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The Synthesis of Ester Compound by Lipase in Organic Solvents

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Abstract

The synthesis of lauryl palmitate from palmitic acid and lauryl alcohol was investigated in organic solvents using lipases. Water-immiscible organic solvent such as hexane, toluene, cyclohexane, and isooctane were found to be suitable for ester synthesis. The effect of water content on the initial rate of conversion was examined. As the water content increased, the reaction rate increased. But addition of water in organic solvent decreased thermostability of enzyme. The best lauryl palmitate synthesis was achieved with water content of 0.2~0.4%, reaction temperature of 40° C and 45° C for *Candida cylindracea* lipase and porcine pancreatic lipase, respectively. When ester synthesis was carried out under the optimum conditions, the conversion yield of palmitate into lauryl palmitate after 70hrs reached 85% and 69% for the *Candida cylindracea* lipase and porcine pancreatic lipase, respectively.

key words : ester synthesis, lipase, organic solvent

INTRODUCTION

Enzyme-catalyzed reactions are generally performed in water. This is attributable to the common idea that an aqueous solution is optimal for preserving the catalytically active conformation of the enzyme protein for binding and catalysis. If water is replaced with an organic solvent, the conformation of the enzyme protein is intensively shifted and after all the catalytic effect of the enzyme is expected to be less optimal than in water. The investigation of enzymes in organic solvents has been disturbed by this recognition. But this notion is wrong. In cells many enzymes or multienzyme complexes, including lipase, esterase, and dehydrogenase *etc.*, were located on the outer or interface of the plasma membrane. Because these enzymes were related to hydrophobic environment, reaction system of which organic solvent is introduced in part or whole as the reaction media serves as an important model in studying enzyme function *in vivo*. There are numerous possible advantages of conducting biocatalytic reaction using enzymes or microorganisms in organic solvents[1] : better solubility of nonpolar substrates ; shift of reaction equlibria in desirable direction such as use of hydrolase for synthetic reactions ; recovery and reusability of enzymes even without immobilization ; enhanced thermostability- ; elimination of microbial contamination. Thus the study of enzyme action in organic solvent has undergone rapid expansion. To investigate enzymatic reaction in organic solvent, some reaction systems have been introduced : aqueous-organic biphasic system [2,3] ; reverse micellar system[4,5] ; nearly anhydrous organic solvents[6]. Klibanov and Zaks[7] have shown that lipase could catalyze the transesterification between tributyrin and various primary and secondary alcohols in 99% organic solvent. It is thought that the amount of water truly necessary for an enzyme to function in organic solvent is a little. Except for this essential water, organic solvent can be substituted for the remainder of the water without detrimental effect on enzymatic activity. Enzymes in organic solvent can catalyze a variety of reactions that are possible only in water-restricted conditions. For example, lipase in organic solvent can catalyze esterification, interesterification, transesterification, aminolysis, thiotransesterification, and oximolysis[8]. In the present study, we examined optimum condition for synthesis of ester compound from fatty acid and alcohol in organic solvent.

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MATERIALS AND METHODS

Materials

Candida cylindracea lipase and porcine pancreatic lipase were purchased from Sigma. These lipases had specific activities of 900 and 15 units/mg of solid, respectively. In all experiments the lipases were used without further purification. The organic solvents were the special grade. Water was removed from organic solvent by shaking them with 3Å molecular sieves.

Enzyme assay

Determination of substrates and products in ester synthesis reaction

Ester synthesis reaction in organic solvent was initiated by the addition of a lipase to a mixture of substrates in organic solvent. The reaction mixture was shaken on shaking water bath at 100rpm and 37° C. Periodically, aliquots were assayed by gas chromatography using a 530μm fused silica capillary column (Hewlett-Packard).

Assay of lipase-catalyzed hydrolysis of lauryl palmitate in water

Lipase activity in emulsion system was determined by the method of Watanabe *et al.*[9]. Lauryl palmitate emulsion was prepared as follows : 10ml of lauryl palmitate solution and 30ml of 2.0% polyvinylalcohol were emulsified by a homogenizer. The reaction mixture of 2.5ml of lauryl palmitate emulsion, 2ml of distilled water, and 0.5ml of 110mM $CaCl_2$ (final concentration : 10mM), and 0.5ml of enzyme solution was incubated at 37° C for 10min. The reaction was stopped by addition of 10ml of acetone-ethanol mixture (1 : 1) and the liberated free fatty acid was titrated with 0.01N NaOH.

RESULTS AND DISCUSSION

Selection of organic solvent

In order to examine the correlation of enzyme function with the nature of the organic solvent and select the solvent suitable for lauryl palmitate synthesis by lipase, lipase activity in a variety of organic solvents was investigated. Palmitic acid and lauryl alcohol were dissolved in organic solvent. Then lipase was added, and the reaction mixture was shaken at 37° C. Periodically lauryl palmitate was assayed by gas chromatography. Enzymatic activities in various organic solvents were seen in Table 1. Lipases are catalytically active in a variety of organic solvents. Dimethylsulfoxide was only solvent in which lipases were completely inactive. Dimethylsulfoxide can dissolve protein, enzymes in it change their conformation and consequently inactive[8]. The catalytic activities of porcine pancreatic lipase (PPL) are similar to different solvents, while those of the *Candida cylindracea* lipase (CCL) vary being somewhat high in water-immiscible solvents (cyclohexane, isooctane) and relatively low in water-miscible ones (dioxane, pyridine). The essential water, which ensures maintenance of catalytically active conformation of the enzyme, for PPL is strongly bound to the enzyme molecule and even polar solvents do not strip it from enzyme. On the other hand, this water for CCL is weakly bound to the enzyme molecule ; therefore, it is divided into hydrophilic (but not hydrophobic) solvents[8]. Hence, It can be concluded that the significant factor in the effect of organic solvent on enzymatic activity exerted more influence on enzyme bound water than with

Table 1. Reaction of the lipase-catalyzed esterification between palmitic acid and lauryl alcohol in different organic solvents

Solvent	Reaction rate, μmol/hr/mg of enzyme	
	Porcine pancreatic lipase	*Candida cylindracea* lipase
Hexane	0.45	1.03
Toluene	0.46	0.41
Cyclohexane	0.31	1.33
Isooctane	0.37	1.42
Benzene	0.21	0.16
Chloroform	0.13	0.08
Diethyl ether	0.29	0.08
Pyridine	0.11	0.08
Tetrahydrofuran	0.17	0.08
Acetone	0.16	0.10
Dioxane	0.16	0.07
Dimethylsulfoxide	0.00	0.00
Cyclohexanone	0.20	0.07
Carbon tetrachloride	0.21	0.17

A lipase powder (10mg) was added to 1ml of an organic solvent containing 0.3mmole palmitic acid and lauryl alcohol. The mixture was shaken at 37° C and the time course of reaction was followed by gas chromatography

enzyme itself. In the case of PPL, hexane and toluene showed relatively high lipase activity. On the other hand, the lipase from *Candida cylindracea* yielded good lipase activity in cyclohexane and isooctane (Table 1). Therefore, these solvents were used for esterification reaction study.

Effect of water content

We examined the dependence of the rate of esterification on water content. Palmitic acid and lauryl alcohol were dissolved in organic solvent, which had been dehydrated by molecular sieves and added a known amount of water. Then a lipase powder was added and the reaction mixture was shaken at 37° C. Fig. 1 shows that enzyme activity from porcine pancrease increased in proportion with water content up to 0.4% and then decresed slightly with the increase of the water content. In the case of CCL, the esterification rate was maximum at 0.4% and 0.2% of water content for cyclohexane and isooctane, respectively. The increase of CCL activity by added water was much more than that of PPL.

Effect of temperature on enzyme activity

We examined the effect of temperature on enzyme reaction in organic solvent and lipase activity in water was studied for comparative purpose. In the case of hydrolytic activity of lipase, an enzyme solution was added to substrate solution at various temperature and the enzyme activity was determined. Esterification reaction was performed by adding the enzyme to organic solvent containing substrate and the known amount of water at a given temperature, and then enzyme activity was measured at various temperature. Fig. 2 shows the effect of temperature on lipase activity. In the case of CCL, enzymatic activity in organic solvent was found to be relatively good at 40° C, while enzyme in water showed high activity at 35° C. In the case of PPL, maximum activity for esterification and hydrolysis was shown at 45° C for both case.

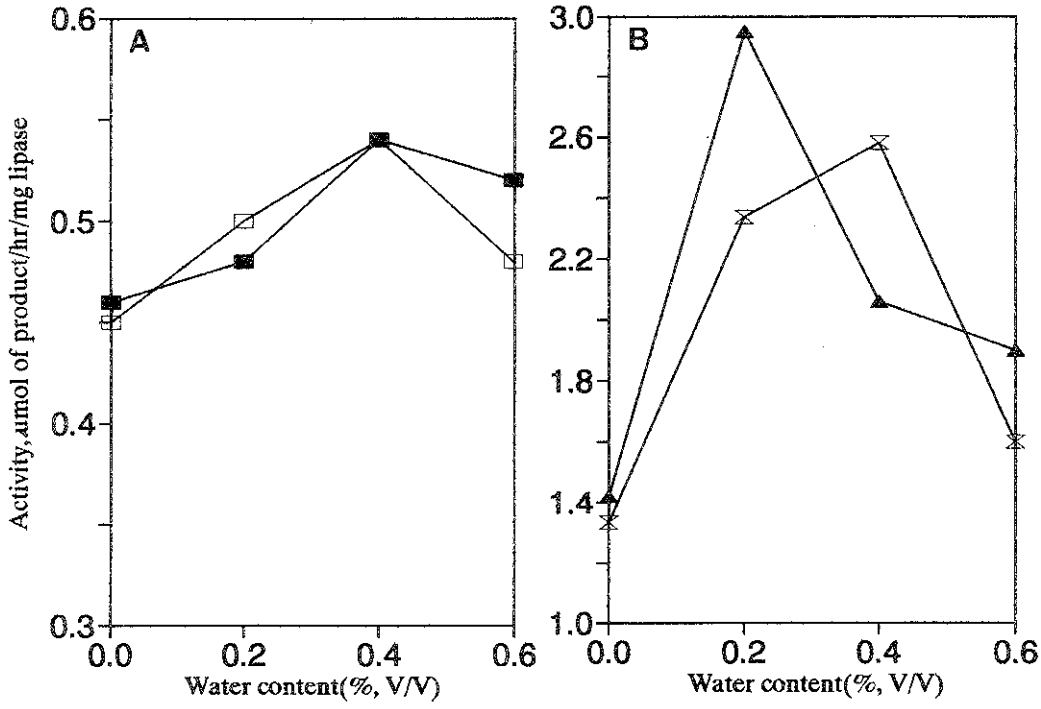

Fig. 1. Lipase-catalyzed esterification reaction between palmitic acid and lauryl alcohol in organic solvent and the effect of added water.

A : porcine pancreatic lipase (PPL) B : *Candida cylindracea* lipase (CCL)
□ : Hexane ■ : Toluene ⧖ : Cyclohexane ▲ : Isooctane

Thermal stability

We compared with effect of organic solvent and water on thermal stability of enzyme. Thermal stability was determined by the assay of residual enzyme activity after preincubation of enzyme at various temperature. Table 2 and 3 show the thermal stabilities of lipases. When water content decreased, enzyme thermostability was enhanced. This result must be due to the fact that water is involved in enzyme thermoinactivation[7].

Time course

Esterification reaction in organic solvent under the optimum conditions was performed (Fig. 3). In the

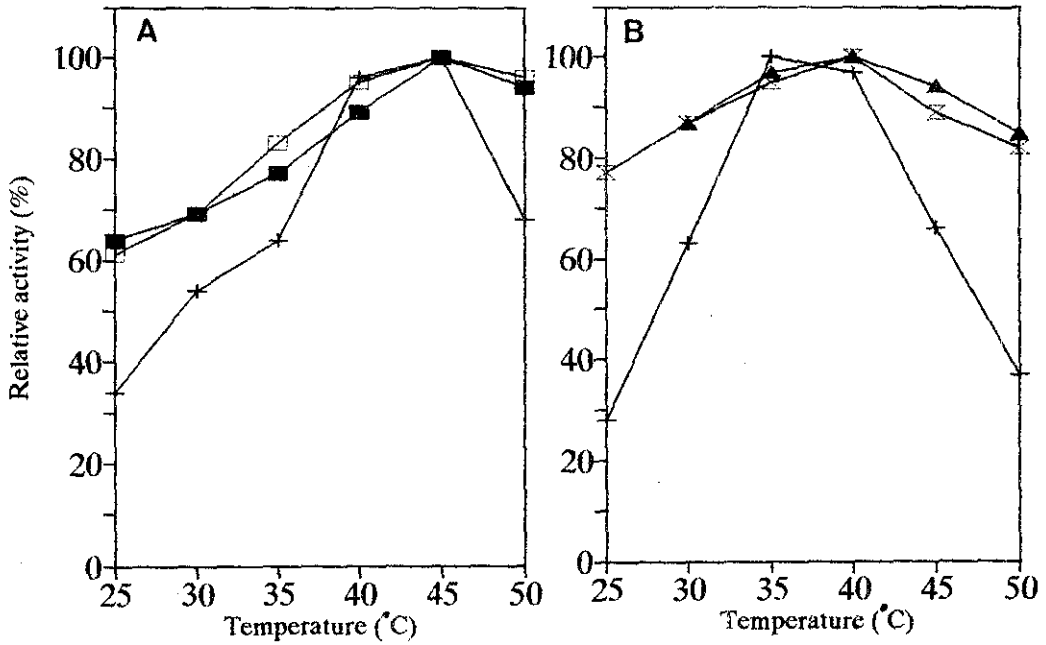

Fig. 2. Effect of temperature on esterification and hydrolysis activity of lipases in organic solvent and water, respectively.
A : porcine pancreatic lipase (PPL) B : *Candida cylindracea* lipase (CCL)
□ : Hexane ■ : Toluene | : Water ⧖ : Cyclohexane ▲ : Isooctane

Table 2. Thermal stability of porcine pancreatic lipase for esterification and hydrolysis reaction in organic solvent and water, respectively

Temperature (°C) \ Solvent	Relative activity (%)				
	Hexane		Toluene		Water
	0[a]	0.4[a]	0[a]	0.4[a]	
25	100	100	100	100	100
35	100	100	100	100	100
45	100	75	100	88	95
55	100	57	100	75	62
65	85	45	90	60	42
75			86	48	11
85			79	20	0
95			70	10	0

[a] water content in organic solvent
Porcine pancreatic lipase in organic solvent and water was incubated at various temperature for 20 min and the residual activity was determined

Table 3. Thermal stability of *Candida cylindracea* lipase for esterification and hydrolysis reaction in organic solvent and water, respectively

Temperature (°C) \ Solvent	Relative activity (%)				
	Cyclohexane		Isooctane		Water
	0[a]	0.4[a]	0[a]	0.4[a]	
25	100	100	100	100	100
35	100	100	100	100	100
45	100	75	100	89	86
55	84	52	100	72	58
65	62	48	89	63	24
75	55	35	65	45	0
85			57	35	0
95			39	28	0

[a] water content in organic solvent
Candida cylindracea lipase in organic solvent and water was incubated at various temperature for 20 min and the residual activity was determined

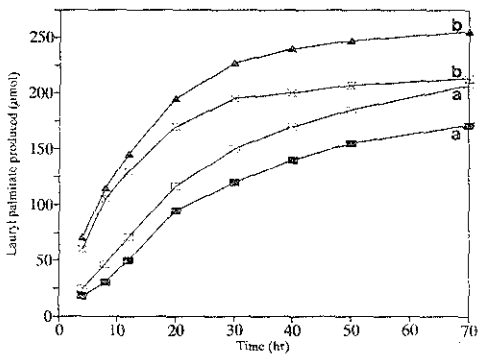

Fig. 3. Time course of esterification reaction between palmitic acid and lauryl alcohol by porcine pancreatic lipase and *Candida cylindracea* lipase.
a : porcine pancreatic lipase (PPL)
b : *Candida cylindracea* lipase (CCL)
□ : Hexane ▨ : Toluene
⧖ : Cyclohexane ▲ : Isooctane

Table 4. The optimum condition for the lauryl palmitate production by esterification of palmitic acid and lauryl alcohol

	Solvent	Water content (% v/v)	Temperature (°C)	Time (hr)	Yield (%)
CCL	Isooctane	0.2	40	70	88
PPL	Hexane	0.4	45	70	69

case of CCL, reaction was performed by adding the enzyme to an organic solvent containing substrate and the given amount of water at 40°C. Conversion yield to lauryl palmitate after 70hrs of reaction was 71%, 85% for cyclohexane and isooctane, respectively. In the case of PPL, enzyme was added to organic solvent containing 0.4% water and substrate and then reaction was continued at 45°C. Conversion yield of palmitate into lauryl palmitate after 70hrs of reaction was 69%, 57% for hexane and toluene, respectively. Table 4 shows optimum condition for the lauryl palmitate production from palmitic acid and lauryl alcohol. PPL in hexane containing 0.4% water at 45°C showed 69 % of conversion yield after 70hrs of reaction. CCL in isooctane containing 0.2% water at 40°C showed 88 % of high conversion yield after 70hrs of reaction. CCL in hexane also showed high conversion yield about 90% (data not shown). In conclusion, it is possible to produce complex substances from simple matter by enzymatic synthesis in organic solvent. Synthesis of novel product should of course be possible if further improvement for activity and stability of enzymes in organic solvents are employed.

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유기용매계에서 리파제에 의한 에스테르 화합물 합성

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요 약

유기용매계에서 리파제를 사용하여 lauryl alcohol과 palmitic acid로 부터 lauryl palmitate를 합성하였다. Hexane, toluene, cyclohexane, isooctane과 같이 물과 혼합할 수 없는 유기용매가 이 합성반응에 유용하였다. Ester 전환 속도에 대한 수분 함량의 영향은 일정범위 까지 수분함량이 증가함에 따라 반응속도가 증가하였다. 반면에 유기용매계의 수분 함량을 감소시키면 효소의 열안정성은 오히려 향상되는 결과가 얻어졌다. Lauryl palmitate 합성속도는 수분 함량 0.2~0.4%, 반응온도 40°C (CCL), 45°C (PPL)에서 가장 양호하게 나타났다. 이 때의 lauryl palmitate로의 전환율은 반응 70시간에서 CCL의 경우 85%, PPL의 경우 69%로 나타나 반응효율이 우수함이 확인되었다.