Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

Effect of Modifier on Enzymatic Function of Poly[(*N*-Acylimino)ethylene]-Modified Lipases in Organic Solvents

Kensuke Naka[a]; Yoshiki Chujo[a]; Masatoshi Miyamoto[b]; Takeo Saegusa[c]
[a] Department of Synthetic Chemistry Faculty of Engineering, Kyoto University, Kyoto, Japan [b] Department of Polymer Science and Engineering Faculty of Textile Science Kyoto, Institute of Technology Matsugasaki, Sakyo-ku, Kyoto, Japan [c] KRI International, Inc., Kyoto, Japan

To cite this Article Naka, Kensuke , Chujo, Yoshiki , Miyamoto, Masatoshi and Saegusa, Takeo(1997) 'Effect of Modifier on Enzymatic Function of Poly[(*N*-Acylimino)ethylene]-Modified Lipases in Organic Solvents', Journal of Macromolecular Science, Part A, 34: 1, 35 — 48
To link to this Article: DOI: 10.1080/10601329708014932
URL: http://dx.doi.org/10.1080/10601329708014932

J.M.S.—PURE APPL. CHEM., A34(1), pp. 35–48 (1997)

EFFECT OF MODIFIER ON ENZYMATIC FUNCTION OF POLY[(*N*-ACYLIMINO)ETHYLENE]-MODIFIED LIPASES IN ORGANIC SOLVENTS

KENSUKE NAKA* and YOSHIKI CHUJO

Department of Synthetic Chemistry
Faculty of Engineering
Kyoto University
Kyoto 606, Japan

MASATOSHI MIYAMOTO

Department of Polymer Science and Engineering
Faculty of Textile Science
Kyoto Institute of Technology
Matsugasaki, Sakyo-ku, Kyoto 606, Japan

TAKEO SAEGUSA

KRI International, Inc.
17 Chudoji-Minami-Machi, Shimogyo-ku, Kyoto 600, Japan

ABSTRACT

Candida cylindracea lipase was modified by carboxylic-acid-terminated polymers having different molecular weights, i.e., poly[(*N*-acetylimino)ethylene] (**2a**), poly[(*N*-propionylimino)ethylene] (**2b**) and poly[(*N*-isobutyrylimino)ethylene] (**2c**). The effect of *N*-acyl substituent of **2a–c** on the enzymatic activity of modified lipase (**4**) for hydrolysis of olive oil and *p*-nitrophenyl propionate in an aqueous solution and for esterification between *n*-amyl alcohol and caprylic acid in chloroform, benzene, toluene, and isooctane was examined by using **2a–c** of a similar MW (5000 ~ 6000). It was found that the active site reactivity of lipase was not as influenced by the chemical modification of lipase in an aque-

ous medium, and that the rates of esterification were remarkably enhanced, especially in benzene and toluene, by modification with **2b** or **2c** compared with the native lipase. The effects of the molecular weight of **2a–c** and the average number of **2a–c** per molecule of lipase on the enzymatic activity were also investigated.

INTRODUCTION

One of the active areas of recent research in biotechnology is the use of enzymes in nonaqueous media to achieve a rate enhancement of common organic reactions such as esterification, transesterification, and amidation. Since it has been commonly known that organic solvents generally denature and inactivate most 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]), some methods have been developed to improve the catalytic activity of enzymes in organic media which involve, for example, immobilization of an enzyme [3], formation of an enzyme–lipid complex [4], a technique for site-directed mutagenesis [5], and a chemical modification of an enzyme with an amphiphilic polymeric modifier [6, 7].

In preceding papers we demonstrated that poly[(*N*-propionylimino)ethylene] is an excellent polymeric modifier for enzymes, bovine liver catalase, and *Candida cylindracea* lipase in comparison with poly(ethylene glycol) (PEG), a commonly used polymeric modifier [8]. The property of the amphiphilic polymeric modifier seems to be very important for controlling the stability of modified enzymes in nonaqueous media. In a series of poly[(*N*-acylimino)ethylene]s (PAIs), the hydrophilicity/lipophilicity is easily controlled by selecting its acyl substituent.

In the present work we examine the effect of *N*-acyl substituent on the enzymatic activity of PAI-modified *Candida cylindracea* lipase (**4**). Three types of polymer–enzyme hybrid, **4a**, **4b**, and **4c**, 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**), respectively. We also investigated the effect of the molecular weight of **2** and of the extent of modification of the resulting hybrid on the enzymatic activity in organic solvents.

EXPERIMENTAL

Materials

Crude lipase OF 360 from *Candida cylindracea* was obtained from Meito Sangyo Co. (Tokyo, Japan), which was purified according to the literature [9]. The preparation of carboxylic-acid-terminated PAIs, i.e., poly[(*N*-acetylimino)ethylene] (**2a**), poly[(*N*-propionylimino)ethylene] (**2b**), and poly[(*N*-isobutyrylimino)-ethylene] (**2c**), and the chemical modification of the polymeric modifier to lipase was carried out as described in preceding papers [10, 11]. Solvents for enzymatic assay were of the best grade, which were treated with an aqueous 0.05 M pH 7.0 phosphate buffer solution at 30 ± 0.01°C to saturate water unless otherwise indicated.

Instrumentation

The number-average molecular weights of the polymeric modifiers (**2**) were determined by GPC measurements with a Shodex A803 column in chloroform. Size exclusion chromatography (SEC) 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 IR spectroscopy, which was performed on a Perkin-Elmer 1640 spectrometer. The polymer contents of **4a**, **4b**, and **4c** were evaluated from the ratios of the absorbances of 1534 and 1420 cm^{-1} (for **4a**), 1534 and 1200 cm^{-1} (for **4b**), and 1534 and 1445 cm^{-1} (for **4c**) by using calibration curves obtained from mixtures of lipase with **2a**, **2b**, or **2c**, respectively. Differential scanning calorimetry was performed on a Seiko DSC calorimeter (I&E Model DSC-200 calorimeter combined with a Seiko SSC/580 thermal controller).

Enzyme Assay

The ester hydrolysis activity in 0.05 M pH 7.0 phosphate buffer was carried out by using two substrates, olive oil and *p*-nitrophenyl propionate (PNP). The activity for olive oil was measured in a heterogeneous system at 40 ± 0.1°C with shaking at 120 strokes/min by using poly(vinyl alcohol) as the emulsifier according to the literature [12]. The activity was calculated from the concentrations of the resulting fatty acids at 10 and 40 minutes of reaction time determined by titration with 0.1 N aq NaOH. The activity for a water-soluble substrate, PNP, was carried out in a homogeneous system at 30 ± 0.01°C. The consumption of PNP was determined by monitoring the production of *p*-nitrophenoxide in UV spectroscopy at 400 nm in cells of 1 cm path length. To 3 mL of 0.05 M phosphate buffer (pH 7.0) in a cuvette, 50 μL of an acetonitrile solution of 152 mM PNP was added and 0.2 mL of 0.05M enzyme solution in phosphate buffer (pH 7.0) were added using a specially designed syringe at 30 ± 0.01°C. The activity was calculated from the slope of the respective time courses.

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 μmol 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 a silicon DC 550 column (1 m). The initial rate of the reaction was taken as the activity of the sample.

RESULTS AND DISCUSSION

Preparation of Poly[(*N*-Alkylimino)ethylene]-Modified Lipase

Carboxyl-terminated PAIs having different molecular weights, 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. The modification of lipase by poly[(*N*-butyrylimino)ethylene] as well as homologues of **2a–c** having longer acyl substituents failed due to poorer

solubilities of these polymers in water. The molecular weights of the polymers and their extents of functionalization (F_n) are summarized in Table 1.

The chemical modification of lipase with **2a–c** was performed according to an analogous procedure described in a preceding paper [11]. (See Scheme 1.) The modified enzyme samples **4** were obtained after dialysis against distilled water using ultrafiltration and the subsequent lyophilization. The successful modification of lipase was confirmed from the SEC measurement using a TSK-Gel G3000SW$_{XL}$ column in a 0.05 M phosphate buffer (pH 7.0). In every case a unimodal peak was observed, and hence, no contaminations of the unmodified lipase and the unreacted modifier in the product were detected. The position of the peak top in the chart shifts to a high molecular weight region with the increase of the feed ratio of the modifier to the lipase or with the increase of the molecular weight of the modifier. The average number of polymeric modifier per lipase molecule was calculated from the polymer content and the molecular weight of the modifier, and is shown in Table 2. In a preceding paper [8] it was shown that the average number of modification by the polymers per molecule of enzyme (NM) could be controlled by changing the molar ratio of **2** to lysyl group. In order to study the effect of the extent of modification on the enzymatic activity in organic solvents, two series of samples were prepared by using **2b-2** (M_n = 2660) and **2b-4** (M_n = 5160) as polymeric modifiers. They were **4b-2**, **4b-3**, and **4b-4** (from **2b-2**), and **4b-6**, **4b-7**, and **4b-8** (from **2b-4**) (Table 2).

TABLE 1. Carboxylic-Acid-Terminated Poly[(*N*-Acylimino)ethylene] (**2**)[a]

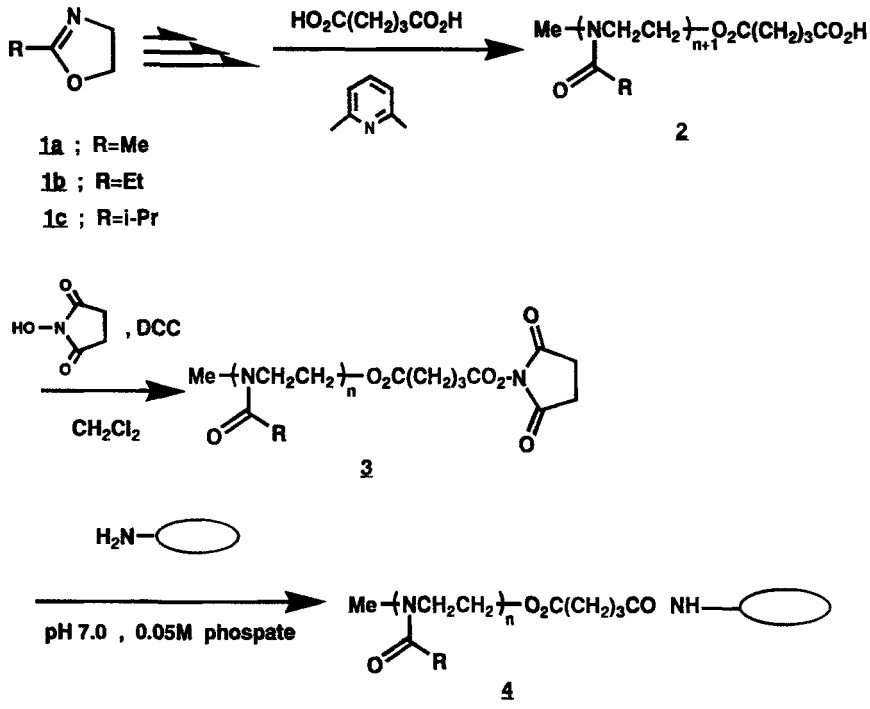
Run	Monomer 1	[1]/[MeOTs]	Product 2	M_n[b]	M_w/M_n[b]	F_n[c]
1	**1a**	10	**2a-1**	780[d]	1.12	1.05
2	**1a**	25	**2a-2**	3140	1.06	1.03
3	**1a**	79	**2a-3**	5660	1.09	0.89
4	**1b**	78	**2a-4**	6370	1.07	0.90
5	**1b**	9	**2b-1**	650[d]		0.60
6	**1b**	20	**2b-2**	2660	1.04	1.28
7	**1b**	38	**2b-3**	3800	1.03	0.85
8	**1b**	50	**2b-4**	5160	1.05	0.70
9	**1b**	60	**2b-5**	5430	1.03	0.67
10	**1c**	47	**2c**	5170	1.05	0.82

[a]Polymerization was carried out using methyl tosylate (MeOTs) as initiator in CH_3CN at 70°C for 48 hours.

[b]Determined by GPC measurement. Each of the calibration curves 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 ^{1}H-NMR measurement.



SCHEME 1.

The Effect of *N*-Acyl Substituent on the Enzymatic Activity of 4

To evaluate the effect of amphilphilic property of the modifier on the enzymatic activity of the hybrid, three samples of **4** with a polymeric modifier of similar MW (5000 ~ 6000) were examined. All of the samples were prepared by reactions with similar modifier/lipase feed ratios, ca. 10. They were **4a-4**, **4b-7**, and **4c**, and the molecular weights of the polymeric modifier in them were 6370, 5160, and 5170, respectively.

First, the enzymatic activities of these samples for ester hydrolysis in an aqueous buffer solution (pH 7.0) were investigated by using olive oil as the hydrophobic substrate in a heterogeneous system and by using *p*-nitrophenyl propionate (PNP) as a water-soluble substrate in a homogeneous system (Table 3). Although the activity of **4a-4** for olive oil was lower than the native enzyme, **4b-7** and, especially, **4c** showed remarkably enhanced activities in comparison with the native enzyme. On the other hand, the activities of **4** for a water-soluble substrate, PNP, were not so different from that of unmodified lipase. It was suggested that the enzymatic function of the lipase moiety of **4** is not much affected by the chemical modification.

In order to estimate the influence of modification by **2**, the kinetic parameters (V_{max} and K_m) of unmodified lipase and **4c** for hydrolysis of PNP were determined by applying Dixon's linear least-squares analysis (Fig. 1). The values of V_{max} and K_m for unmodified lipase were 1.9 mM·min^{-1}·(nmol of lipase)$^{-1}$ and 0.36 mM, respectively, and those for **4c** under the same condition were 1.5 mM·min^{-1}·(nmol of lipase)$^{-1}$·(V_{max}) and 0.63 mM (K_m). Generally, the chemical modification of

TABLE 2. Characterization of Modified Lipase (4)

Modifier	Feed ratio[a]	Modified lipase 4	GPC elution volume,[b] mL	Polymer content,[c] wt%	NM[d]
—	—	Unmodified	10.68	0	0
2a-1	10	**4a-1**	9.62	4	2.6
2a-2	10	**4a-2**	9.33	13	2.4
2a-3	12	**4a-3**	8.86	17	1.9
2a-4	10	**4a-4**	8.13	26	2.7
2b-1	10	**4b-1**	10.00	4	3.1
2b-2	3.0	**4b-2**	9.67	13	2.8
2b-2	10	**4b-3**	9.63	15	3.2
2b-2	10 × 2	**4b-4**	9.14	21	5.0
2b-3	12	**4b-5**	9.03	21	3.5
2b-4	3.6	**4b-6**	9.92	18	2.1
2b-4	14	**4b-7**	8.67	27	3.2
2b-4	14 × 3	**4b-8**	8.40	33	4.8
2b-5	13	**4b-9**	8.56	26	3.2
2c	10	**4c**	8.70	18	1.7

[a]Feed ratio of the modifier to the total lysyl amino residues, [2]/[Lys].
[b]Position of the peak top of GPC curve.
[c]Determined from IR measurement.
[d]Average number of polymer molecules per molecule of lipase. Calculated based on the value of polymer content in **4** determined from IR spectrum.

TABLE 3. Ester Hydrolysis Activity of Modified Lipase (4)

Modified lipase, 4	Activity (μmol/min/mg of lipase)[a]	
	Olive oil	*p*-Nitrophenyl propionate
Unmodified	4.7	124
4a-4	1.6	130
4b-7	13	177
4c	27	97

[a]Conditions are described in the Experimental Section. Activity was estimated per mg of lipase.

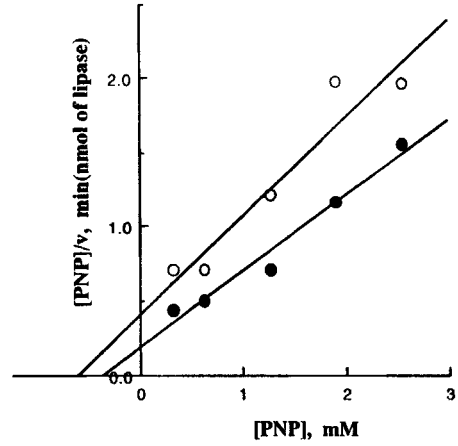


FIG. 1. Dixon's linear least-squares analysis for **4c** (○) and for unmodified lipase (●).

enzyme lowers the enzymatic activity unless any special effect by the modification takes place. For example, the acetylated *Candida cylindracea* lipase (NM = 11) was one-twelfth as active as the native one [11]. In our former work using catalase it was also shown that the activity of the **2a**-modified catalase decreased with increasing NM value [8]. The fact that the difference of V_{max} is smaller than that of K_m indicates that the active site reactivity was not as influenced by the chemical modification in a homogeneous system by **2c**. Although V_{max} and K_m values could not be determined, the remarkable rate enhancement for olive oil by the chemical modification with **2b** or with **2c** may be ascribed to the lipophilic property of the polymer chain on the enzyme surface which enhances the affinity toward the hydrophobic substrate.

The enzymatic esterification between *n*-amyl alcohol and caprylic acid catalyzed by **4a-4**, **4b-7**, **4c**, and unmodified lipase was investigated in chloroform, benzene, toluene, and isooctane. The results are summarized in Table 4. Although

TABLE 4. Enzymatic Esterification Activity of the Modified Lipase

	Activity (μmol/h/mg of lipase)[a]			
Modified lipase	In chloroform	In benzene	In toluene	In isooctane
Unmodified	5.7	1.6	0.5	140
4a-4	12	7.7	13	149
4b-7	12	38	59	384
4c	8.0	40	81	479

[a]Activity was estimated per molecule of lipase. All solvents were saturated with water at 30 ± 0.01°C.

the activity of **4** in chloroform was not as strongly influenced by modifications, especially in benzene and in toluene, the activity of **4b-7** and **4c** was remarkably enhanced.

To investigate the influence of the polymeric modifier on the enzyme function, the effect of the molecular weight of the modifier was examined in four representative solvents: isooctane, benzene, toluene, and chloroform. Figure 2(A) shows the relationship between the esterification activity of **4b** samples with different molecular weights by using *n*-amyl alcohol and *n*-caprylic acid as the substrate in water-saturated isooctane. Obviously, the activity of **4b** increased proportionally with an

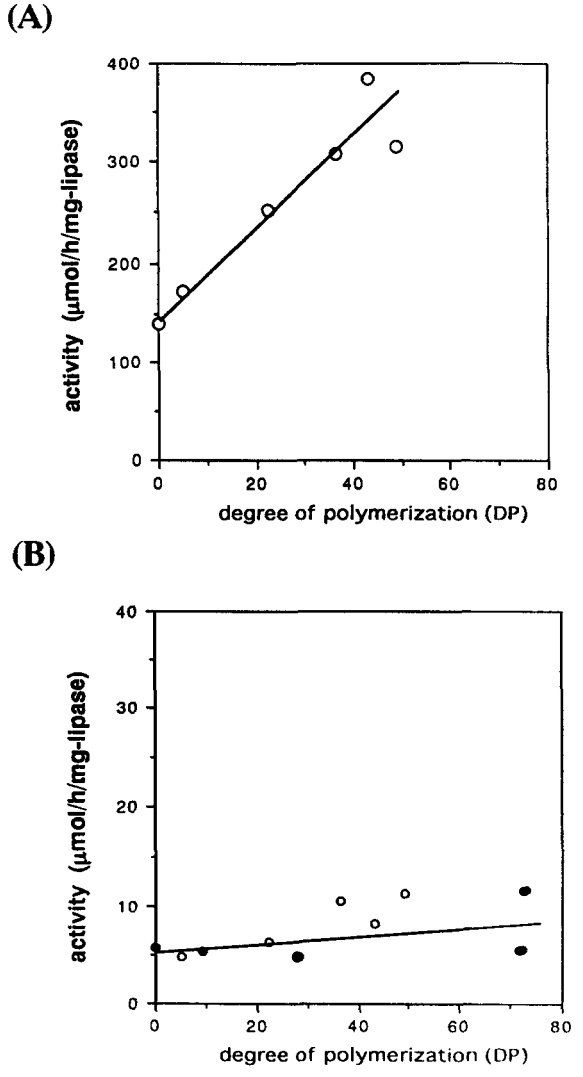


FIG. 2. Enzymatic esterification activity of **4a** (●) and **4b** (○) against the degree of polymerization (DP) in water-saturated isooctane (A), chloroform (B), benzene (C), and toluene (D) at 40 ± 0.01°C.

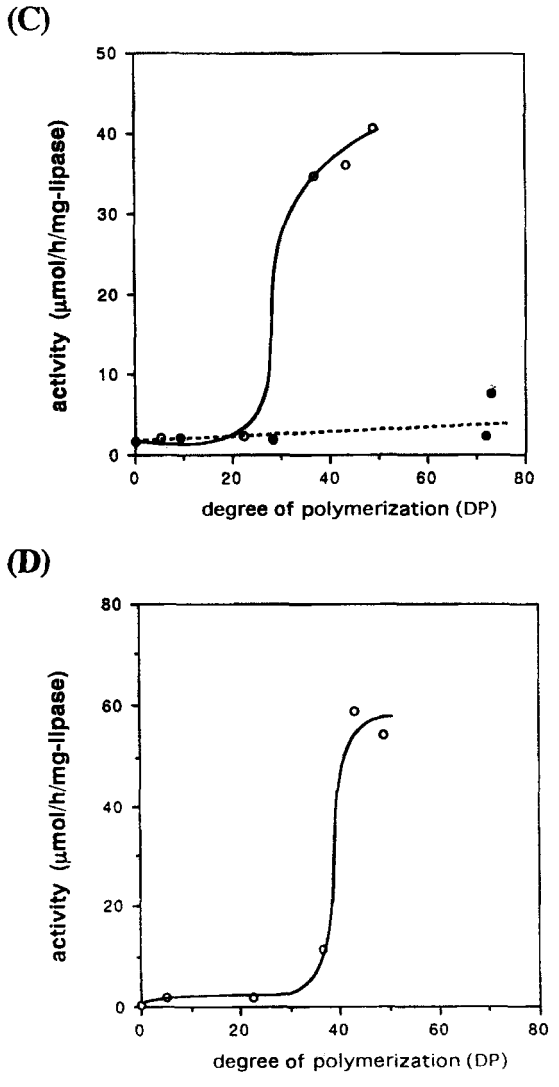


FIG. 2. Continued.

increasing degree of polymerization of the polymeric modifier. Since the unmodified lipase was active enough in moisturized isooctane, the main reason for the improved activity of **4b** is that the introduction of the hydrophobic polymer to the enzyme surface enhances the dispersibility of the hybrid enzyme in the solvent, and the dispersibility of the hybrid enzyme increased with increasing polymer content. In chloroform, however, the effect of the polymeric modifier, **2a** and **2b**, was hardly observed (Fig. 2B). On the other hand, both in benzene and in toluene, the activities of **4b** were dramatically enhanced when **2b** with DP > 40 was introduced to the enzyme. However, neither a significant relationship between DP with the activity nor a dramatic rate enhancement of the activity was observed in the series of **4a**

(Figs. 2C and 2D). This suggests that an appropriate amount of **2b** is required to stabilize the enzyme and to exhibit its esterification activity in these solvents.

Kinetic analysis of the esterification between *n*-amyl alcohol and caprylic acid by the unmodified lipase and **4b** in water-saturated benzene was carried out by changing the alcohol concentration of *n*-amyl alcohol from 0.1 to 0.9 M (Fig. 3). Although alcohol in high concentrations inhibited the reaction, the data obtained in low alcohol concentrations (<0.4 M) fitted well the Lineweaver–Burk plot (Fig. 4). Kinetics parameters V_{max} and K_m for unmodified lipase were 2.5 μmol/h and 0.87 M, respectively. V_{max} and K_m for **4b** were 54 μmol/h and 0.2 M, respectively. The above results of the difference of V_{max} suggest that the reaction site of the lipase moiety is strongly activated by chemical modification with **2b**.

Figure 5(A) shows plots of the esterification activity of these samples in water-saturated benzene against NM values. The activities were increased with increasing NM value in both series. Although the polymer content of **4b-4** was almost the same as that of **4b-8**, the activity of the latter was twice as high as the former. Obviously, the polymeric modifier with a higher molecular weight was more favorable than the modifier with a lower MW. It is considered that chemical modification of enzyme decreases the enzymatic activity, especially in the case of low MW modifiers, since the activity of the acetylated lipase (NM = 11) decreased to one-tenth as much as that of the native one in phosphate buffer. Probably low MW modifiers may react more readily with inside ϵ-amino groups and disrupt the hyperstructure of the enzyme.

The activities in water-saturated isooctane were decreased with increasing NM value in the series of **2b-2** modified samples. On the other hand, NM does not influence the activity of samples derived from **2b-4** (Fig. 5B). Although the modified polymer keeps water around an enzyme, which is indispensable for an enzyme to reveal its activity, the chemical modification of lysine residues partially destroys the hyperstructure of an enzyme. The negative effect may balance the positive one in the case of **2b-2** modification since the negative effect becomes significant as the

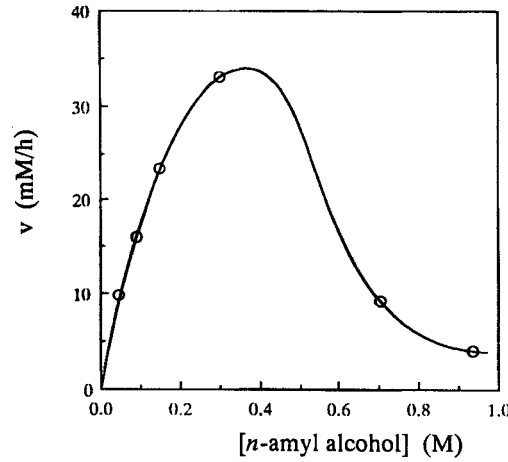


FIG. 3. Effect of alcohol concentration on the rate of esterification catalyzed by **4b** in water-saturated benzene at 40°C.

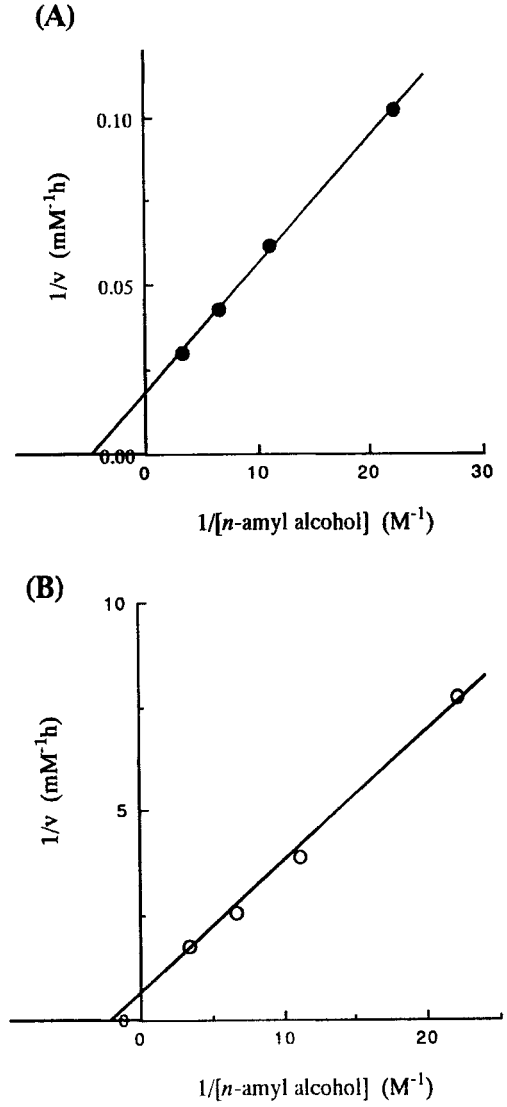


FIG. 4. Lineweaver-Burk plot for **4b** (A) and for unmodified lipase (B) in low alcohol concentration (< 0.4 M).

activity of the native lipase increases as in isooctane. It may balance the positive effect in the case of the **2b-2** modification as the positive effect becomes less dominating with the modifier having a lower molecular weight.

Thermal Stability of Modified Lipase

The thermal stabilities of lipase and the modified lapases were investigated by using PNP as substrate. The enzyme solution (0.11 mM in 0.05 M phosphate buffer, pH 7.0) was kept at a desired temperature for 10 minutes, then cooled rapidly to

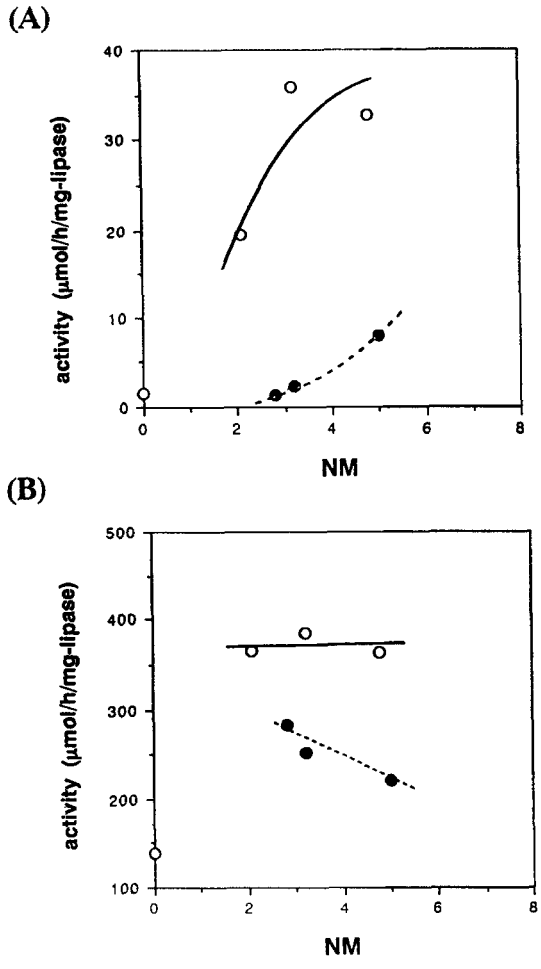


FIG. 5. Enzymatic esterification activity of **4b-2, 3, 4** (●) and **4b-6, 7, 8** (○) in water-saturated benzene (A) and isooctane (B) at 40 ± 0.01°C against the average number of polymer molecules per lipase (NM).

room temperature, and assayed as described in the Experimental Section at 30 ± 0.01°C. The results indicate that the lipase and **4b** begin to lose activity at about 55°C while the deactivations of **4a** and **4c** started at lower temperatures (Fig. 6). To detect the conformational changes of these samples at a selected temperature, DSC analysis of aqueous solutions (pH 7.0) of the samples was carried out. In every DSC chart a broad endothermal peak due to denaturation of the enzyme moiety of the sample was observed. The starting temperature of the denaturation of the lipase, **4a**, **4b**, and **4c** were 53, 46, 55, and 49°C, respectively. These results indicate that chemical modification with **2a** or **2c** reduces the thermal stability of the hyperstructure of lipase to some extent.

The thermal profiles of the unmodified lipase, **4b-7**, and **4c** were also investigated in water-saturated isooctane (Fig. 7). The temperatures of the maximum

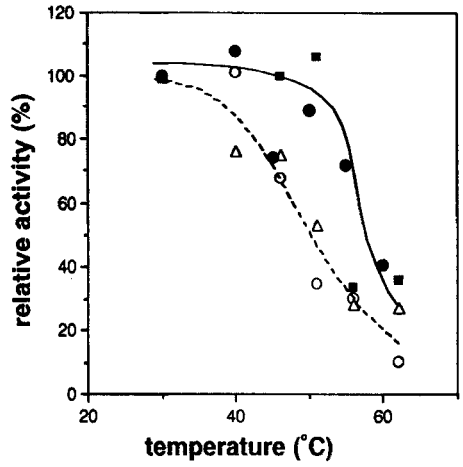


FIG. 6. Thermal stability of unmodified lipase (■), **4a-4** (△), **4b-7** (●), and **4c** (○) in aqueous solution containing 0.05 M phosphate pH 7.0. Samples were placed in a water bath of a specific temperature. After 10 minutes the samples were cooled to room temperature and assayed at 30 ± 0.01°C.

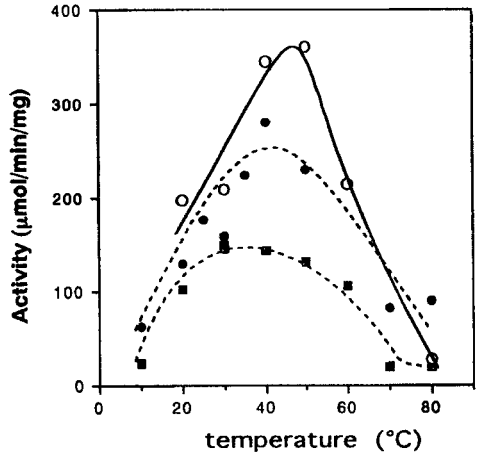


FIG. 7. Enzymatic activity of **4b-7** (●), **4c** (○), and native lipase (■) in water-saturated isooctane at various temperatures.

esterification activity (T_{max}) of **4b-7** and **4c** are higher than that of the native lipase. This indicates that modification with **2b-4** or **2c** results in a higher thermal stability than that of the native lipase.

REFERENCES

[1] A. Zaks and A. M. Klibanov, *J. Biol. Chem., 263*, 3194 (1988).
[2] A. Zaks and A. M. Klibanov, *Proc. Natl. Acad. Soc. USA, 82*, 3192 (1985).
[3] S. Fukui and A. Tanaka, *Annu. Rev. Microbiol., 26*, 145 (1982).
[4] Y. Okahata and K. Ijiro, *J. Chem. Soc., Chem. Commun.*, p. 1392 (1988).
[5] C.-H. Wong, S.-T. Chen, W. J. Hennen, J. A. Bibbs, Y.-F. Wang, J. L.-C. Liu, M. W. Pantoliano, M. Whitlow, and P. N. Bryan, *J. Am. Chem. Soc., 112*, 945 (1990).
[6] K. Takahashi, T. Nishima, Y. Saito, and Y. Inada, *Biochem. Biophys. Res. Commun., 121*, 261 (1984).
[7] M. W. Baillargeon and P. E. Sonnet, *J. Am. Oil Chem. Soc., 65*, 1812 (1988).
[8] M. Miyamoto, K. Naka, M. Shiozaki, Y. Chujo, and T. Saegusa, *Macromolecules, 23*, 1604 (1990).
[9] M. Kawase and A. Tanaka, *Enzyme Microbiol. Technol., 11*, 44 (1989).
[10] M. Miyamoto, K. Naka, M. Tokumizu, and T. Saegusa, *Macromolecules, 22*, 1604 (1989).
[11] N. Miyamoto, K. Naka, Y. Chujo, and T. Saegusa, Submitted to *Biocatalysis*.
[12] N. Tomizuka, Y. Ota, and K. Yamada, *Agric. Biol. Chem., 30*, 576 (1966).

Received March 5, 1996