Effect of solvent polarity on enzymatic function of poly [(*N*-acylimino)ethylene] modified lipase

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(Communicated by Seizo OKAMURA, M. J. A., March 12, 1999)

Abstract: The effects of the polarity of various water-immiscible organic solvents on the enzymatic esterification activities of three modified *Candida cylindracea* lipases, which were prepared by the chemical modification of lipase with poly[(*N*-acetylimino)ethylene] (**2a**), poly[(*N*-propionylimino)ethylene] (**2b**) and poly[(*N*-isobutyrylimino)ethylene] (**2c**), were examined and their enzymatic activities were tried to correlate to the logarithm of the partition coefficient of the solvent between water and 1-octanol (log P). It was found that although the activity increased with increasing the hydrophobicity of the solvent and with increasing the hydrophobicity of the *N*-substituent of **2**, a great rate enhancement of the enzymatic activity was achieved in benzene and toluene by the chemical modification by **2b** or **2c**, while unmodified lipase and **2a**-modified one were almost inactive in them. Moreover, the effect of water concentration in organic solvent was investigated.

Key words: Modified enzyme; lipase; poly [(*N*-acetylimino)ethylene]; organic solvents.

Introduction. The use of enzyme in non-aqueous media is one of the active areas of recent research in enzyme technology. There are numerous advantages of using biocatalyst in organic solvents in comparison with water. However, the use of organic solvents in biocatalyst system is seriously limited since it has been commonly known that organic solvents generally denaturize and inactivate most of enzymes (recent works have revealed that several native enzymes, for example, lipase, subtilisin and α -chymotrypsin, are still active in certain organic solvents.^{1),2)} Laane and co-worker proposed general rules for the optimization of different biocatalytic systems in various types of non-aqueous media.³⁾ Using the logarithm of the partition coefficient, log P, as a quantitative measure of solvent polarity, it was shown that the activity is low in relatively polar solvents, having a log P < 2; is quite variable in solvents having a log P between 2 and 4; and is high in apolar solvents having a log P > 4.

Further, it is said that water is required for enzymatic function, and removal of water should drastically distort that conformation and inactivate the enzyme. In water-immiscible organic solvents, a trace of water is absolutely necessary for enzymes to be active.^{2),3)} It is important for the enzymatic reaction to control water concentration in organic solvents.

In preceding papers, we demonstrated that poly[(*N*-acylimino)ethylene] (PAI) is an excellent polymeric modifier for enzymes such as bovine liver catalase and *Candida cylindracea* lipase in comparison with poly(ethylene glycol) (PEG), a commonly used polymeric modifier.⁴⁾⁻⁷⁾ The most important property of the amphiphilic polymeric modifier, the hydrophilicity/lipophilicity balance, is easily controlled in the case of PAI by selecting its acyl substituent, and the PAI modified enzymes are found to be more active in organic solvents, benzene and chloroform, than unmodified lipase and PEG modified one having a similar extent of modification.

In the present paper, we examined the effect of the properties of various water-immiscible organic solvents on the enzymatic activities of PAI modified *Candida cylindracea* lipases, **4a**, **4b**, and **4c**, which were prepared by the chemical modification of lipase with poly[(*N*-acetylimino)ethylene] (**2a**), poly[(*N*-propionylimino)ethylene] (**2b**) and poly[(*N*-isobutyrylimino)

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ethylene] (**2c**), respectively, and their enzymatic activities for esterification were tried to correlate to the solvent polarity, log P. Furthermore, we investigated the effect of water concentration in organic solvent.

Results and discussion. *Poly[(N-acylimino)ethylene] modified lipase.* Three carboxylic acid terminated PAIs having different amphiphilic properties, i.e., poly[(N-acetylimino)ethylene] (**2a**), poly[(N-propionylimino)ethylene] (**2b**), and poly[(N-isobutyrylimino)ethylene] (**2c**) were chosen as the polymeric modifiers for *Candida cylindracea* lipase, and the molecular weights of the polymeric modifiers in them were 6,370, 5,160, and 5,170, respectively (Table I).

Three samples of modified lipase having a similar length of the polymeric modifier were prepared by the reactions with similar modifier / lipase feed ratios, ca. 10. They were **4a**, **4b**, and **4c**. The chemical modification of lipase with **2** was performed according to an analogous procedure described in the preceding paper.⁷⁾ The modified enzyme samples **4** were obtained after dialyza-tion against distilled water using ultrafiltration and the subsequent lyophilization. The successful modification of lipase was confirmed from the GPC measurement using a TSK-Gel G3000SW_{XL} column in a 0.05 M pH 7.0 phosphate buffer. In every case a unimodal peak was observed, and no contamination of the unmodified lipase and the unreacted modifier in the product was confirmed. The average number of polymeric modifier per lipase molecule was calculated from the polymer

Table I. Carboxylic acid terminated poly[(N-acylimino)ethylene] (**2**)^a

Monomer	[1]	Product			
1	[MeOTs]	2	M_n^b	M_w/M_n^b	F_n^c
1a	78	2a	6,370	1.07	0.90
1b	50	2b	5,160	1.05	0.70
1c	47	2c	5,170 ^d	1.05	0.82

^a Polymerization was carried out by using methyl tosylate (MeOTs) as initiator in CH₃CN at 70°C for 48 h. ^b Determined by GPC measurement. Each calibration curve was obtained by using standard samples of **2a** and **2b**, respectively. ^c Average of the extent of functionalization per molecule, determined by alkali titration. ^d Determined by ¹H-NMR measurement.

content together with the molecular weight of the modifier. These values are summarized in Table II.

Effect of water in non-aqueous media. Previously, it has been reported that water plays an important role for the revelation of the enzymatic activity of hybrid in organic media.^{2),3)} To confirm the effect of water on the enzymatic activity of the hybrid in the present case, the enzymatic activities of **4b** in benzene with various concentration were measured.

Fig. 1 shows time-conversion curves of the substrates catalyzed by **4b** in absolute benzene (a) and in water saturated one (b) (ca. 30 mM H₂O determined by Karl-Fisher method).

The esterification reaction effectively proceeded in water saturated benzene, while no reaction occurred in

Table II. Characterization of modified lipase (**4**)

Modifier	Feed ratio	Modified lipase 4	GPC elution volume ^a (ml)	Polymer content (wt%)	NM ^c
—	—	unmodified	10.68	0	0
2a	10	4a	8.13	26	2.7
2b	14	4b	8.67	27	3.2
2c	10	4c	8.70	18	1.7

^a Position of the peak top of GPC curve. ^b Determined from IR measurement. ^c Average number of the polymer molecule per lipase. Calculated based on the value of polymer content in **4** determined from IR.

Fig. 1. Time conversion curves of the esterification catalyzed by **4b** in water saturated (○) and absolute (●) benzene. (A) Molecular sieves 3 Å was added to the reaction mixture after 8 h. (B) Small amount of water corresponding to water saturated concentration was added to the reaction mixture after 8 h.

absolute benzene. After eight hours, an enough amount of molecular sieves 3 Å to dehydrate the solvent was added to the moistured mixture. At this point the progress of esterification was almost completely stopped. On the contrary, the addition of water to the absolute benzene system up to saturation completely restored the esterification activity of the hybrid. It was suggested that the inactivation of **4b** by dehydration in benzene was reversible.

Fig. 2 shows a plot of enzymatic activity of **4b** (the activities were calculated from the concentration of caprylic acid after 20 h) against the concentration of water in benzene. The enzymatic activity of **4b** was not so much influenced by the concentration of water if it was below 10 mM. Then, activity rapidly increased with increasing the water concentration to 30 mM, which corresponded to saturation. As water was added further, the activity decreased slowly. This inactivation at a higher

Fig. 2. Plots of enzymatic esterification activity versus the concentration of water in benzene. The vertical dashed line represented the water saturated concentration at $30 \pm 0.01^\circ\text{C}$ in benzene (ca. 30 mM). Activities were estimated by the conversion of the substrate after 20 h.

water concentration region was not caused by the reversed reaction, an ester hydrolysis reaction, since it was confirmed that the rate of hydrolysis of the product, *n*-amyl caprylate, was negligible under these conditions. The main reason for the inactivation at higher water concentration would be ascribed to the decrease of the local concentrations of the substrates around the hybrid since an aqueous layer forms around the enzyme and the substrate tends to distribute in the benzene phase.

The role of water for our modified lipases in organic media has been explained as follows: Anhydrous organic solvents strip off the water layer around the hybrid, which is required for the revelation of enzyme functions in the organic solvent and hence deactivate the hybrid. On the other hand, water saturated organic solvents lose such the water-stripping ability and do not effect on the enzyme function.

Table III. Enzymatic ester synthetic activity of modified lipase (**4**) in various water-immiscible organic solvents^a

Solvent ^c	log P ^d	Activity ($\mu\text{mol}/\text{min}/\text{mg}$ of lipase) ^b			
		Lipase	4a	4b	4c
Cyclohexanone	0.96	5.3	20.5	16.6	18.7
2-Heptanone	1.8	1.6	2.6	9.8	7.8
Propyl ether	1.9	3.5	11.2	15.1	18.7
Chloroform	2.0	5.7	11.8	11.5	8.0
Benzene	2.0	1.6	7.7	37.7	39.8
Toluene	2.5	0.5	12.8	58.9	80.6
Carbon tetrachloride	3.0	114	86.9	113	156
Cyclohexane	3.2	157	182	246	418
Hexane	3.5	103	239	376	345
Isooctane	4.5	140	149	384	479

^a Ester synthesis activity was measured by using *n*-amyl alcohol and *n*-caprylic acid as substrates at $40 \pm 0.1^\circ\text{C}$. ^b Solvents were all saturated with water at $30 \pm 0.1^\circ\text{C}$. ^c Activity was estimated per lipase. ^d The logarithm of the partition coefficient between 1-octanol and water.³⁾

Effect of solvent polarity on the enzymatic function of the hybrid. For esterification in various organic solvents, three samples, **4a**, **4b**, and **4c**, were investigated. *n*-Amyl alcohol and caprylic acid were chosen as substrate because of the easy determination of conversion by the GLC analysis. Water plays an important role for the revelation of the enzymatic activity of hybrid in organic media previously described. With considering the above findings water saturated organic solvents at 30°C were used for the enzymatic assay described below.

The effects of the polarity of solvent on the esterification abilities of three **4** samples were examined. The logarithm of the partition coefficient of the solvent between water and 1-octanol (log P) was chosen as an intimation for the polarity of the solvent according to former literature.³⁾ The esterification activities of the samples as well as those of the unmodified lipase in water-immiscible organic solvents are summarized in Table III. The data clearly show the polarity of the solvent has a great effect on the enzymatic activity of the samples. The comparison of these data reveals that all modified lipases are generally more effective biocatalysts than the unmodified lipase in organic media. Concerning the activity of the lipase in organic solvent, a previous work by Laane *et al.* has shown a similar relationship between log P and the enzymatic activity for the transesterification of tributyrin with *n*-heptanol in the cases of *Candida cylindracea* and *Mucor sp.* lipases.³⁾

In the solvents whose log P values were less than 2.0, i.e., cyclohexanone, 2-heptanone, *n*-propyl ether, and chloroform, the activities of all samples were quite

low. Although all of the modified lipases were a little active than the native lipase in any of these solvents, no clear effect of *N*-acyl substituent of **4** on the catalytic activity of the hybrid was found.

In aromatic hydrocarbons, benzene and toluene, whose log P values were 2.0 and 2.5, respectively, a great rate enhancement of the enzymatic activity was achieved by the chemical modification by **2b** or **2c**, while the unmodified enzyme and **4a** in the aromatic hydrocarbons were almost inactive.

In non-polar solvents whose log P values were higher than 3.0, carbon tetrachloride and three aliphatic hydrocarbons, all of the samples showed excellent activities. In these solvents the activities of **4b** and **4c** increased with increasing the hydrophobicity of the *N*-substituent of PAI. The rate enhancement of the activity by the chemical modification was not so high in comparison with that of the unmodified enzyme itself. The unmodified lipase showed significantly high activities in these non-polar solvents. It can be explained that these solvents do not remove the aqueous layer around the native enzyme.³⁾

Although the high activity of the samples in these non-polar solvents, a more polar solvent is preferable for the practical usage because of the poor solubility of substrates in these solvents. Hence, the high activity of the **4b** and **4c** samples in aromatic hydrocarbons are considered to be quite significant.

Experimental section. *Materials.* Crude lipase OF 360 from *Candida cylindracea* was obtained from Meito Sangyo Co. (Tokyo, Japan), which was purified according to a literature.⁸⁾ The preparation of carboxylic acid terminated PAIs, i.e., poly[(*N*-acetylrimino)ethylene] (**2a**), poly[(*N*-propionylimino)ethylene] (**2b**) and poly[(*N*-isobutyrylimino)ethylene] (**2c**),⁹⁾ and the chemical modification of the polymeric modifier to lipase⁷⁾ were carried out as described in the preceding papers. Solvents for enzymatic assay were of best grade, which were treated with an aqueous 0.05 M pH 7.0 phosphate buffer solution at $30 \pm 0.01^\circ\text{C}$ to saturate water unless otherwise indicated. Absolute benzene was prepared by drying over calcium hydride and the subsequent distillation under nitrogen. The substrates were dried over molecular sieves $3\text{\AA}$ before the enzymatic assay in absolute benzene.

Instrumentation. The number-average molecular weights of the polymeric modifiers (**2**) were determined by the GPC measurements with Shodex A803 column in chloroform. The GPC measurements of the polymer-enzyme hybrids were performed by using

TSK-GEL G3000SW_{XL} in 0.05 M pH 7.0 phosphate buffer at room temperature. The polymer contents of the hybrids were determined by the IR spectroscopy, which were performed on a Perkin-Elmer 1640 spectrometer. Namely, the polymer contents of **4a**, **4b**, and **4c** were evaluated from the ratios of the peaks of 1534 cm⁻¹ and 1420 cm⁻¹ (for **4a**), 1534 cm⁻¹ and 1200 cm⁻¹ (for **4b**), and 1534 cm⁻¹ and 1445 cm⁻¹ (for **4c**) by using calibration curves obtained from mixtures of lipase with **2a**, **2b**, and **2c**, respectively.

Enzyme assay. The esterification activity of the samples in organic media was assayed by using *n*-caprylic acid and *n*-amyl alcohol as substrates. To 2 ml of an organic solution containing 450 mmol of each substrate was added 1 mg of enzyme. Then, the reaction mixture was incubated at 40 ± 0.01°C with shaking at 120 strokes/min. The conversion of the substrates was determined by GLC using silicon DC 550 column (1 m). The initial rate of the reaction was taken as the activity of the sample.

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