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Chemical Modification of Lipase with Poly[(*N*-Acylimino)ethylene]s Having a Hydrophobic Component at the Polymer End

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CHEMICAL MODIFICATION OF LIPASE WITH POLY[(*N*-ACYLIMINO)ETHYLENE]S HAVING A HYDROPHOBIC COMPONENT AT THE POLYMER END

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ABSTRACT

Novel amphiphilic polymeric modifiers, i.e., block copolymers of 2-oxazolines containing hydrophobic and hydrophilic components (**2**), and homopolymers of 2-oxazolines having one hydrophobic unit at the polymer end (**5**), were prepared. Each of the polymeric modifiers was coupled with *Candida cylindracea* lipase by using a carbodiimide. It was shown that a 2-oxazoline block copolymer having both a hydrophobic and a hydrophilic component remarkably enhanced the thermal stability of lipase in organic solvents. It was also found that the introduction of a hydrophobic unit at the polymer end gave a significant activation of the

enzyme functions in organic solvents, even when poly[(*N*-acetylimino)-ethylene], which was not a suitable modifier for enzyme use in an organic solvent, was employed.

INTRODUCTION

The conjugation of a bipolymer with a synthetic polymer is a quite attractive and important way to achieve improvement of the original properties of the bipolymer or to prepare a novel functional hybrid. The conjugation of an enzyme with poly(ethylene glycol) (PEG) has been intensively investigated because the resulting hybrid is useful as a biocatalyst for organic synthesis in nonaqueous media as well as a drug with decreased antigenicity and the enhanced stability in vivo [1, 2].

In preceding papers we investigated the chemical modification of lipase from *Candida cylindracea* with poly[(*N*-acylimino)ethylene]s and found the resulting modified lipase, for example, poly[(*N*-propionylimino)ethylene]-modified lipase, shows higher activity in nonaqueous media than PEG-modified lipase [3]. We also found that the nature of the polymeric modifier leads to a significant influence of the enzyme activity in organic solvents [4].

For the purpose of additional improvement of the enzymatic functions by means of chemical modification, we synthesized block copolymers of 2-oxazolines containing hydrophobic and hydrophilic components (**2**), and homopolymers of 2-oxazolines having one hydrophobic unit at the polymer end (**5**), as novel amphiphilic polymeric modifiers, and examined the influences of these modifiers on lipase functions.

EXPERIMENTAL

Materials

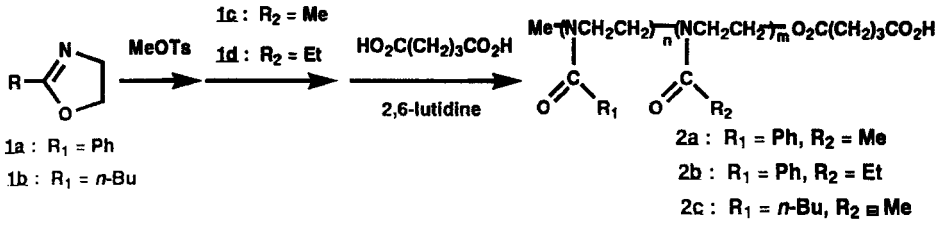
Carboxylic-acid-terminated 2-oxazoline block copolymers (**2**) were prepared according to the method described in a preceding paper [5]. 2-*n*-Butyl-3-methyl-2-oxazolinium tosylate (**4**) was prepared according to the method reported in a previous paper [6]. Crude lipase OF 360 from *Candida cylindracea* was obtained from Meito Sangyo Co. (Tokyo, Japan) and purified by the method of Tanaka et al. [7]. The chemical modification by the polymeric modifier of lipase was carried out according to an analogous procedure described in preceding papers [3, 4].

Instrumentation

A GPC analysis of the modified enzymes was performed by using a TSK-GEL G3000SW_{XL} column in 0.05 M pH 7.0 phosphate buffer at room temperature. IR spectroscopy was carried out with a Perkin-Elmer 1640 spectrometer.

Enzyme Assay

The esterification activity of the modified lipase 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 μmol of each substrate was added 1 mg of enzyme. Then



SCHEME 1.

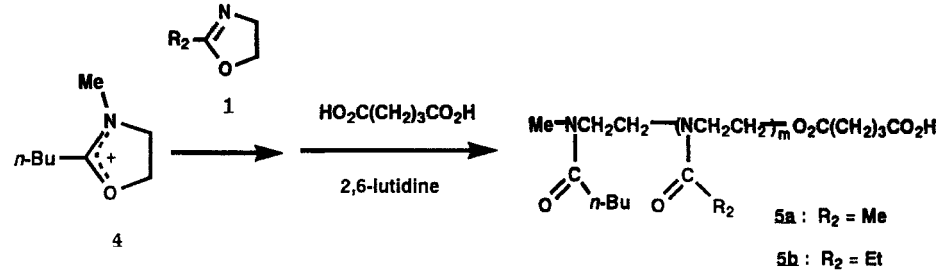
the reaction mixture was incubated at 40 ± 0.01°C with shaking at 120 strokes/min. The conversion of the substrates was determined by gas chromatography using a silicon DC 550 column (1 m). The activity was estimated by the initial rate of the reaction.

RESULTS AND DISCUSSION

Preparation of the Modified Lipase

Carboxylic-acid-terminated 2-oxazoline block copolymers consisting of hydrophobic and hydrophilic blocks (**2**) were prepared by a so-called one-pot two-stage block copolymerization between two oxazoline monomers [8]. The propagating end of the block copolymer was reacted with a large excess of glutaric acid in the presence of 2,6-lutidine to introduce the carboxylate functional group to the polymer end [5]. (See Scheme 1.) One of the carboxylic acid groups of the dibasic acid was used in esterification, and the other remained unreacted. The introduction of one hydrophobic monomeric unit at the initiating end of poly[(*N*-acetylimino)ethylene] and poly[(*N*-propionylimino)ethylene] was carried out by using 2-*n*-butyl-3-methyl-2-oxazolinium tosylate (**4**) as initiator [6]. (See Scheme 2.) The unit ratio, the molecular weight, and the extent of functionalization of the polymers obtained are summarized in Table 1.

To couple lipase with the above polymeric modifiers chemically, the modifiers were converted to their activated esters by the reaction with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in CH_2Cl_2. (See Scheme 3.) Then a large excess amount of the activated polymer (the molar ratio of the polymer to lysine residues



SCHEME 2.

TABLE 1. Preparation of Carboxylic-Acid-Terminated Block Copolymer and Homopolymer from 2-Oxazolines[a]

		1st stage		2nd stage,	Block copolymer				
M_1	M_2	Initiator	$[M_1]$/[I]	$[M_2]$/[I]	Structure	Unit ratio[a] (m:n)	M_n[b]	M_w/M_n[b]	F_n[c]
1a	**1c**	MeOTs	9.5	38.2	**2a**	6.0:34	3240	1.47	0.91
1a	**1d**	MeOTs	10.5	39.3	**2b**	6.6:40	4590	1.51	1.01
1b	**1c**	MeOTs	10.2	32.7	**2c**	12:41	5110	1.07	0.76
—[e]	**1c**	4[d]	—	34.9	**5a**	1:30	2540	1.15	0.43
—[e]	**1d**	4[d]	—	34.7	**5b**	1:39	3880	1.05	0.68

[a]Determined by ^{1}H-NMR which was recorded on a 60 MHz Hitachi R-600 NMR spectrometer. $CDCl_3$ was used as solvent.

[b]Determined by GPC measurement. GPC analysis was performed by using a TSK-GEL G2500H column in DMF containing 0.4% triethylamine at 50°C. The calibration curve was obtained by using standard samples of poly[(*N*-acetylimino)ethylene] which were prepared by the ring-opening polymerization of **1c**.

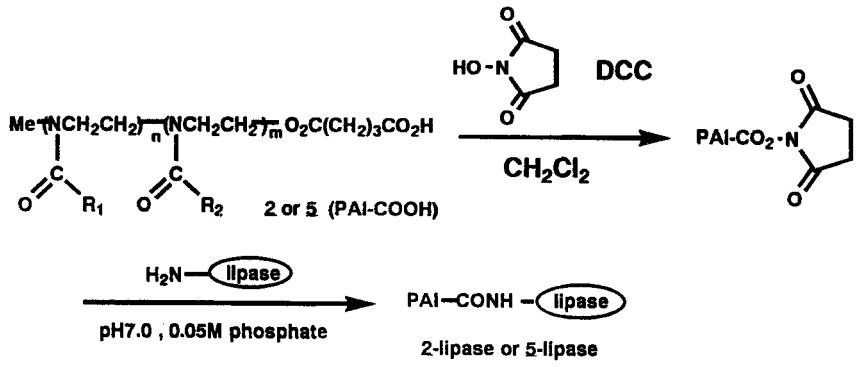
[c]Average degree of functionalization per molecule was determined by alkaline titration.

[d]2-*n*-Butyl-3-methyl-3-oxazolinium tosylate. A 1:1 adduct of **1a** with MeOTs.

[e]Homopolymerization of the hydrophilic monomer (M_2).

in the enzyme was 10) was reacted with lipase in 0.05 M phosphate buffer (pH 7.0) at 25 ± 1°C. The hybrid was obtained as white powders after dialysis against MeOH/H_2O (1/4 v/v) for **2** and against distilled water for **5** using ultrafiltration and the subsequent lyophilization.

The polymer contents of modified lipase were estimated from the peak ratios of the absorbances of 1539 and 1366 cm^{-1} in the IR spectra by using calibration curves prepared from mixtures of lipase with the corresponding modifier. The data



SCHEME 3.

are shown in Table 2. The polymer content of **2b**-lipase was almost equal to that of poly[(*N*-propionylimino)ethylene] (M_n = 5160) (**3b-1**) modified lipase of whose properties were reported previously [3]. On the contrary, polymer contents of **2a**-lipase and **2c**-lipase were remarkably higher than those of **2b**-lipase and **3b-1**-lipase. The polymer contents of **5a**-lipase and **5b**-lipase were almost the same as those of the corresponding homopolymers, **3a**-lipase and **3b-2**-lipase, respectively.

Naka et al. reported an aggregate of **2c** and lipase is formed by mixing **2c** with lipase [9, 10]. GPC analysis shows that **2c**-lipase aggregate is an extraordinary high molecular weight complex ($>10^8$). The aggregate is soluble in water and in chloroform, and reveals quite excellent enzymatic activities in these media. The polymer content of **2c**-lipase aggregate was determined to be 83%. In the present study the higher polymer contents of **2a**-lipase and **2c**-lipase in comparison with the others may be ascribed to an aggregation of the unreacted amphiphilic modifier to the resulting chemically modified lipase. The GPC measurement of **2a**-lipase and **2c**-lipase in 0.05 M phosphate buffer (pH 7.0) clearly showed that they were quite different from that of **2c**-lipase aggregate, i.e., **2c**-lipase consisted of the covalently bound polymer–enzyme hybrid and the unreacted modifier. An aggregate with a very high molecular weight was not formed. The unreacted modifier may be bound to the modified lipase weakly or could not be separated by ultrafiltration.

Covalent bond formation between the modifier and lipase was further confirmed from the thermal stability of **2c**-lipase. Both **2c**-lipase and **2c**-lipase aggregate suspensions (2 mg in 1.5 mL of water-saturated toluene) were incubated at 80°C for 4 hours. The GPC analysis of the samples revealed that no significant decomposition of **2c**-lipase did not occur, while **2c**-lipase aggregate was completely decomposed to the components, lipase and **2c**.

TABLE 2. Polymer Content of Modified Lipase

Modifier	Modified lipase	Polymer content, wt%[a]
None	Unmodified lipase	0
2a	**2a**-lipase	70
2b	**2b**-lipase	28
2c	**2c**-lipase	62
3a[b]	**3a**-lipase	13
3b-1[c]	**3b-1**-lipase	27
3b-2[d]	**3b-2**-lipase	21
5a	**5a**-lipase	13
5b	**5b**-lipase	21

[a]Determined from IR measurement.

[b]Carboxylic-acid-terminated poly[(*N*-propionylimino)ethylene] (M_n = 5160).

[c]Carboxylic-acid-terminated poly[(*N*-acetylimino)ethylene] (M_n = 3140).

[d]Carboxylic-acid-terminated poly[(*N*-propionylimino)ethylene] (M_n = 3800).

Enzymatic Activity of Modified Lipase with 2 in Nonaqueous Media

The enzymatic esterification between *n*-amyl alcohol and caprylic acid was investigated in water-saturated benzene and in water-saturated toluene at 40 ± 0.01°C. The results are summarized in Table 3. To compare the effect of the polymeric modifier on lipase functions, the activities of the hybrid were calculated per milligram of lipase moiety. Although the activities of **2a**-lipase and **2c**-lipase were significantly higher than those of the native lipase and **2b**-lipase, **2c**-lipase aggregate showed the highest activity among the samples in organic media. The higher activity of **2a**-lipase and **2c**-lipase in comparison with the native enzyme may be ascribed to the higher dispersibility of the modified lipase in organic media. The reason of the lower activity of **2b**-lipase is not clear. Perhaps the poly[(*N*-propionylimino)ethylene] block of **2b** inactivates lipase functions.

As described above, **2c**-lipase aggregate was completely decomposed at 80°C while **2c**-lipase was stable at that temperature. Therefore, the thermal profiles of the hybrids were investigated to clarify the difference between the enzymatic functions of **2c**-lipase and **2c**-lipase aggregate. The thermal stabilities of hybrids were tested by two methods. In the first method the enzymatic esterification activity was measured at various temperatures in water-saturated toluene. The results are summarized in Table 4. The activities of both **2a**-lipase and **3b-1**-lipase at 60°C were reduced to 50% of the activities at 40°C, and only 10% of the activity remained at 80°C. The activity of **2c**-lipase at 60°C was higher than that at 40°C, and 40% of its original activity was kept even at 80°C. Contrary to **2c**-lipase, **2c**-lipase aggregate

TABLE 3. Enzymatic Esterification Activity of Modified Lipase with **2**

Modified lipase	Activity, μmol/h/mg of lipase[a]	
	In benzene[b]	In toluene[b]
Unmodified lipase	0.6	0.5
2a-lipase	47	147
2b-lipase	0.1	0.1
2c-lipase	37	113
3a-lipase[c]	1.8	—
3b-1-lipase[d]	38	59
3b-2-lipase[e]	35	—
2c-lipase aggregate	103	255

[a]Activity was estimated per lipase.

[b]Solvents were all saturated with water at 30 ± 0.01°C.

[c]Poly[(*N*-propionylimino)ethylene] (M_n = 5160) (**3a**) modified lipase.

[d]Poly[(*N*-acetylimino)ethylene] (M_n = 3140) (**3b-1**) modified lipase.

[e]Poly[(*N*-propionylimino)ethylene] (M_n = 3800) (**3b-2**) modified lipase.

TABLE 4. Esterification Activity of the Sample in Water-Saturated Organic Media at Varying Temperatures

	Activity, μmol/h/mg of lipase moiety					
	In toluene			In isooctane		
Sample	40°C	60°C	80°C	40°C	60°C	80°C
Unmodified	–	–	–	143	105	19
2a-lipase	147	63	14	293	293	113
2c-lipase	113	150	47	226	474	447
3b-1-lipase	59	30	8	384	292	123
2c-lipase aggregate	255	–	7	–	–	–

kept only 3% of its original activity at 80°C. These results indicate that chemical modification with **2c** prevented the reversible thermal inactivation of the hyperstructure of lipase moiety.

In the second method the toluene suspension of the sample was held at 80°C for a desired time, cooled rapidly to room temperature, and assayed at 40°C. Plots of the enzymatic activity versus incubation time are shown in Fig. 1. Although **3b-1**-lipase was markedly inactivated within 30 minutes, the thermal stability was increased in **2a**-lipase and **2c**-lipase, especially after 4 hours of incubation at 80°C for **2c**-lipase, after which more than 80% of its original activity remained. On the contrary, **2c**-lipase aggregate showed 30% of its original activity after 4 hours of incubation at 80°C. The time courses of inactivation of **2a**-lipase and **3b-1**-lipase at

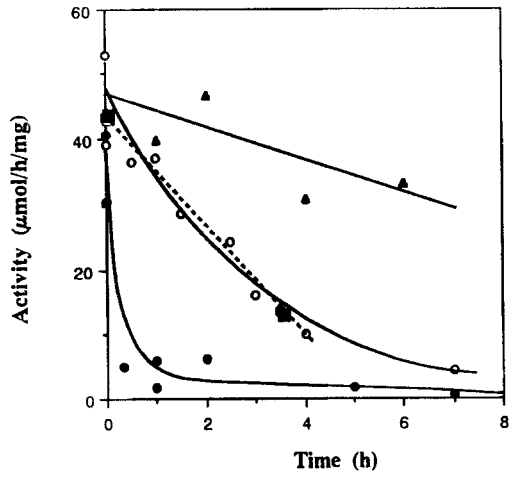


FIG. 1. The time courses of thermal inactivation of **2a**-lipase (○), **2c**-lipase (▲), **3b-1**-lipase (●), and **2c**-lipase aggregate (■) at 80°C in water-saturated toluene. Samples were periodically assayed at 40°C.

40°C in water-saturated toluene were also measured (Fig. 2). Although the activity was reduced by half after 2 hours, inactivation of **2a**-lipase was hardly recognized even after 6 hours. These results indicate that the introduction of **2a** or **2c** to the lipase surface kept the enzymatic ability in toluene and prevented it from irreversible inactivation. The thermal stability of **2c**-lipase was higher than that of **2c**-lipase aggregate in toluene. To keep the stability at a higher temperature, the modifier should be bound covalently to the enzyme.

The enzymatic esterification activities of the hybrids were also measured at various temperatures in water-saturated isooctane. Table 4 shows that the thermal stabilities of both **2a**-lipase and **2c**-lipase were higher than those of unmodified lipase and **3b-1**-lipase. Surprisingly, the activity of **2c**-lipase at 60°C was more than two times that at 40°C, and even at 80°C the drop of activity was hardly recognized. The esterification reaction rate catalyzed by **2c**-lipase was accelerated more than 20 times faster than by unmodified lipase at 80°C. The activity of **2a**-lipase at 60°C was equal to that of 40°C, although the activities of unmodified lipase and **3b-1**-lipase at 60°C were decreased to about 70% of their original activities at 40°C.

These observations show that 2-oxazoline block copolymer with both hydrophobic and hydrophilic components, especially **2c**, remarkably enhanced the thermal stability of lipase in organic solvents. It is said that thermal unfolding of an enzyme molecule seems to be a universal and general stage in enzyme thermoinactivation [11]. It can therefore be presumed that the introduction of **2c** prevented thermal unfolding of the enzyme structure in organic solvents.

Enzymatic Activity of Modified Lipase with 5 in Nonaqueous Media

Although the activity of **3a**-lipase was almost the same as that of unmodified lipase, **5a**-lipase enhanced the activity in all solvents, which were the same level of that of **3b-2**-lipase (Table 5). These results suggest that the introduction of a hydrophobic unit at the polymer end gave a significant activation of the enzyme

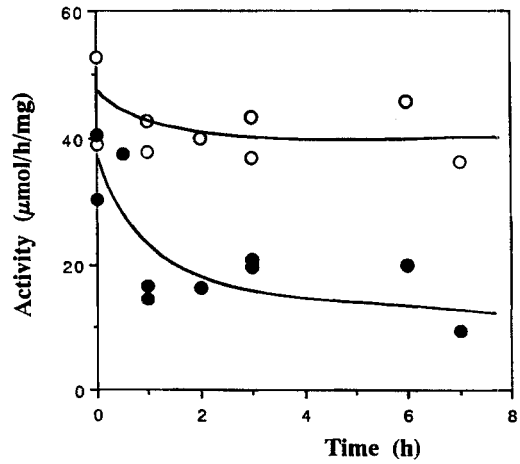


FIG. 2. The time courses of thermal inactivation of **2a**-lipase (○) and **3b-1**-lipase (●) at 40° C in water-saturated toluene. Samples were periodically assayed at 40°C.

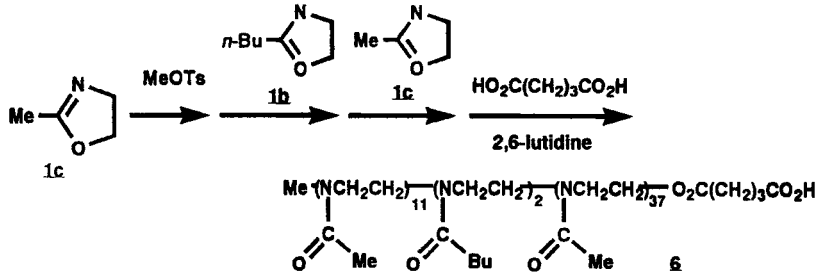
TABLE 5. Enzymatic Esterification Activity of Modified Lipase with **5**

Modified lipase	Activity, μmol/h/mg of lipase[a]		
	In benzene[b]	In chloroform[b]	In isoctane[b]
Unmodified lipase	0.6	5.7	140
5a-lipase	22	11	333
5b-lipase	34	15	329
3a-lipase	1.8	4.8	126
3b-2-lipase	35	11	306
6-lipase	7.6	6.6	216

[a]Activity was estimated per lipase.
[b]Solvents were all saturated with water at 30 ± 0.01°C.

functions in these organic solvents, even when poly[(*N*-acetylimino)ethylene], which was not a suitable modifier for enzyme use in an organic solvent, was employed.

To make sure of the effect of the polymer end, poly[(*N*-acetylimino)ethylene] component was introduced to the polymer end of **5a**. For this purpose a carboxylic-acid-terminated ABA-type triblock copolymer (**6**) was prepared via one-pot three-stage copolymerization by using **1c** for a hydrophilic chain (A block) and **1b** for a hydrophobic chain (B block). (See Scheme 4.) The number-average molecular weight of **6** was estimated to be 4380. The unit ratio of **6** was found to be 11 (**1c** unit):2 (**1b** unit):37 (**1c** unit). The carboxylate end group per molecule was estimated to be 0.77 based on the result of alkaline titration together with the molecular weight. The polymer content of **6** modified lipase (**6**-lipase) was 17%, which was almost equal to those of **5a**-lipase and **3a**-lipase. The activity of **6**-lipase was lower than that of **5a**-lipase. These results indicate that the hydrophobic unit was required at the polymer end, especially for poly[(*N*-acetylimino)ethylene], to activate the lipase functions in organic solvents.



SCHEME 4.

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