetate was purchased from Alfa Aesar, USA. Lipase WGL (wheat germ) was from Sigma. PS IM (an immobilized lipase from *Pseudomonas cepacia*), Lipase AK 20 (*Pseudomonas fluorescence*), Lipase AY 30G (*Candida rugosa*), Newlase F (*Rhizopus niveus*), Lipase AYS (*C. rugosa*), Lipase R (*Penicillium roqueforti*), Lipase G 50 (*Penicillium camemberti*), Lipase A 6 (*Aspergillus niger*), and lipase FAP-15 (*Rhizopus oryzae*) were from Amano Enzyme Inc., Japan. Lipase

PPL (porcine pancreas) was from Shanghai Kaiyang Co., Ltd. China. All other chemicals were commercially available and of high purity.

General Procedure for Enzymatic Kinetic Resolution [15]

The transesterification reaction was carried out in a 25-ml conical flask capped with a stopper and stirred at 200 rpm and at 35°C. The reaction mixture was made of 10 ml organic solvent, *(R,S)*-1-phenylethanol (1 mM), vinyl acetate (1.5 mM), and lipase (50 U/ml). Samples were withdrawn at regular intervals from the reaction mixture and analyzed by GC.

Control of the Water Activity

The solvents were dried by gentle shaking with 4 Å molecular sieves overnight. Two control method were followed: method I: the water activities (a_w) of the anhydrous solvents, the substrates, and the enzymes were controlled by gaseous equilibrium with different saturated salt solutions in separate closed containers for 72 h at room temperature. The following salts were used: LiCl (a_w = 0.11), CH_3COOK (a_w = 0.23), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75), K_2SO_4 (a_w = 0.97) [16]. Method II: the water activity (a_w) of the reaction mixture was controlled by adding 1 g/l of particular salt hydrates [7] to the reaction mixture in a separate closed container for 24 h with stirring at 200 rpm and at 35°C.

Analysis

A HP 7890 GC was used to analyze chiral sec-alcohol and its acetic ester in the reaction mixture. The products were separated on a Chiraldex™ G-TA column (30 m× 0.25 mm). The temperature program used was to keep the column oven at 80°C for 5 min, then increase the temperature to 110°C at 10°C/min and finally maintain the oven at this temperature for 18 min, giving a total run time of 26 min. The split ratio was 1:50 and the injector and flame ionization detector temperature were set at 250°C and 300°C, respectively.

Results and Discussion

Effect of Addition Concentration of Salt Hydrates

Although Halling and his co-worker had done some work on the method II, there is still has suspicion that the salt hydrates themselves affect the reaction. So the concentration of added salt hydrates should be kept minimal. According to the work by Ching-Shih Chen [17], the common addition concentration is between 5–50 g/l. So the concentration gradients were set as 0.1, 1, 10, 100, and 500 g/l.

Figure 1 shows that there was little difference in the conversion ratio with the change of addition concentration; the better result is achieved at 1 and 10 g/l. When the concentration is up to 100 g/l, the conversion ratio decreased. The possible reason is that the density of system and mass transfer are changed. So the optimum concentration of 1 g/l was used in the following investigation.

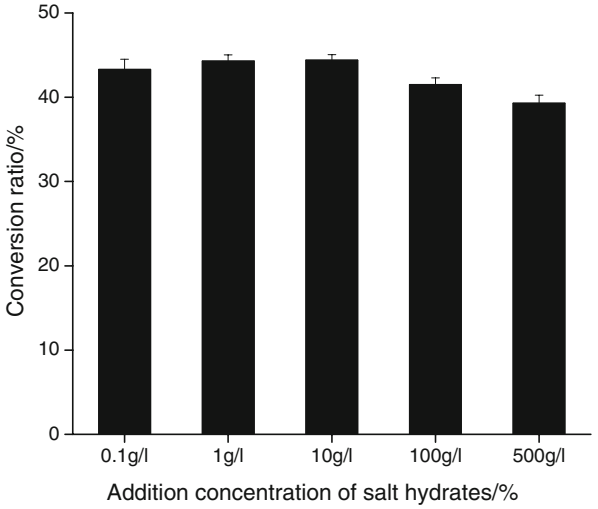

Fig. 1 Effect of addition concentration of salt hydrates (Na_2HPO_4 7/2). Reaction conditions: a mixture of (*R,S*)-1-phenylethanol, vinyl acetate, and lipase AK 20 was shaken in *n*-hexane at 35°C for 48 h

Effect of Solvent

The hydrophobicity of solvent plays important role to the water level in biocatalysis. Commonly, the high hydrophobic solvents are considered the most suitable solvent for biocatalysis [18]. In addition to solvent hydrophobicity, the molecular structure of the solvent and the solubility of substrate in solvent are also important [4, 19]. To date, there is no ideal principle to guide the selection of organic solvent for any particular process.

Different kinds of organic solvents were screened for the kinetic resolution of *rac*-1-phenylethanol (Table 1). The highest conversion ratio (44.0%) was achieved in *n*-hexane using lipase AK 20. Although the nature of solvent affects the catalytic activity greatly, it seems to have no influence with the enantioselectivity of lipase ($E > 200$). The enantioselectivity of the lipases seems to be independent of organic solvent with log *P* values from 0.49 to 4.5. The possible reason is that solvents interact less with the protein structure, so they have little effect on the enantioselectivity of lipase. On the other hand, solvents have different ability to solvate the substrates and this influences the

Table 1 Effect of solvents.

Organic media	logP	Conversion ratio (%)	ee_p (%)	*E*
n-Hexane	3.5	44.0	99	>200
Toluene	2.5	40.3	99	>200
THF	0.49	25.2	99	>200
Dichloromethane	1.75	20.5	99	>200
Carbon tetrachloride	2.86	34.2	99	>200
Isooctane	4.5	38.6	99	>200
i-PrO_2	1.9	42.4	99	>200

Reaction conditions: a mixture of (*R*,*S*)-1-phenylethanol, vinyl acetate, lipase AK 20, and 1 g/l salt hydrates was shaken with a water activity of 0.56 at 35°C for 48 h

thermodynamic activity of the substrates and thereby the measured lipase activity. (*R,S*)-1-phenylethanol has a high solubility in *n*-hexane and *n*-hexane is a hydrophobic solvent, it has fewer tendencies to strip essential water from lipase. So *n*-hexane was selected to be the reaction medium in the following reactions.

Water Activity Dependence of Various Lipases

The influence of water activity on the reaction rates of lipases has been studied [4, 20]. The results show that lipases respond differently to increasing water activity. Some show activity optimum at low water activity, some have intermediate profiles with broader optima. Most other types of enzymes require relatively high water activity to express good catalytic activity, for example esterase, β-glucosidase, and tyrosinase [21]. But no detailed work has reported on the difference between water activity dependence of lipases.

We have selected 11 lipases and only PS IM is an immobilized one, some from microorganism, one from animal, and one from plant. As Figs. 2, 3, and 4 show, they can be divided into three types. The first type is lipase that has low water activity dependence and can work in low water activity, its performance changes little with the change of water activity, such as lipase Newlase F (*R. niveus*), lipase FAP-15 (*R. oryzae*). Their optimum water activity is 0.19 and the minimum relative initial rate is about 47.2%. It has been reported that RAL (*Rhizopus arrhizus*) has low water activity dependence too [22], so the lipase from *Rhizopus* sp. may all has low water activity dependence.

The second type is lipase that has medium water activity dependence and their activity change with the change of water activity, most lipases belongs to this type. As Fig. 3 shows, the favorite water activity of lipases in this type is nearly the same and which is about 0.60. Their performance changes with the change of water activity. Such as lipase AK 20 (*P. fluorescence*), lipase AY 30G (*C. rugosa*), lipase AYS (*C. rugosa*), lipase R (*P. roqueforti*), lipase G 50 (*P. camemberti*), lipase A 6 (*A. niger*), and lipase PPL (porcine pancreas).

As Fig. 4 shows, the third type is lipase that has a high water activity dependence and works only in a high water activity (a_W>0.75), such as lipase WGL (wheat germ). Its

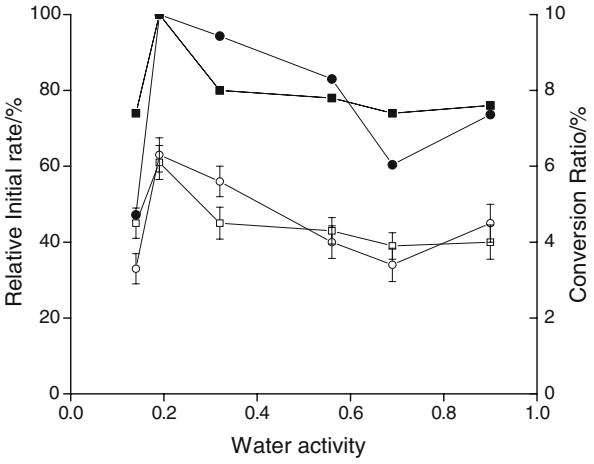

Fig. 2 Water activity dependence of various lipases; type I. Reaction conditions: a mixture of (*R,S*)-1-phenylethanol, vinyl acetate, and 1 g/l salt hydrates was shaken in *n*-hexane at 35 °C for 48h. *Filled square* is relative initial rate of lipase FAP 15, *filled circle* is relative initial rate of lipase Newlase F, *unfilled square* is conversion ratio of lipase FAP 15, *unfilled circle* is conversion ratio of lipase Newlase F

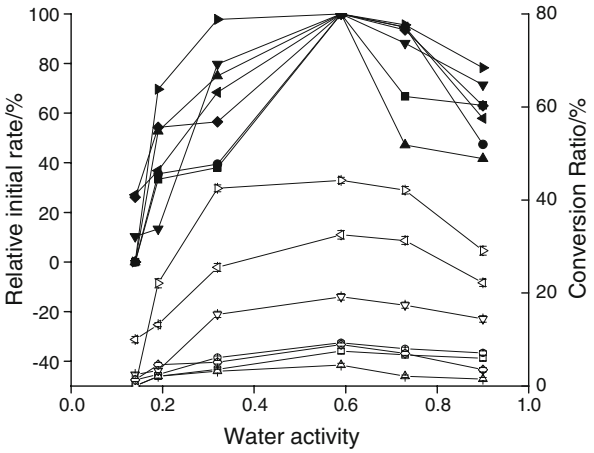

Fig. 3 Water activity dependence of various lipases; type II. Reaction conditions: a mixture of (*R,S*)-1-phenylethanol, vinyl acetate, and 1 g/l salt hydrates was shaken in *n*-hexane at 35°C for 48 h. *Filled square* is relative initial rate of lipase 30 G, *filled circle* is relative initial rate of lipase R, *filled triangle* is relative initial rate of lipase 50, *unfilled triangle* is relative initial rate of lipase AYS, *filled left-facing triangle* is relative initial rate of lipase 6, *filled right-facing triangle* is relative initial rate of lipase 20, *filled diamond* is relative initial rate of lipase PPL; *unfilled square* is conversion ratio of lipase 30 G, *unfilled circle* is conversion ratio of lipase R, *unfilled triangle* is conversion ratio of lipase 50, *unfilled inverted triangle* is conversion ratio of lipase AYS, *unfilled left-facing triangle* is conversion ratio of lipase 6, *unfilled right-facing triangle* is conversion ratio of lipase 20, *unfilled diamond* is conversion ratio of lipase PPL

performance changes greatly with the change of water activity. WGL only shows high activity with a water activity of 0.90. PS IM (*P. cepacia*) is an immobilized enzyme, its performance changes little with the water activity, it maybe because the carrier has enough binding water. Edlund et al. [23] also reported that the performance of immobilized enzyme was much less sensitive to water activity than crude enzyme.

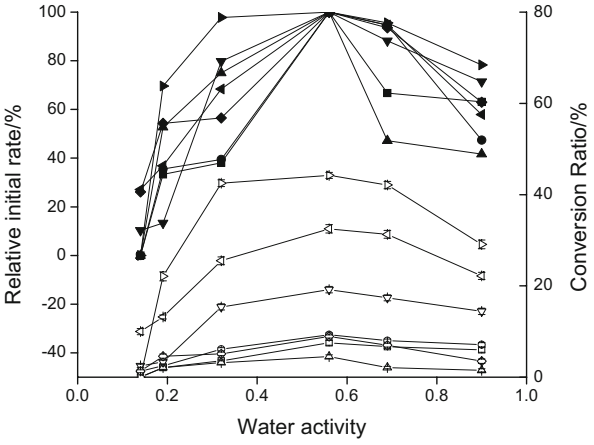

Fig. 4 Water activity dependence of various lipases; type III. Reaction conditions: a mixture of (*R,S*)-1-phenylethanol, vinyl acetate, 1g/l salt hydrates was shaken in *n*-hexane at 35°C for 48h. *Filled square* is relative initial rate of lipase WGL, *filled circle* is relative initial rate of lipase PS IM, *unfilled square* is conversion ratio of lipase WGL, *unfilled circle* is conversion ratio of lipase PS IM

The reason of the difference between lipases is the structure, the hydration state of enzyme, and water molecule in the binding pocket [24]; so the water activity dependence is various. Also with WGL, because it has similar structure with esterase, so it is the possible reason of high water activity dependence.

The Relation Between Water Activity and Enantioselectivity

There are also many articles on the relation between water activity and enantioselectivity, the result is rather contradictory—increase, decrease, or no variation with the change of water activity. Also some articles claim that it has been demonstrated specifically that variations of the water activity or the water content of the solvents had no effect on enzyme enantioselectivities [24].

As Table 2 shows, the enantioselectivity of various lipases did not change when they were at their optimum water activity, all enzymes have a high E value (E>200). So the water activity has no effect on the enantioselectivity in this reaction. It maybe because the enantioselectivity is determined by the protein structure of transition sate [25], the hydration level of lipase has little effect on it.

Difference Between Two Control Methods

The control method commonly are two, one is by the method of pre-equilibrate via the vapor phase with saturated salt solutions of known water activity (method I). However, some report said that a_w will change from the pre-set value with the reaction proceed [4]. The second is by the method of add solid salt hydrates to the reaction mixture, Halling and his co-worker has done a lot of work on it and proved it can control the water activity perfectly throughout the reaction (method II) [3, 5, 7].

The reaction we chose is a transesterification reaction, so there is not water consumed or produced in the reaction. As Fig. 5 shows, method II is better than method I, both in the initial rate and conversion ratio. The maximal initial rate of method I is 3.4 μM/h and the

Table 2 Maximal observed initial rates of each lipase.

Lipase	Maximal initial rate/mM/h	α_w	Conversion ratio (%)	E
30 G	0.84	0.56	7.5	>200
R	0.76	0.56	9.3	>200
50	0.36	0.56	4.5	>200
AYS	2.0	0.56	19.2	>200
A 6	3.8	0.56	32.5	>200
WGL	1.4	0.90	15.9	>200
20	4.6	0.56	44.2	>200
Newlase F	0.53	0.19	6.3	>200
PS IM	25.0	0.69	50.0	>200
PPL	0.46	0.69	8.9	>200
FAP-15	0.50	0.19	6.1	>200

Reaction conditions: a mixture of alcohol, vinyl acetate, and 1 g/l salt hydrates was shaken with a water activity of 0.59 at 35°C for 48 h

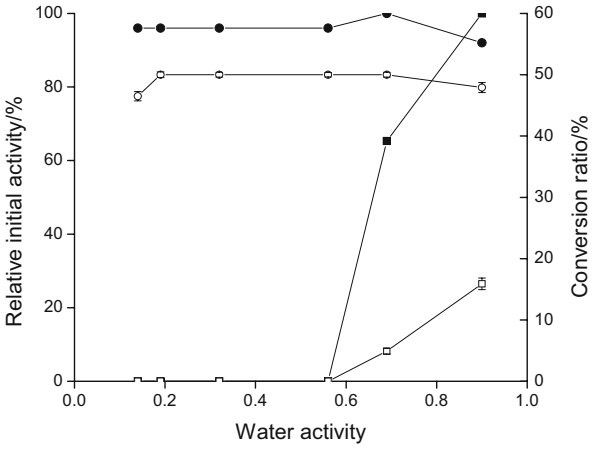

Fig. 5 Difference of two control methods. Reaction conditions: a mixture of alcohol, vinyl acetate, lipase A 6, and 1 g/l salt hydrates was shaken with a water activity of 0.56 at 35°C for 48 h. *Filled square* is relative initial rate of method I, *filled circle* is relative initial rate of method II, *unfilled square* is conversion ratio of method I, *unfilled circle* is conversion ratio of method II

maximal initial rate of method II is 3.8 μM/h. The maximal conversion of method I is 16.2% and the maximal conversion ratio of method II is 32.5. The optimum water activity of method I is 0.53 and 0.56 for method II. The initial rate and conversion ratio of method I change sharply than method II with the change of water activity. So it can be concluded that method II is more stable in the control of water activity throughout the reaction.

Conclusion

In conclusion, various lipases have been screened; the effects of organic solvent, addition concentration of salt hydrates, difference between two control methods are investigated. From the results above, the final conclusion was drawn:

1. Lipase can be divided into three types according to the water activity dependence. The first is lipase have low water activity dependence, the second is lipase have medium water activity dependence and the third is lipase has high water activity dependence. Most of lipases have medium water activity dependence and the optimum water activity is about 0.60.
2. The enantioselectivity of lipases in the reaction seems to be independent of organic solvent and water activity.
3. It was found the method that add solid salt hydrates to the reaction mixture (method II) is more stable in the control of water activity than the method that pre-equilibrate via the vapor phase (method I). The addition concentration of salt hydrates has little influence to the catalysis; the optimum concentration is 1 g/l.

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