Amidination of Lipase with Hydrophobic Imidoesters

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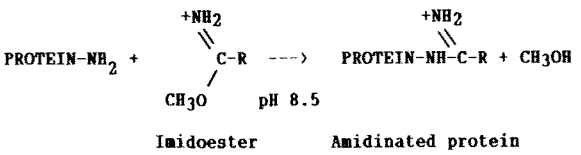
Lipase from *Candida rugosa* was chemically modified by amidination with imidoester hydrochlorides of different hydrophobicity. The modified enzyme showed a higher ester synthesis activity but a lower ester hydrolysis activity compared with the native enzyme. The maximum specific activity of the modified enzyme depended on its degree of derivatization. Benzene was found to be the best solvent for the synthesis reaction. The optimal temperature for the reaction was not affected by modification of the lipase. The modified lipase was more thermostable and solvent-stable than the native enzyme. When fatty acids of different carbon chainlength were tested as substrates in the synthesis of esters with the modified lipase, the highest activity was observed with myristic acid and propanol.

KEY WORDS: Amidination, ester hydrolysis, ester synthesis, lipase.

The alteration of native properties of enzymes used in biotechnological applications can be achieved by chemical modification (1,2). For example, the covalent attachment of hydrophobic residues to the free amino groups of enzymes enhanced their catalytic activity and stability (3), and this allowed them to be incorporated more readily and strongly onto liposome membranes (4) and hydrophobic supports (5). Such modification allowed them to perform better in a hydrophobic environment.

Modification of protein by amidination with imidoesters, as shown below,



offers several advantages over other approaches to amino group derivatization. The reaction conditions are relatively mild and the reaction is specific for the epsilon aminolysil and alpha-amino residues of proteins. The amidine linkages formed are stable over a wide range of experimental conditions (6). The derivatized protein retains its positive charges, thus minimizing any changes in its physical and biological properties related to total net charge (7). Imidoesters of different hydrophobicity are easily synthesized in high yield, and they are generally water soluble.

We reported recently that lipase from *Candida rugosa* modified with polyethylene glycol (8) and aldehydes (3) showed increased catalytic activity and stability in organic solvents at high temperature. For this paper, a study on the activity of lipase from *Candida rugosa* modified with several hydrophobic imidoesters was carried out.

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EXPERIMENTAL PROCEDURES

Lipase from *Candida rugosa* (Sigma, Type IV) was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Purification of lipase. The *Candida rugosa* lipase was purified by water extraction and gel filtration on a Superose 6 column with a Pharmacia Fast Performance Liquid Chromatography system (Pharmacia, Inc., Uppsala, Sweden). A twelve-fold increase in purity was obtained.

Synthesis of imidoesters. Imidoester hydrochlorides I (methyl acetimidate), II (methyl benzimidate), III (methyl 4-biphenyl benzimidate), IV (methyl n-dodecanimidate), V (methyl 3-phenylpropionimidate) and VI (methyl 4-phenylbutyrimidate), were synthesized from their corresponding nitriles according to the procedure of Hunter and Ludwig (6). All products were dried over silica gel and sodium hydroxide pellets *in vacuo*. High yields (95–98%) of imidoesters were obtained. The purity of the imidoesters was tested by melting point determination in the electrothermal digital melting point apparatus, H-NMR (nuclear magnetic resonance) studies in a Bruker WP 80SY NMR spectrophotometer with deuterodimethylsulphoxide-d_6 as solvent. Infrared (IR) studies were performed in a Beckman Acculab 7 infrared spectrophotometer (Beckman Co., Palo Alto, CA).

Amidination of lipase. Lipase was amidinated by the procedure adapted from Wofsy and Singer (9). The imidoester hydrochloride (0.1–0.3 g total) was added in several small increments (5–10 mg) at intervals of 10–20 min to a stirred solution of lipase in 0.1 M borate buffer (1% protein) at pH 8.5 and 0°C. After each addition, the pH was readjusted to 8.5 with 5 M NaOH. The reaction was terminated after 2 h by adjusting the pH to 7. All excess imidoesters and their insoluble hydrolysis products were removed by centrifugation. The samples were dialyzed at 4°C overnight, lyophilized in the cold and stored at −50°C prior to use. Lipase was derivatized to different degrees by varying the molar ratio of the imidoester with respect to the enzyme. A four-fold molar excess of the imidoesters to the free amino groups of the enzyme generally produced about 40–50% modification.

Protein assay. The amount of protein was determined by titration of the amino acids with trinitrobenzene sulfonate (TNBS) following the hydrolysis of the enzyme or its derivatives (10). The extent of protein modification was determined by comparing the number of amino acid groups that reacted with TNBS in the modified and unmodified protein (11).

Activity assay. The synthetic reaction system consisted of solvent (0.5 mL), propanol (1.0 mmol), fatty acid (0.35 mmol) and unmodified or modified lipase (5 mg). The mixture was incubated at 37°C for 24 h with continuous shaking at 200 rpm. The reaction was terminated by dilution with 3.5 mL of ethanol/acetone (1:1, vol/vol). The remaining free fatty acid was determined by titrating the mixture with 0.05 M NaOH with an automatic titrator (ABU 90, Radiometer, Copenhagen) to an end point of pH 9.5.

Specific activity was expressed as μmol free fatty acid/min/mg protein. Products of the synthetic reaction were also examined by thin-layer chromatography (TLC) on precoated silica gel plates (60 F_{254}, Merck, Darmstadt, Germany). The mobile phase consisted of petroleum ether, diethyl ether and glacial acetic acid (80:30:1, vol/vol/vol).

The hydrolytic reaction with olive oil emulsion was carried out as reported previously (8).

RESULTS AND DISCUSSION

The characteristics of the synthesized imidoesters are summarized in Table 1. Synthesis of the imidoesters from their corresponding nitriles was followed by the disappearance of the -C≡N stretch band (2260–2240 cm^{-1}) and the appearance of a -C=NH absorption band at 1600–1670 cm^{-1}. All of the imidoesters had a narrow melting point range, indicating a high degree of purity. The $-OCH_3$ protons of the imidoesters showed an H-NMR chemical shift of approximately 4.10 ppm. The yield was generally about 95–98%.

These imidoesters were extremely hygroscopic. Thus, they were kept in a vacuum desiccator over silica gel and NaOH pellets. They were quite stable when stored in this manner and could amidinate protein effectively even after six months of storage. The quality of imidoesters was tested periodically by determining their melting points, IR spectra and their ability to modify amino groups of bovine serum albumin. Because of their relatively short half-life [for example, methyl acetimidate has a half life of about 27 min at pH 8.0 and 25°C (6)], the imidoesters were added in small increments (about 10 mg) to the amidination mixture at 10–20-min intervals for about 2 h to ensure that they were present at all times to react with the lipase. Amidination was carried out at pH 8.5 because our initial investigation showed that lipase was relatively stable under such conditions (unpublished data).

Table 2 showed the synthetic and hydrolytic activities of the lipase modified with different imidoesters. The hydrolytic activity of the lipase was substantially reduced after derivatization. Our prior investigation showed that derivatization of the lipase did not alter its ability to catalyze the hydrolysis of both the primary and secondary esters of triacylglycerols (unpublished data). The synthetic activities of all the modified lipase preparations were higher than that of the native enzyme. Similar enhancement of the esterification activity of the lipase from *Candida cylindracea (rugosa)* was reported after modification of lysine residues with pyridoxal 5′-phosphate (12). Lipase that was modified with the high molecular weight imidoesters, such as III, IV, V and VI, showed a higher activity as compared with those modified with the low molecular weight imidoesters (I and II). Figure 1 shows a thin-layer chromatogram of the synthetic reaction of the

TABLE 1

Characteristics of Imidoesters

Imidoesters	R	m.p. (°C)[a]	H-NMR δ(s,OCH_3)[b] (ppm)	IR (-C=NH) (cm^{-1})
I	CH_3-	86–87	4.15	1640
II	C_6H_5-	122–123	4.12	1640
III	$(C_6H_5)_2$-	215–217	4.35	1600
IV	$CH_3(CH_2)_{10}$-	91–92	4.13	1640
V	$C_6H_5(CH_2)_2$-	71–72	4.12	1640
VI	$C_6H_5(CH_2)_3$-	106–107	4.08	1640

[a] Determined with an electrothermal digital melting point apparatus.
[b] Deuterodimethylsulphoxide-d_6 was used as a solvent.

TABLE 2

Activities of Modified and Unmodified Lipases

Imidoesters	% Modification[a]	Activity (%) Synthetic[b]	Activity (%) Hydrolytic[c]
—	0	100	100
I	41	314	30
II	45	362	7
III	40	374	7
IV	54	607	10
V	42	530	3
VI	40	656	2

[a] Determined with TNBS (ref. 11).
[b] The ester synthesis was followed by the rate of disappearance of oleic acid from the reaction mixture containing propanol and oleic acid as substrates in benzene. Titration of the remaining acid was with 0.05 M NaOH. Activity was expressed as % of the unmodified lipase activity.
[c] Activity was expressed as % of the hydrolysis of olive oil emulsion by the unmodified lipase. The rate of appearance of acid in the reaction mixture was followed by titration with 0.05 M NaOH.

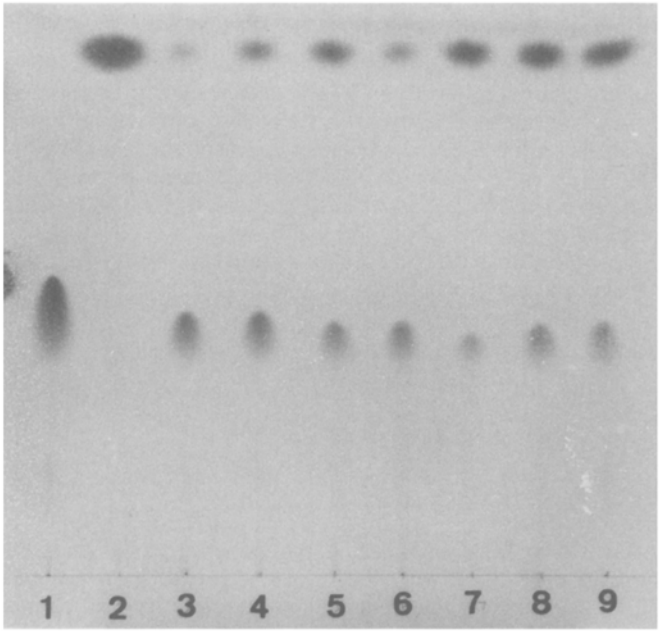

FIG. 1. A thin-layer chromatogram showing the synthesis of propyl oleate from oleic acid and propanol in benzene in the presence of native and modified lipases: 1, Heat-denatured lipase; 2, propyl oleate standard; 3, native lipase; 4, imidoester I lipase; 5, imidoester II lipase; 6, imidoester III lipase; 7, imidoester IV lipase; 8, imidoester V lipase; and 9, imidoester VI lipase. The reaction mixture contained 1.0 mmol propanol, 0.35 mmol oleic acid and 5 mg enzyme in 0.5 mL benzene. The mixture was incubated at 37°C, 200 rpm and 24 h.

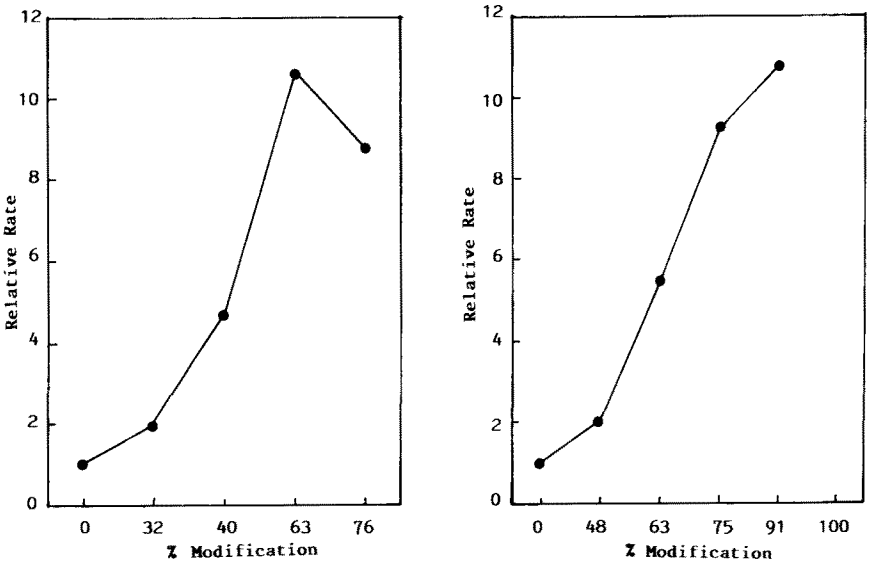

FIG. 2. Effect of increasing degree of modification on the esterification activity of lipase derivatized with imidoester VI (A) and imidoester I (B). The reaction conditions were as in Figure 1. Activity was expressed as the rate of disappearance of oleic acid from the reaction medium, followed by titration with 0.05 M NaOH to pH 9.5. Relative rate is calculated by dividing the specific activity of the modified lipase at the indicated degree of modification with the specific activity of the unmodified lipase.

different amidinated lipases. The ester, propyl oleate, was detected when either the modified or unmodified lipase was used as a catalyst. Because the degree of modification was similar for all of the modified enzymes, the higher activities of the preparations of the larger imidoesters are associated with increased hydrophobicity. Modification alters the microenvironment at or near the active site, facilitates the interaction of the substrates with the enzyme and the release of products. A similar finding was reported for alkylated lipase (3).

The effect of increasing the degree of lipase modification by methyl acetimidate hydrochloride (I) and methyl-3 phenylpropionimidate hydrochloride (VI) on ester synthesis activity was studied (Fig. 2). Maximal activity was shown to be dependent on the degree of modification by the respective imidoesters. To achieve maximal activity, a lower degree of modification (63%) was needed with the more hydrophobic imidoester VI as compared to imidoester I (about 90%). As the degree of modification with imidoester VI was further increased beyond 63.%, the modified enzyme showed a decrease in ester synthesis activity.

The activities of the amidinated lipases in various organic solvents of different polarities are listed in Table 3. The modified lipase preparations were active in all solvents tested. Their activities were higher in the less polar solvents, such as benzene, hexane and carbon tetrachloride. These findings, in agreement with those of Laane *et al.* (13), demonstrate that the esterification activities of lipases can be enhanced either by modification of the enzymes with the appropriate imidoesters or by changing the reaction media. Together, the above approaches can be used to generate a more active enzyme system to catalyze chemical transformations more efficiently.

TABLE 3

Specific Ester Synthesis Activity (μmol/min/mg protein) in Various Organic Solvents[a]

Solvent[b]	NL[c]	Amidinated lipase[d]					
		I	II	III	IV	V	VI
Benzene	0.59	1.86	2.13	2.21	3.58	3.12	3.87
Hexane	0.43	0.60	0.91	1.05	1.30	1.17	1.48
CCl_4	0.27	0.44	0.50	0.76	0.65	0.57	0.73
$CHCl_3$	0.39	0.56	0.65	0.74	0.60	0.65	0.73
CH_2CCl_3	0.05	0.11	0.26	0.28	0.18	0.22	0.26
DMF[c]	0.06	0.16	0.28	0.34	0.26	0.29	0.33
Pyridine	0.14	0.35	0.50	0.54	0.43	0.53	0.56

[a]The rate of disappearance of oleic acid from a reaction mixture containing propanol and oleic acid in benzene is followed by titration with 0.05 M NaOH. The specific ester synthesis activity is expressed as μmol acid reacted/min/mg protein.

[b]The organic solvents were dried over molecular sieve 3°A before use (ref. 16).

[c]NL, native lipase; DMF, dimethylformamide.

[d]The % modification of amidinated lipase is as in Table 2.

The optimal temperature (37°C) of the hydrolytic and synthetic reaction was not affected by modification of the lipase (Fig. 3). The stability of the native enzyme and lipase modified with imidoester I and VI incubated in benzene at room temperature over a period of eight days was investigated (Fig. 4). The modified lipases were more stable than the native lipase. The half-lives of the enzyme modified with imidoester I and VI were 5.6 and 6.3 days, respectively, while the native lipase had a half-life of only 3.7 days. In addition, modified lipase was more stable than

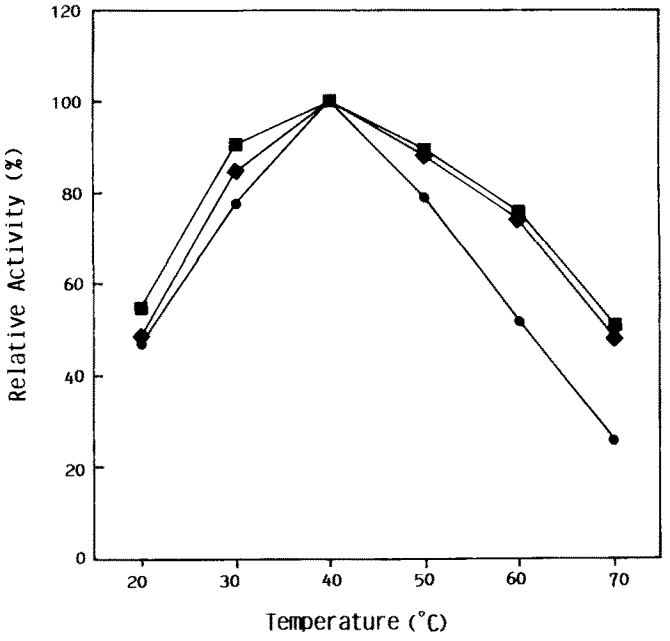

FIG. 3. Optimum esterification temperature of native lipase (— ● —) and lipase modified with imidoester I (—◆—) and imidoester VI (— ■ —). The reaction conditions and esterification activity determination were as in Figures 1 and 2.

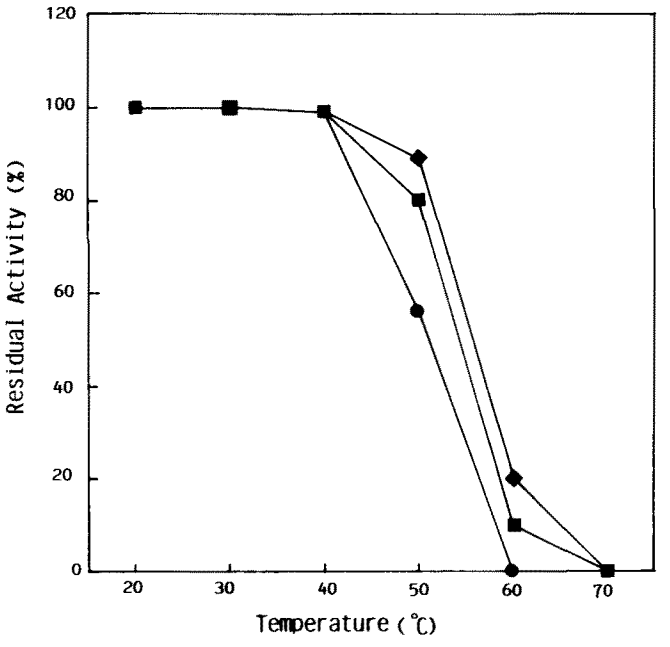

FIG. 5. Thermostability of native lipase (— ● —) and lipase modified with imidoester I (— ■ —) and imidoester VI (—◆—). Samples were incubated at various temperature and the activity was determined after 1 h. Details of the reaction conditions and activity determination were as in Figures 1 and 2.

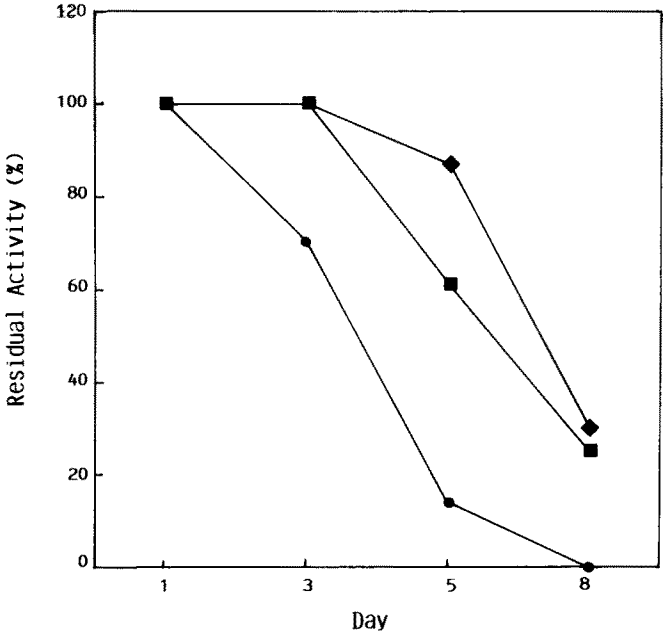

FIG. 4. Stability of native lipase (— ● —) and lipase modified with imidoester I (— ■ —) and imidoester VI (—◆—) in benzene incubated at room temperature for various time intervals. The reaction conditions and activity determination were as in Figures 1 and 2.

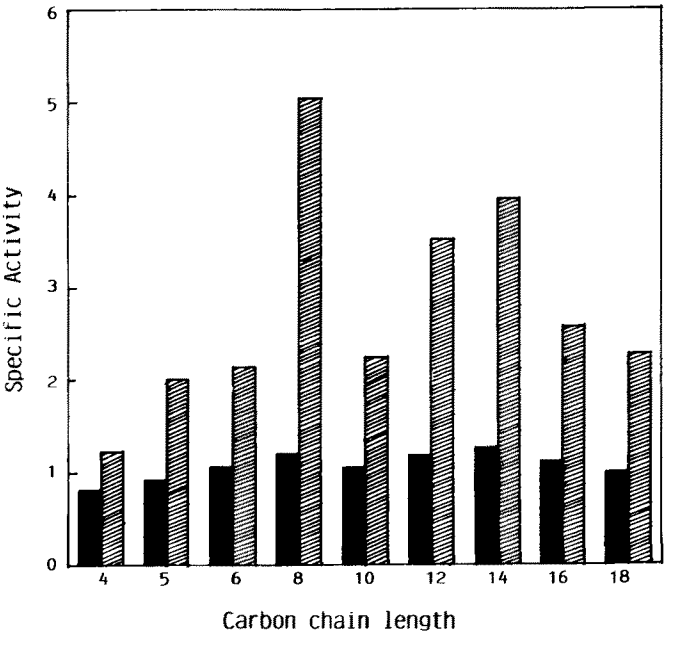

FIG. 6. Fatty acids specificity of native lipase (closed box) and lipase modified with imidoester VI (striped box). Details of the reaction conditions and activity determination were as in Figures 1 and 2.

native lipase when incubated for 1 h at temperatures from 20 to 70°C (Fig. 5). Similar findings were previously reported by Ampon *et al.* (3). Studies by Volkin *et al.* (14) showed that chemical modification of the lysine residues of ribonuclease was found to increase heat stability due to a decrease in the intermolecular crosslinking caused by transamidation and intermolecular disulfide interchange. This crosslinking was shown to cause thermal aggregation, which correlated with the loss of enzymatic activity.

The effect of varying the carbon chainlength of the fatty acid used as a substrate is shown in Figure 6. The activity trends of the modified and native lipases on the fatty acid

substrates were similar, but the specific activity of the modified lipase was considerably higher. The specific activity of the modified enzyme was highest when caprylic acid (C_8) was used as the substrate, followed by myristic acid (C_{14}) and lauric acid (C_{12}). Similar studies on the synthesis of oleyl esters by Okumura *et al.* (15) with microbial lipases showed that the lipases from *Aspergillys niger* and *Rhizopus delemar* showed no selectivity with regard to the type of fatty acids used. The lipases from *Geotrichum candidum* and *Penicillium cyclopium* synthesized oleyl esters only from medium- or long-chain fatty acids.

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