Enzymic Synthesis of Fatty Esters by Hydrophobic Lipase Derivatives Immobilized on Organic Polymer Beads

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ABSTRACT: Lipase from *Candida rugosa* was modified with several hydrophobic modifiers before being adsorbed onto organic polymer beads. The effects of different enzyme modifiers, supports, solvents, reaction temperatures, fatty acids, and alcohols on the activity of the immobilized enzyme were investigated. The immobilized lipases were good biocatalysts for esterification reactions in organic solvents. They exhibited high activities in all solvents tested, including polar solvents. The activity seemed to depend on the type of support rather than on the modifier of the enzyme. The medium polar support, XAD7, appeared to be the best for the modified lipases. The immobilized lipase favored the medium-chain fatty acids rather than the long-chain fatty acids as acyl donors. The alcohol selectivity of the enzyme was unchanged upon immobilization. The native and immobilized lipases favored the short-chain and terpene alcohols as nucleophiles.

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Many novel ideas for using lipases to produce oleochemicals from fats and oils have been investigated *via* hydrolysis, ester synthesis, and interesterification reactions (1). Esters, the main products of esterification reactions, can be used for a diverse range of applications. Monoglycerides, glycolipids, phospholipids, and alkyl esters can be used in emulsification of foods, feeds, pharmaceuticals, and personal hygiene products. Alkyl esters and terpene esters, such as ethyl butyrate, citronellyl butyrate, and geranyl butyrate, are used in the flavor and fragrance industries for food, feed, and perfumes.

The increasing emphasis on the use of biocatalysts for their favorable properties, such as their mild and environmentally friendly reactions and their high specificities, has also resulted in increasing use of immobilized enzymes. Immobilization of an enzyme facilitates its recovery for possible reuse and continuous application, and improves its stability, as well (2). In addition, the interactions between the support and the enzyme may favorably alter the chemical or physical properties of the enzyme, and often a new property is generated that can be exploited for industrial purposes.

Recently, we described a novel method for immobilizing lipase onto polymer beads (3). It consisted of attaching various hydrophobic groups onto the enzyme molecule (4–6) and then adsorbing the hydrophobic enzyme derivatives thus formed onto several organic polymers. In this article, the immobilized lipases prepared in this manner were investigated by following their esterification reactions. Their activities in various organic solvents were examined with respect to the different types of modifiers and polymer beads used. The effects of temperature and different types of fatty acids and alcohols on the activity of the immobilized enzyme were also investigated.

EXPERIMENTAL PROCEDURES

Materials. Lipase from *Candida rugosa* (Type IV), Amberlite XAD2, and XAD7 were obtained from Sigma Chemical Co. (St. Louis, MO). The polycarboxylic acid (RCOOH) was prepared in our laboratory by hydrolyzing poly(ethyl acrylate–divinyl benzene) (20 g) in 100 mL sodium hydroxide (10 M) for 6 h at 95°C. Poly(ethyl acrylate–divinyl benzene) was synthesized from 120 mL ethyl acrylate and 12.5 mL divinyl benzene solution by suspension polymerization at 85°C. All polymer beads were washed with distilled water until no chloride was detected, and then dried overnight at 40°C before being used for immobilization. All other reagents were of analytical grade. The organic solvents and substrates were dried over molecular sieve 3Å before use.

Purification of lipase. Commercial lipase from *C. rugosa* (2 g) was dissolved in distilled water (10 mL). The mixture was stirred at 4°C for 1 h and centrifuged at 12,000 rpm for 10 min. The supernatant (5 mL) was passed through a permeation chromatography column, Superose 6 on a Pharmacia fast performance liquid chromatography system (Pharmacia, Uppsala, Sweden). About 11–12-fold purification and an overall yield of 60% were obtained.

Modification of lipases. Purified lipase was modified with monomethoxy–polyethylene glycol of molecular weight 1900

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(PL1900) as described by Basri *et al.* (4). Reductive alkylation of lipase with acetaldehyde (AL) was as described by Ampon *et al.* (5). Amidination with methyl 4-phenylbutyrimidate [IL(VI)] was according to the method of Wofsy and Singer (7). The amount of modifier added to the enzyme was adjusted so that each derivatized enzyme was modified to a similar extent (40–50% modification).

Immobilization of lipase. The lipase solutions (1% wt/vol, 10 mL) and polymer beads (1 g) were mixed at room temperature by shaking at 100 rpm in a sealed vial for 1 h. The beads were separated from the supernatant by filtration through Whatman no. 1 filter paper. They were then washed by resuspending and filtering three times each with 20 mL of buffered 1 M KCl, pH 7 and 50% ethylene glycol, and once with 20 mL distilled water. The beads were lyophilized, kept in sealed vials, and stored at 0°C prior to use. The polymer beads used for immobilization of the enzyme were Amberlite XAD2 (nonpolar), Amberlite XAD7 (medium polar), and polycarboxylic acid, RCOOH (polar).

Protein assay. The amount of protein was determined by titration with trinitrobenzene sulfonate (TNBS) of the amino acids produced after hydrolysis of the enzyme or its derivatives (8). For the immobilized enzyme, 0.5-g samples were suspended in 2 mL of 6 M HCl. Each sample was then sealed inside an evacuated glass tube and heated for 24 h at 110°C. The cooled samples were then filtered by passage through a plug of glass wool, and the soluble fractions were subjected to reaction with TNBS. 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 (9).

Activity assay. The reaction system consisted of hexane (0.5 mL), propanol (2.67 mmol), oleic acid (0.35 mmol), and immobilized lipase (0.3 g). The mixture was incubated at 28°C (unless otherwise stated) for 5 h with continuous shaking at 150 rpm in a horizontal shaker waterbath. The reaction was terminated by dilution with 3.5 mL of ethanol/acetone (1:1, vol/vol), and the remaining free fatty acid in the reaction mixture was determined by titration with 0.05M NaOH in an automatic titrator (ABU 90; Radiometer, Copenhagen, Denmark) to an endpoint of pH 9.5. Specific activity of the enzyme is expressed as μmol/min/mg protein. Relative activity (%) was expressed as activity of the enzyme in various organic solvents compared to its activity in hexane (unless otherwise stated).

RESULTS AND DISCUSSION

Esterification by immobilized lipases. The results of the esterification reactions with the immobilized lipases are shown in Table 1. Immobilization of enzyme onto polymer beads showed increased esterification activity compared to free enzyme. The amount of bound protein ranged from 7.5 to 24.3 mg/g polymer. Immobilization of native lipase on XAD7 polymer beads showed a 105-fold increase in esterification activity. Immobilization of modified lipase, however, showed between 168- to 184-fold activation, which was much higher than that observed for immobilization of native enzyme. When the same modified lipase (PL) was immobilized onto several types of polymer beads, those adsorbed to XAD7 exhibited the highest esterification activation (184 ×), followed by RCOOH (169 ×), and XAD2 (127 ×).

TABLE 1
Esterification Activities of Immobilized Lipases[a]

Immobilized lipases	Bound protein (mg/g carrier)	Esterification activity	Activation factor[b]
Purified lipase	—	0.43	1
PL	—	1.29	3
XAD7NL[c]	20.1 ± 1.44	45.17 ± 3.51	105
XAD7PL	24.3 ± 2.98	79.42 ± 3.01	184
XAD7IL	22.2 ± 1.24	72.97 ± 2.98	170
XAD7AL	20.6 ± 0.98	72.33 ± 4.23	168
XAD2PL	7.5 ± 1.65	54.67 ± 3.35	127
RCOOHPL	21.9 ± 2.13	72.60 ± 3.89	169

[a]Mean value ± standard deviation based on three samples. The activities are expressed as μmol/min/mg protein. The ester synthesis is followed by the rate of disappearance of oleic acid from the reaction mixture containing propanol and oleic acid. The percent modification of the modified enzymes were: PL1900, 45%; IL (VI), 40%; and AL, 40%; lipase modified with monomethoxypolyethylene glycol (PEG1900)—PL1900; lipase modified with acetaldehyde—AL; lipase modified with methyl 4-phenylbutyimidate—IL (VI); NL—native lipase or unmodified lipase (purified lipase).

[b]Activation factor against free lipase.

[c]Immobilized directly to the support.

Unlike many other immobilized lipase preparations reported to date (10), immobilization of hydrophobic lipase reported here showed considerable increase in esterification activity in organic solvents. This is presumably due to the increased hydrophobicity of lipase and its hydrophobic milieu (1). The local concentration of hydrophobic substrates around the enzyme may be higher and more accessible to the active site. Furthermore, the diffusion of hydrophobic substrates is not restricted, because the enzyme modifiers are both hydrophobic and flexible in organic solvents.

Esterification activity in the presence of organic solvents. The activities of the different modified lipases adsorbed onto XAD7 in various organic solvents are shown in Table 2. The immobilized lipases can catalyze ester synthesis in all organic solvents investigated. Their activities were generally high in the nonpolar solvents (log $P > 2$) (11) such as benzene, hexane, and carbon tetrachloride. However, the activity of the immobilized lipase in DMF (log $P < 2$) was also high, whereas in pyridine (log $P < 2$) the activity was considerable.

The activities of the PL1900 preparation, which was adsorbed onto different supports in various organic solvents, are also shown in Table 3. They can also catalyze ester synthesis efficiently in the organic solvents tested.

The high esterification activities in all solvents tested may be due to the increased stability of the enzyme upon immobilization (12). Although the more polar solvents tend to strip water from the enzyme molecule (13), the supports may trap and prevent the disruption of the enzyme-bound water essen-

TABLE 2
Esterification Activities of XAD7 Immobilized Lipases in Various Organic Solvents

		Activity[a] of immobilized lipases (%)[b]				
Solvent	Log P[c]	Purified lipase	PL1900	XAD7PL1900	XAD7IL(VI)	XAD7AL
Hexane	3.5	0.43 (100)	1.29 (100)	79.42 (100)	72.97 (100)	72.33 (97)
CCl_4	3.0	0.27 (63)	0.81 (63)	72.43 (91)	42.79 (59)	71.85 (99)
Benzene	2.0	0.59 (137)	1.77 (137)	79.41 (100)	72.97 (100)	74.76 (103)
$CHCl_3$	2.0	0.39 (91)	1.17 (91)	46.09 (58)	41.44 (57)	62.62 (95)
CH_2CCl_3	na[d]	0.05 (12)	0.15 (12)	46.50 (59)	36.49 (50)	61.17 (85)
Pyridine	0.7	0.14 (33)	0.42 (33)	41.98 (53)	31.08 (43)	34.95 (48)
DMF[e]	−1.0	0.06 (14)	0.18 (14)	72.84 (92)	45.95 (63)	52.91 (73)

[a]Activities are expressed as *mmol/min/mg* protein. The ester synthesis is followed by the rate of disappearance of oleic acid from the reaction mixture containing propanol and oleic acid. The % modification of the *modified enzymes* were as in Table 1.

[b]Percent activities are expressed as a percentage of the esterification activity of immobilized lipase in hexane.

[c]From Laane *et al.* (Ref. 11).

[d]Not available.

[e]DMF, dimethylformamide.

TABLE 3
Esterification Activities of PL1900 Immobilized on Three Types of Polymer Beads in Various Organic Solvents

		Activity[a] of immobilized lipases (%)[b]		
Solvent	Log P[c]	XAD2PL1900	XAD7PL1900	RCOOHPL1900
Hexane	3.5	54.67 (100)	79.42 (100)	72.60 (100)
CCl_4	3.0	53.33 (100)	72.43 (91)	71.23 (61)
Benzene	2.0	53.33 (98)	79.42 (100)	72.60 (100)
$CHCl_3$	2.0	44.00 (81)	46.09 (58)	63.93 (88)
CH_2CCl_3	na[d]	45.33 (83)	46.50 (59)	57.08 (79)
Pyridine	0.7	38.67 (71)	41.98 (53)	44.75 (62)
DMF	−1.0	41.33 (76)	72.84 (92)	53.88 (74)

[a]Activities are expressed as μmol/min/mg protein. The ester synthesis is followed by the rate of disappearance of oleic acid from the reaction mixture containing propanol and oleic acid. The percent modification was as in Table 1. Abbreviation as in Table 2.

[b]Percent activities are expressed as a percentage of the esterification activity of immobilized lipase in hexane.

[c]From Laane *et al.* (Ref. 11).

[d]Not available.

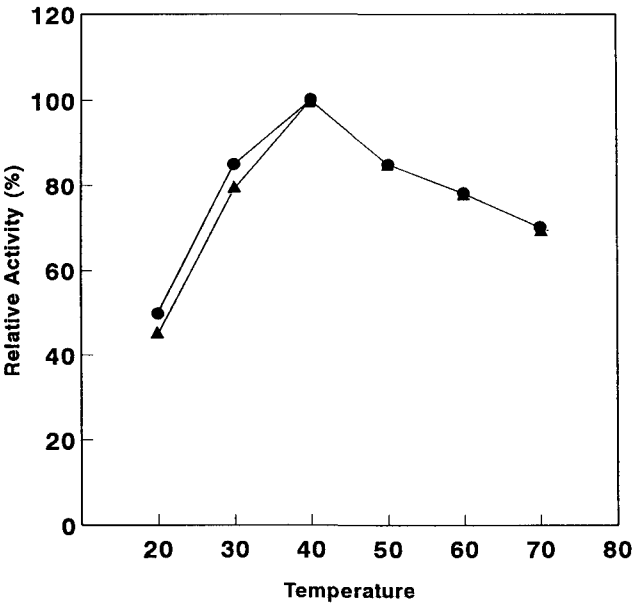

FIG. 1. The effect of temperature on the esterification reaction by immobilized lipase. Symbols: Purified lipase (▲), XAD7PL (●).

tial to maintain the three-dimensional structure of the enzyme for catalysis. In our experiment, the activity of the immobilized lipase in all solvents seemed to depend on the type of support rather than on the modifiers used. The enzyme immobilized on polar organic polymer beads seemed to exhibit higher activities when compared to preparations immobilized on less polar supports. This may be due to their water-retaining ability, although the identity of the solvent also plays a role in the reaction.

The effect of temperature on esterification activity. The effect of temperature on the esterification reaction of immobilized lipases is shown in Figure 1. Modification of the enzymes did not alter the optimum temperature (40°C) of the reaction. This trend was also observed with trypsin immobilized by diazo binding with amino acid copolymer (14) and with other examples of immobilized enzymes (15).

Fatty acid selectivity. The effect of increasing the carbon chainlength of the fatty acids used as substrates is presented

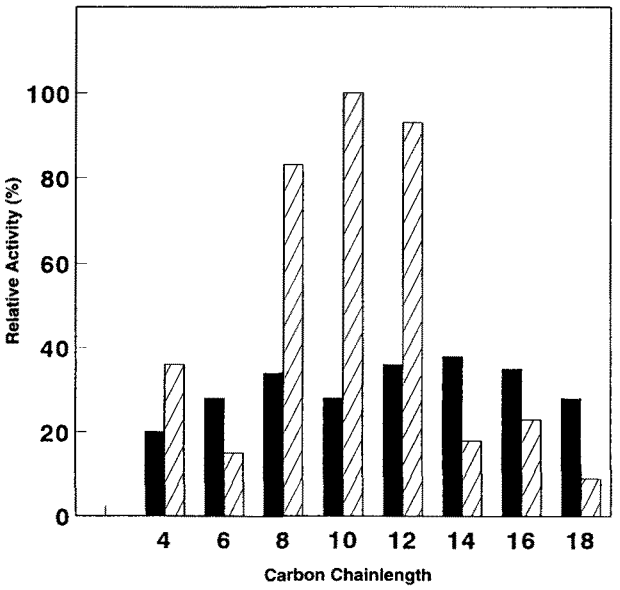

FIG. 2. The effect of chainlength of fatty acids as acyl donor in the esterification reaction by immobilized lipase. Symbols: purified lipase (black box), XAD7PL (striped box).

TABLE 4
Alcohol Selectivity in the Esterification Reaction by Immobilized Lipase

	Activity[a] lipases (%)	
Alcohol	Purified lipase	XAD7PL
Methanol	14.60 ± 1.22	23.47 ± 1.45
Ethanol	29.21 ± 0.89	21.43 ± 1.38
1-Propanol	60.91 ± 1.89	62.85 ± 1.14
1-Butanol	100.00 ± 1.44	100.00 ± 1.26
1-Pentanol	63.45 ± 2.01	69.46 ± 1.83
1-Hexanol	47.34 ± 1.65	59.18 ± 1.92
1-Heptanol	48.02 ± 1.32	63.88 ± 1.63
1-Octanol	35.38 ± 1.22	65.75 ± 1.21
1-Nonanol	36.59 ± 1.53	60.19 ± 2.34
1-Decanol	36.49 ± 0.98	67.92 ± 1.75
1-Undecanol	31.71 ± 1.18	68.99 ± 1.27
1-Dodecanol	40.10 ± 1.24	50.11 ± 1.18
3-Methoxy-1-propanol	25.91 ± 2.12	46.98 ± 1.95
3-Butoxy-1-propanol	30.12 ± 1.87	42.97 ± 1.56
N-Methylpropanol	33.43 ± 1.89	46.93 ± 1.98
N-Ethylpropanol	34.81 ± 1.74	48.23 ± 2.03
3-Bromopropanol	3.24 ± 1.32	4.11 ± 1.65
3-Chloropropanol	4.91 ± 1.56	5.14 ± 1.48
Geraniol	73.67 ± 1.67	81.63 ± 1.25
Citronellol	79.61 ± 1.81	89.93 ± 1.89
Farnesol	77.69 ± 1.93	100.00 ± 1.37

[a]Relative activity is calculated by dividing the specific activity of the enzyme in the esterification reaction for the indicated alcohol as the nucleophile with the specific activity of the enzyme with butanol as the nucleophile (mean value ± standard deviation based on five samples). The percent modification was as in Table 1.

in Figure 2. The specific activity of the immobilized enzyme was highest when capric acid (C_{10}) was used as the substrate, followed by lauric acid (C_{12}) and caprylic acid (C_8). Generally, free lipase favored the esterification of fatty acids with a chainlength above 12 (6,16,17). Upon immobilization, the lipase favored fatty acids with medium chainlength (C_8–C_{12}). This could be due to the fact that the more rigid nature of the immobilized enzyme might restrict the longer substrates from reaching the active site. A similar observation had been reported by Cabezas *et al.* (18), who showed that immobilization of α-chymotrypsin with a hydrophobic polymer altered the substrate specificity of the enzyme.

Alcohol selectivity. Immobilization did not alter the alcohol selectivity of the enzyme (Table 4). Both lipases showed moderately high activities when longer-chain alkyl alcohols and alcohols with electron-donating groups were used as nucleophiles. High activities were exhibited when butanol and terpene alcohols were used as the nucleophiles. Reports by Hills *et al.* (19) showed that butanol and pentanol were esterified most efficiently, and alcohols with longer chains were esterified more slowly by lipase from oilseed rape. The electron-donating alcohols 3-methoxy-1-propanol, 3-butoxy-1-propanol, *N*-methylpropanol, and *N*-ethylpropanol served as better nucleophiles when compared to electron-withdrawing alcohols (3-bromopropanol and 3-chloropropanol).

The mode of action of fatty acid and alcohol selectivity studies can be explained by the mechanism of the lipase reaction as reported by Pan *et al.* (20): An acyl donor attacks lipase and forms an acyl–enzyme, then an alcohol reacts with the acyl–enzyme to yield an ester. The studies suggest that the acylation process is affected by immobilization, and the deacylation process of the acyl–enzyme intermediate was not changed by immobilization. The high activities exhibited when terpene alcohols were used has potential in the field in biotransformation because terpene esters are in great demand in the cosmetic industries.

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