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Modified Enzymes for Reactions in Organic Solvents

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Abstract

Recent studies on biocatalysis in water–organic solvent biphasic systems have shown that many enzymes retain their catalytic activities in the presence of high concentrations of organic solvents. However, not all enzymes are organic solvent tolerant, and most have limited and selective tolerance to particular organic solvents. Protein modification or protein tailoring is an approach to alter the characteristics of enzymes, including solubility in organic solvents. Particular amino acids may play pivotal roles in the catalytic ability of the protein. Attaching soluble modifiers to the protein molecule may alter its conformation and the overall polarity of the molecule. Enzymes, in particular lipases, have been chemically modified by attachment of aldehydes, polyethylene glycols, and imidoesters. These modifications alter the hydrophobicity and conformation of the enzymes, resulting in changes in the microenvironment of the enzymes. By these modifications, newly acquired properties such as enhancement of activity and stability and changes in specificity and solubility in organic solvents are obtained. Modified lipases were found to be more active and stable in organic solvents. The optimum water activity (a_w) for reaction was also shifted by using modified enzymes. Changes in enantioselective behavior were also observed.

Index Entries: Modified enzymes; organic solvents; polyethylene glycol; lipase.

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Introduction

Most organic syntheses are carried out in organic solvents. As such, the use of enzymes is rather limited in this area. In most organic syntheses, the enzymes are poorly soluble in water and are often reported to be unstable in organic solvents *(1)*. Enzymes often form aggregates and denature in organic solvents. However, a few enzymes are found to be functional in organic solvents. A solvent is necessary for solubilizing the substrates, and in synthetic reactions, the use of high ratios of organic/water phase is found to be beneficial. Litjens et al. *(2)* proposed a quantitative equilibrium model that adequately described such interplay in the promotion of hydrolysis or ammoniolysis. High ratios of organic/water phase can be sustained by continuous removal of water to accelerate the synthetic reaction *(3)*. Quiros et al. *(4)* showed that by choosing the appropriate solid-state buffer or organic base, the enantioselectivity of *Candida antarctica* B (CALB) in the alcoholysis of (+/–)-2-phenyl-4-benzyloxazol-5(4H)-one could be selectively tuned. Similarly, Kwon et al. *(5)* identified isooctane as the best reaction medium for enantiomeric esterification of (*S*)-2-methylbutanoic acid methyl ester, an important compound for apple and strawberry flavoring.

A strategy to improve the functionality of enzymes in organic solvents is through chemical modification of the enzyme's molecules. Specifically, certain amino acid residues within the protein's molecules, which play pivotal roles in protein conformation and catalysis, are derivatized with certain modifiers that may affect the enzymic properties. Enzymes have been chemically modified by polyethylene glycol (PEG) *(6)*, reductive alkylation *(7)*, and amidation by imidoester *(8)*. Since enzymes are naturally hydrophilic molecules, it is thought that by chemical modification, the hydrophobicity of the molecules can be altered without causing a big loss in activity. Modification techniques can alter and improve proteins' native properties and endow them with new functions *(9)*.

A solvent is necessary for solubilizing substrates and for partitioning the substrates and products in different phases *(10)*. It can be used to increase enzyme stability *(11)*. Because many hydrolytic enzymes are used in industry, hydrolysis can be avoided by tilting equilibrium toward synthetic activity by using organic solvents *(12)*. Apparently solvents can change the enzyme specificity and enantioselectivity *(13)*. Solvents can cause changes in protein dynamics *(14)*, and, obviously, the choice of different solvents may affect protein conformation *(15)*.

The effect of solvents on enzyme activity, specificity, and stability has been studied, and many solvent parameters have been investigated to develop a strategy for choosing an appropriate solvent system for a particular enzyme and reaction *(16)*. By combining solvent engineering and thermodynamics, lipase-catalyzed synthesis can be selectively enhanced *(17)*. These strategies would be an indispensable tool for expanding the utilization of enzymes in organic synthesis.

Table 1
Organic Solvents and log *P* Values

Solvent	Log *P*
Acetonitrile	0.33
Diethylether	0.85
Dichloromethane	1.3
Chloroform	2.0
Toulene	2.5
Carbon tetrachloride	3.0
Hexane	3.5
n-Heptane	4.0
Octane	4.5
Isooctane	4.5
Hexadecane	8.8

Materials and Methods

Reagents and Solvents

Commercial lipase from *Candida rugosa* (Type VII) and aldehydes were obtained from Aldrich (Milwaukee, WI). Imidoesters, PEG-5000 and *p*-nitrophenyl chloroformate were purchased from Sigma (St. Louis, MO). (±)-Menthol, butyric anhydride, and ethyl caproate were from Fluka (Buchs, Switzerland). All the reagents and solvents used were of analytical grade. The solvents selected for study are given in Table 1.

Preparation of Native Lipase

Commercial lipase from *C. rugosa* (5.0 g) was dispersed in distilled water (100.0 mL). This mixture was stirred using a magnetic stirrer, centrifuged at 2353*g* for 10 min, and the supernatant was frozen and lyophilized.

Modification of Lipase

The reductive alkylation of lipase was carried out according to Means and Feeney *(18)*, and the chemical modification of lipase by PEG was performed according to the procedure developed by Basri et al. *(19)*. The fatty imidoester was prepared according to Basri et al. *(8)*. For all types of modified lipases used in these experiments, the degree of modification was between 40 and 50%.

Determination of Protein

Protein concentration was determined by the Bradford Coomassie blue assay procedure *(20)* using bovine serum albumin as the standard.

Table 2
Salts Used
to Obtain Specific a_w

Salt	a_w
LiCl	0.12
$MgCl_2$	0.32
$Mg(NO_3)_2$	0.55
KI	0.68
KCl	0.86
KNO_3	0.95

Determination of Degree of Modification

The content of free lysine (Lys) before and after modification was determined by reacting the lyophilized lipase and PEG-lipases with 2,4,6-trinitro benzene sulfonic acid *(21)*.

Esterification Assay

The reaction mixture comprised propanol (1 mmol) (2.0 mL) and oleic acid (0.35 mmol) in the specified organic solvent. Unless otherwise stated, the organic solvent used was hexane. The reaction was carried out in 20 × 125 mm screw-capped culture tubes in a 30°C shaking water bath (Hotech Shakerbath Model 903) for 1 h. Native lipase (10.0 mg) and an equivalent amount containing the same protein concentration of modified-lipases were used as biocatalysts and added immediately before incubation. A control without the presence of lipase was simultaneously incubated. The unreacted fatty acid was titrated using an autotitrator (Radiometer, ABU90) using 0.05 *N* NaOH to an end point of 9.5.

Enantioselective Assay

For enatioselective reaction, the conditions for reaction were similar except the substrates were composed of (±)-menthol (1 mmol) and butyric anhydride (1 mmol) in hexane. The reaction was carried out for 48 h. Enantioselective analysis of menthyl esters was carried out using chiral gas chromatography *(22)*.

Water Activity Studies

The reaction was monitored by direct esterification. The reaction mixture and the enzymes were equilibrated separately for at least 16 h in salt hydrate environments, before being mixed to initiate the reaction. The salts used (by preequilibrium) are listed in Table 2. Supersaturated solutions of these salts were placed in airtight bottles. Reaction mixtures and enzyme were preequilibrated separately in these bottles for at least 16 h at room temperature.

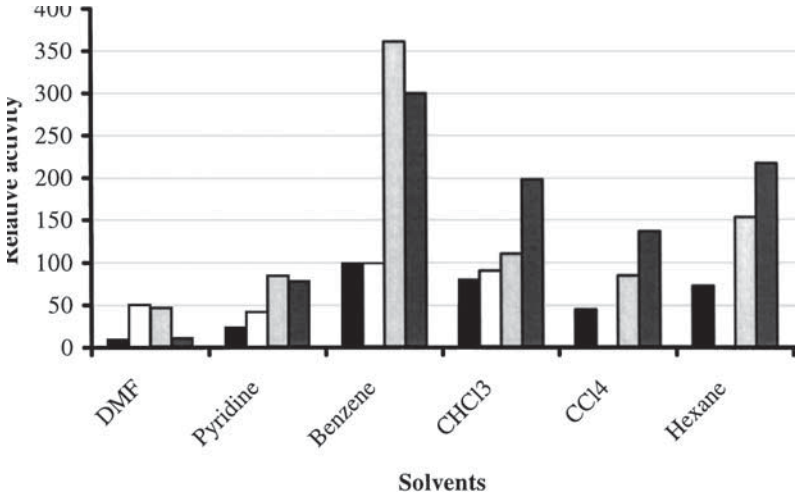

Fig. 1. Esterification of activity of native (■), dodecyl-lipase (□), phenylimido-esterified-lipase (▨), and PEG2000-lipase (▩) in organic solvents. The activity for dodecyl-lipase in hexane and CCl_4 was not determined. The log *P* of the solvents increases from left to right on the *x*-axis.

Results and Discussion

Esterification Activity of Modified Lipases in Organic Solvents

It is well known that the activity of enzymes in enzymatic reactions is affected by the nature and polarity of organic media in which the reactions take place *(16,23)*. Log *P*, the logarithm of partition coefficient of a given solvent between water and 1-octanol, is a useful parameter to predict enzyme activity. Generally, enzymes show higher activity in apolar solvents with high log *P*. This trend was also exhibited by the modified enzyme (Fig. 1). Modified lipases exhibited higher esterification activity compared with native lipase. Chemically modified lipases probably are able to maintain their conformation, since the solvents tend to limit the flexibility of the enzyme molecules. Another factor to be considered is that although the modified enzyme is not totally soluble, it is better dispersed as compared to native lipase, which is totally insoluble and tends to stick to vial walls. Ampon et al. *(24)* reported that the degree of modification and chain length of aldehydes affected the activity of lipase in aqueous solution or organic solvent. Apparently, modification may increase surface hydrophobicity and increase the activity *(25)*. Jasmani et al.'s *(22)* findings supported the fact that a higher degree of modification increases the esterification activity. Earlier, Basri et al. *(6)* showed that specific synthetic activity was increased up to sevenfold as the degree of modification was increased from 0 to 95%. Note that in most cases, hydrolytic activity of modified lipases was reduced *(26)*. Alkylated trypsin was also shown to be active in organic solvents *(24)*.

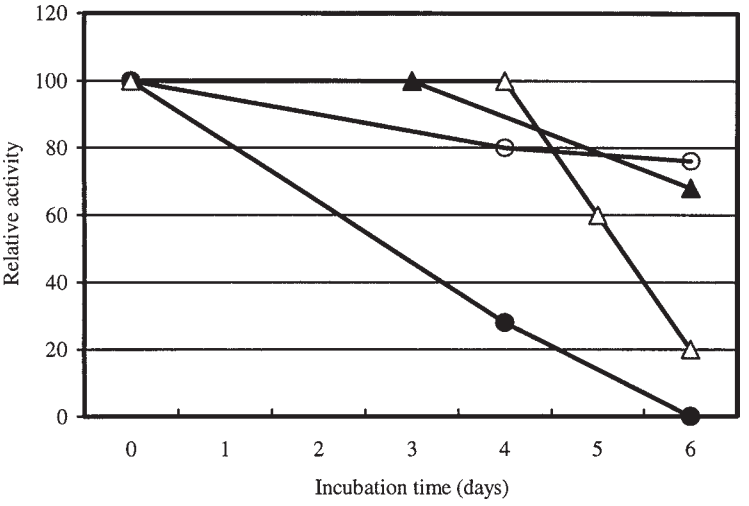

Fig. 2. Stability of modified lipases in benzene at 30°C. The enzymes were incubated at various time intervals in benzene before being assayed. The enzymes used were native (●), dodecyl-lipase (○), phenylimidoesterified-lipase (▲), and PEG2000-lipase (△).

Stability of Modified Lipases in Organic Solvents

Modified lipases are generally more stable than native lipases (Fig. 2). The modification seems to protect the enzyme from denaturation caused by the presence of the organic solvent. The modification may induce the surrounding water, which is necessary for activity, to be tightly bound, and thus not easily stripped by the solvents. Apparently, enzymes tend to be more stable in apolar solvents; again, the argument is that apolar solvents do not strip water from within the protein molecules, thereby keeping its integrity and allowing sufficient flexibility for substrate-enzyme interaction. According to Longo and Combes *(27)*, an increase in enzyme surface hydrophobic character induced a remarkable improvement in thermostability, whereas the increase in hydrophilic character produced the opposite effect. Ampon et al. *(7)* showed that alkylated lipase was slightly more stable at 60°C or higher compared with native lipase, whereas Hernaiz et al. *(28)* reported that PEG-semipurified lipases were more stable in isooctane at 50°C.

Effect of Modified Lipases on Optimum Water Activity for Esterification Reaction

Zaks and Klibanov *(29)* demonstrated that it was the water bound to the enzymes, rather than the water content of the system, that determined enzyme activity. Halling *(30)* proposed that the thermodynamic water activity (a_w) is the parameter that can be used to quantify the water level associated with the enzyme. The water activity governs the degree of hydration of enzymes and gives a direct indication of the mass action of

Table 3
Optimum Water Activity for Esterification Reaction
in Different Organic Solvents

		Optimum a_w				
Solvent	Log P^a	Native	Octyl	Dodecyl	PEG2000	PEG5000
Diethyl ether	0.85	0.68	0.55	0.95	0.86	0.95
Hexane	3.5	0.68	0.55	0.68	0.68	0.86
Isooctane	4.5	0.68	0.86	0.86	0.68	0.86
Hexadecane	8.8	0.68	0.12	0.86	0.55	0.95

[a]Log *P* is the logarithm of partition coefficient of a given solvent between water and 1-octanol *(18)*.

water *(31)*. In the present study, the effect of different solvents in combination with the effect of chemical modification to the enzyme molecule on the optimum a_w were investigated.

For diethyl ether, the presence of modifiers seems to affect the optimum a_w (Table 3). Octaldehyde may improve solubility of the enzyme molecule, and the water requirement is thought to be reduced. However, higher molecular modifiers may have the opposite effect, whereby the a_w increases. Large modifiers may render the lipase less soluble and thus increase its water requirement.

A similar trend was observed in hexane. However, being more nonpolar, the a_w was lower in dodecyl, PEG2000- and PEG5000-lipases. For isooctane, there seems to be a masking effect since there was little effect except for PEG2000-lipase. Basri et al. *(32)* reported that PEG2000-lipase was more soluble than alkylated lipases. Higher solubility may reduce the water layer requirement of enzyme and thus decrease the $a_{w.}$

On the other hand, the trend in hexadecane was different from the other solvents. The solubility effect as well as the high hydrophobicity of the modifiers may cause dehydration of the enzyme molecule, whereas the higher amphiphilic nature of PEG may result in greater binding of water.

However, it is very difficult to predict the nature of interaction between the solvents and the enzyme molecules. This problem is further enhanced by the characteristics of each modifier used.

Effect of Modified Lipases on Enantioselective Esterification

Studies on the enantioselectivity effect of organic solvents and modification of the enzyme molecules were conducted only on PEG-lipase. Enantioselectivity was markedly enhanced with PEG-lipases (Fig. 3). Enantioselectivity was not exhibited in the reactions catalyzed by native lipase in all solvents except in the two extremes, solvents with the lowest (CH_3CN) and highest (octane) log *P*. In contrast to the reactions catalyzed by native lipase, regardless of the solvents, PEG-lipases were very enantioselective although the percentage of enantiomeric excess decreased in hexane, *n*-heptane, and, isooctane.

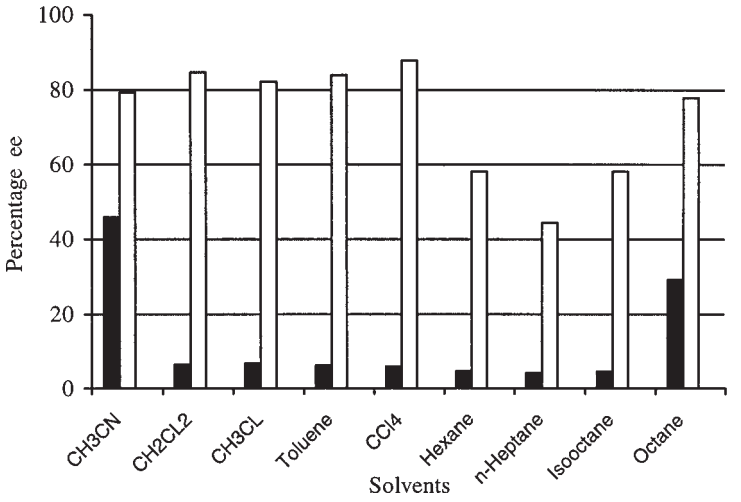

Fig. 3. Effect of solvents on percentage of enantiomeric excess (ee) on esterification of (±)-menthol with butyric anhydride, catalyzed by different enzyme preparations: native lipase (■) and PEG2000-lipase (□). The yield (%) of menthyl esters is defined as the percentage of millimoles of ester formed compared to the initial millimoles of menthol used in the reaction.

Reports seemed to indicate that the degree of modification influenced enantioselectivity. An increase in degree of modification enhanced the percentage of enantiomeric excess of the enzyme such as reported in our earlier work in the enantioselective esterification of (*R,S*)-2-(4-chlorophenoxy)propanoic acid *(33)*. The effect of the degree of modification on enantioselectivity was less pronounced as the effect on percentage of yield. The percentage of enantiomeric excess for PEG-lipases 41% and 50% was comparable although PEG-lipase 80% exhibited a higher value *(22)*. Basri et al. *(33)* showed that alkylated lipase produced higher enantiomeric excess toward the esterification of (*R,S*)-2-(4-chlorophenoxy)propanoic acid compared with the native lipase.

Conclusion

Chemical modification of enzymes, in particular lipases, can alter and improve the native properties of the enzymes and can endow them with novel functions. The facts that lipases react with water-insoluble substrates and that synthetic reactions are favored under low aqueous environments, studies on modified enzymes and solvent or medium engineering should be simultaneously addressed. Modification of enzyme and modification of the immediate environment, in this case through solvent selection, may provide a better microenvironment for the enzymes. The ultimate objective is to enhance the performance of the enzyme and therefore expand the potential use of biocatalysts in chemical processing.

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