Characterization of a Chemically Conjugated Lipase Bioreactor

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Lipase from *Candida cylindracea* was immobilized on glass beads using the biospecific and high-affinity avidin−biotin interaction. Biotinylated lipase and glass beads were prepared by reactions of lipase and 3-aminopropyl glass beads with sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin). Avidin and biotinylated lipase were sequentially adsorbed to the biotinylated glass beads. Biotinylated lipase in solution retained about 63% of the hydrolytic specific activity of native lipase when an average 3 mol of biotin was incorporated/mol of lipase. Nonporous glass beads contained more biotin and protein (avidin and lipase) per unit of surface area, followed by 302 nm mean pore diameter controlled-pore glass beads (CPG-3000) and 198 nm mean pore diameter controlled-pore glass beads (CPG-2000). The hydrolytic specific activity of lipase immobilized on CPG-3000 and on nonporous beads was essentially the same as that for the biotinylated free enzyme, whereas that immobilized on CPG-2000 was about 50% less. The long spacer of NHS-LC-biotin (22.4 Å maximum length) and avidin (70 Å diameter) reduced steric hindrances with emulsified substrates on the matrix surface, resulting in a higher hydrolytic activity as compared with lipase immobilized via covalent linkages. The interesterification activity was 4-fold greater for immobilized lipase than for free lipase.

Keywords: *Biotinylated lipase; immobilized lipase; immobilized avidin; interesterification*

INTRODUCTION

Triacylglyerol hydrolases (EC 3.1.1.3), also known as neutral lipases, catalyze the hydrolysis of ester linkages in neutral lipids with the resulting release of the free fatty acids and glycerol. Hydrolysis of triacylglyerols by lipases is a reversible reaction; hence, the direction of the lipase-catalyzed reaction depends on the water content in the reaction mixture. By using organic solvents and limiting water content in the reaction medium, lipase-catalyzed hydrolysis of lipids can be minimized and interesterification becomes the dominant reaction (Macrae, 1983; Sonnet *et al.*, 1986; Lilly and Dunnill, 1987; Chulalaksananukul *et al.*, 1990; Elliott and Parkin, 1991; Lie and Molin, 1991; Miller *et al.*, 1991; Haas *et al.*, 1993, 1995; Triantafyllou *et al.*, 1993; Valivety *et al.*, 1994). Microbial lipase-catalyzed hydrolysis and interesterification have been used to produce valuable flavor compounds, lipids with improved functional properties, and racemic compounds (Sreenivasan, 1978; Macrae, 1983; Neidleman and Geigert, 1984; Kirchner *et al.*, 1985; Harwood, 1989).

Advantages of using immobilized enzymes include (1) ease of automation and continuous processing, (2) precise control of the extent of reaction without a downstream enzyme inactivation step, and (3) reusability of the enzyme, thereby reducing the production cost (Swaisgood and Horton, 1989). Lipases have been immobilized by acetone precipitation (Macrae, 1983; Wisdom *et al.*, 1984), adsorption (Chulalaksananukul *et al.*, 1990; Lie and Molin, 1991), covalent linkage (Lieberman and Ollis, 1975; Stark and Holmberg, 1989), entrapment (Yokozeki *et al.*, 1982), and microencapsulation (Braun and Olson, 1986a,b). Immobilization via noncovalent interactions is relatively easy, but the enzyme does not have long-term processing stability due to slow desorption. On the other hand, covalently immobilized enzymes are very stable but complicated chemical modifications and derivatizations are usually required.

Avidin, a basic glycoprotein with a molecular weight of 67 000 (Meland and Green, 1963), exhibits an affinity for biotin that is the one of the strongest known noncovalent biological interactions ($K_D = 10^{-15}$ M; Green, 1975). The interaction between avidin and biotin is resistant to strong denaturing reagents, such as 9 M urea and 3 M guanidinium chloride, extremes of pH and temperature, and organic solvents (Green, 1963b). Because only the bicyclic rings of biotin are required for avidin binding (Green, 1963a, 1975), the carboxyl group of the valeric acid side chain can be modified to react with specific functional groups of proteins. To reduce steric hindrance at the biotin-binding site of avidin and to improve accessibility of the biotinylated enzymes to their substrates, a longer spacer arm has been incorporated on the valeric acid side chain of biotin. The stability of avidin−biotin interaction in organic solvents is another advantage for enzyme immobilized via this biospecific adsorption because many lipase-catalyzed reactions are conducted in organic solvents.

In this study, *Candida cylindracea* lipase was immobilized on controlled-pore glass beads of various pore sizes using an avidin−biotin spacer for biospecific adsorption and by covalent attachment to a succinamidopropyl chain. The effects of the pore size and the avidin−biotin spacer on lipase-catalyzed triacylglycerol hydrolysis and interesterification were determined.

MATERIALS AND METHODS

Derivatization of Glass Beads. Controlled-pore glass beads with a particle size of 120/200 mesh and mean pore diameters of 198 or 302 nm (CPG-2000 and CPG-3000, respectively) were obtained from CPG Inc. (Fairfield, NJ). Nonporous glass beads with a mean particle diameter of 106

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μm were purchased from Sigma Chemical Co. (St. Louis, MO). The beads were derivatized according to the methods of Janolino and Swaisgood (1982). After the beads were cleaned in concentrated nitric acid at 100 °C for 1 h and washed with distilled water, silanization was achieved by adding 10% (v/v) (3-aminopropyl)triethoxysilane, adjusted to pH 4, heating at 70 °C for 3 h, decanting the liquid by filtration, and drying overnight at 110 °C. Fines were removed by washing the beads with a large volume of distilled water. After another drying at 110 °C, the presence of amino groups on the surface of the glass beads was determined by adding 1 mL of 0.1 M sodium tetraborate and 3 drops of 3% 2,4,6-trinitrobenzenesulfonate (TNBS) to 1 mg of beads. A yellow color after a 2-h incubation indicated the completion of silanization.

The aminopropyl glass beads were succinylated by adding 4 volumes of 10% (w/v) succinic anhydride and 1% (v/v) triethylamine in acetone to the beads and allowing reaction to proceed at room temperature. Completion of succinylation was determined by a white appearance of the beads in the TNBS test. Succinamidopropyl beads were washed extensively with acetone and dried at 70 °C.

Preparations of Avidin−Biotin Glass Beads. Sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) (Pierce, Rockford, IL) was dissolved in 50 mM sodium bicarbonate buffer, pH 8.5, and the NHS-LC-biotin solution was circulated through the aminopropyl glass beads at 4 °C overnight. The beads were successively washed with 2 M urea, 2 M NaCl, and finally 50 mM sodium phosphate, pH 7, containing 0.02% sodium azide as a preservative. The amount of biotinylation of the glass beads was determined by a specific binding method (Green, 1965). Briefly, a small amount of the biotinylated glass beads was added to a mixture composed of 3 mL of avidin (0.5 mg/mL in 50 mM sodium phosphate, pH 6, containing 0.9% NaCl) and 75 μL of 2-(4′-hydroxyazobenzene)benzoic acid (HABA, 10 mM dissolved in 10 mM NaOH). A decrease of absorption at 500 nm was observed when avidin-bound HABA was displaced by biotin. The amount of biotin on the glass beads was calculated by comparing the absorption changes with a standard curve generated by known quantities of pure avidin, biotin, and HABA. The biotinylated glass beads were stored at 4 °C.

Avidin (4 mg/mL) in 50 mM sodium phosphate buffer, containing 0.9% NaCl, pH 6, was circulated through the biotinylated glass beads at 4 °C overnight. Unbound avidin was removed by washing the beads successively with 2 M urea, 2 M NaCl, and finally 50 mM phosphate buffer containing 0.02% sodium azide, pH 7. The avidin−biotin glass beads were stored at 4 °C.

Biotinlyation of *C. cylindracea* Lipase. *C. cylindracea* lipase, obtained from Sigma, was dissolved in 50 mM sodium bicarbonate, pH 8.5, and filtered through Whatman No. 1 filter paper to remove any undissolved cell debris. The enzyme concentration was determined by a Coomassie Blue-binding method (Bradford, 1976) with bovine serum albumin as a standard. NHS-LC-biotin was added to the lipase solution, and the mixture was incubated at 4 °C for 2 h. After the incubation, unbound biotin was removed by centrifugation using an Amicon Centricon 30 ultrafiltration membrane with a molecular weight exclusion of 30 000. The extent of biotinylation of the lipase was determined by the HABA test as previously described for biotinylated glass beads. Before a small aliquot of biotinylated lipase was added to the avidin−HABA reaction mixture for assay of the bound biotin, the biotinylated lipase was heated at 56 °C for 10 min and digested with 1% Pronase overnight to prevent any steric hindrance of the avidin−biotin interaction.

Immobilization of Lipase via Biospecific Adsorption. Biotinylated *C. cylindracea* lipase was circulated through the avidin−biotin glass beads at 4 °C overnight. After immobilization, unbound lipase was removed by successively washing the beads with 2 M urea, 2 M NaCl, and finally 0.2 M Tris buffer, pH 8. The immobilized lipase were stored at 4 °C with 0.02% sodium azide as a preservative.

Immobilization of Lipase via Covalent Linkage. Lipase was immobilized covalently using the sequential activation/immobilization procedure (Janolino and Swaisgood, 1982). The succinamidopropyl glass beads were incubated with 0.1 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) dissolved in 0.2 M sodium phosphate, pH 4.75, under continuous stirring with bubbling nitrogen gas. After a 30-min incubation, the EDC-activated beads were rapidly washed with ice-cold 47 mM sodium phosphate, pH 7, and then immediately *C. cylindracea* lipase solution, which had been filtered through Whatman No. 1 paper and adjusted to a final concentration of 5 mg/mL in 50 mM sodium phosphate buffer, pH 7, was circulated through the EDC-activated beads at 4 °C overnight. Unbound lipase was removed by successively washing the beads with 2 M urea, 2 M NaCl, and finally 0.2 M Tris-HCl buffer, pH 8. The immobilized lipase was stored at 4 °C with 0.02% sodium azide as a preservative.

Determination of Lipase Hydrolytic Activity. The hydrolytic activity of *C. cylindracea* lipase was determined by titration of free fatty acids released from an olive oil substrate. Three milliliters of 50% olive oil emulsion (Sigma) was mixed well with 1 mL of 0.2 M Tris-HCl buffer, pH 8, and 2.5 mL of distilled water. Either 0.1 mL of soluble lipase or immobilized lipase beads was added to the reaction mixture. The hydrolytic reaction was conducted at 37 °C with continuous mixing by inversion in a 15-mL test tube attached to a rotating wheel. A 30-min incubation was used for lipase biospecifically adsorbed to avidin−CPG, while a 6-h incubation was used for lipase adsorbed on avidin−nonporous beads or for covalently immobilized lipase. At the end of incubation, 3 mL of 95% ethanol was added to stop the reaction. The free fatty acids released from olive oil by the yeast lipase were titrated with 0.05 N NaOH, and the hydrolytic activity was calculated from the difference in required titrant between the assay solution and a blank without enzyme.

Determination of Lipase Interesterification Activity. A model substrate mixture composed of tricaprylin and oleic acid was used to determine the interesterification activity of *C. cylindracea* lipase. Wet immobilized lipase beads were placed on Whatman No. 1 filter paper to remove excess Tris buffer. The beads and the filter paper were dried in a chamber containing calcium sulfate. Either 0.2 g of commercial *C. cylindracea* lipase or 0.3 g of beads with lipase immobilized via biospecific adsorption was added to 60 mL of a substrate solution that had been prepared to contain 18.9 mM of tricaprylin and various concentrations (from 18.9 to 94.5 mM) of oleic acid dissolved in hexane. The reaction mixtures were incubated in a 37 °C shaker (200 rpm; New Brunswick Scientific Inc., Edison, NJ). A small amount (∼60 μL) of sample was withdrawn every 24 h for the immobilized lipase and every 30 min for the first 8 h, then every 24 h, for the free lipase. The samples were filtered through a 0.2-μm filter disk, dried under nitrogen gas, redissolved in acetone/acetonitrile, and analyzed by a reversed-phase HPLC. The HPLC system was composed of an SSI 222B HPLC pump (SSI Scientific System, Inc., State College, PA), two in-series 250-mm × 4.6-mm Zorbax ODS columns (Mac-Mod Analytical Inc., Chadds Ford, PA), and a Waters differential refractometer R401 (Millipore Corp., Milford, MA). The mobile phases used were acetone and acetonitrile (63.5:36.5) at a flow rate of 1 mL/min (El-Hamdy and Perkins, 1981). The equivalent carbon number (ECN = total carbon number of the fatty acid on triacylglycerol − 2 × the number of double bonds in the fatty acids) of interesterification products was determined by comparing their elution times with those of triacylglycerol standards (Sigma) as described by El-Hamdy and Perkins (1981). The kinetic parameters (V_{max} and K_M) of lipase-catalyzed interesterification were determined from double-reciprocal plots of initial rate data and the oleic acid concentrations.

***o*-Phthaldialdehyde (OPA) Spectrophotometric Assay.** The amount of lipase immobilized on CPG-3000 via covalent linkage was determined by an OPA spectrophotometric assay (Church *et al.*, 1983; Thresher, 1989). Briefly, 1 mL of 6 M HCl was added to about 1 mL of dried beads, vacuum-sealed in a glass vial, placed in a heat block, and incubated at 100 °C overnight. The OPA reagent was freshly prepared each day by combining 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20% (v/v) SDS, 40 mg of OPA dissolved in 1 mL of methanol, and 100 μL of β-mercaptoethanol. About 50 μL of

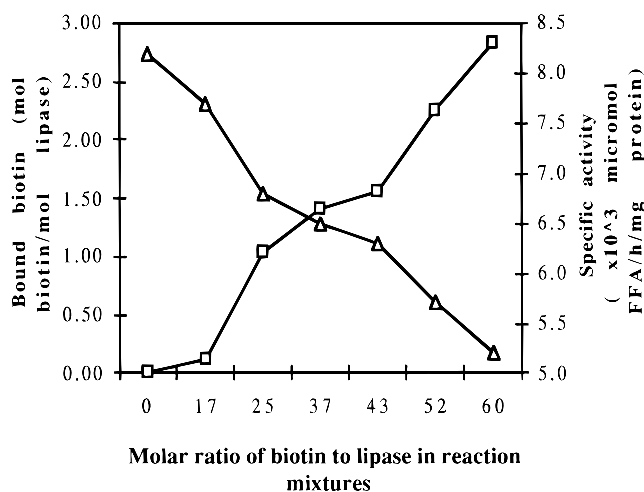

Figure 1. Effect of molar ratio of biotin to *C. cylindracea* lipase in the biotinylation reaction mixture on the extent of lipase biotinylation (□), as determined by a HABA method (Green, 1965), and the specific activity (△) of the biotinylated lipase as a function of the extent of biotinylation.

a dilution of the acid hydrolysate was added to 3 mL of the OPA reagent and incubated for 2 min prior to absorbance reading at 340 nm.

Amino Acid Analysis. The amounts of lipase immobilized on the glass beads (CPG-3000, CPG-2000, and nonporous beads) via biospecific adsorption were determined by amino acid analysis as described by Bidlingmeyer *et al.* (1984) and Walsh and Swaisgood (1993). The protein hydrolysates were derivatized with phenyl isothiocyanate (PITC; Pierce, Rockford, IL), and the phenylthiocarbamoyl (PTC) derivatives of amino acids were separated by HPLC. The HPLC system consisted of two LDC/Milton Roy pumps, a 250-mm × 4.6-mm Beckman C_{18} column, and an Anspec UV−vis detector. The column temperature was maintained at 45 °C with a Bio-Rad column heater. The mobile phase consisted of two solvent mixtures: (A) 0.014 M sodium acetate, pH 4.8, containing 0.125 mL/L triethylamine and (B) 65% acetonitrile in water. An Axxiom data system was used to deliver a linear gradient of solvent B from 10 to 90% in 57 min for the amino acid separation. Elutions of the PTC-amino acid derivatives were monitored at 254 nm with the UV−vis detector, and quantities of amino acid derivatives were calculated by comparing peak areas with those of amino acid standards (Beckman, Fullerton, CA). The amino acid compositions of avidin, lipase, and a 50:50 mixture of avidin and lipase were used to generate a linear regression equation for estimating unknown percentages of these proteins in mixtures of avidin and lipase. Quantities of lipase immobilized on the glass beads were estimated by fitting the amino acid analysis data to the linear regression equation.

RESULTS

Immobilization of *C. cylindracea* Lipase. Determination of the extent of biotinylation of CPG-3000 and CPG-2000 by replacement of HABA bound to avidin by the immobilized biotin yielded an average of 0.2 and 1 μmol of biotin/g of beads for CPG-3000 and CPG-2000, respectively. The relationship between the molar ratio of biotin to *C. cylindracea* lipase in the biotinylation reaction and the extent of lipase biotinylation is shown in Figure 1. *C. cylindracea* lipase required a molar ratio of biotinylation reagent to lipase of 50−60 in the reaction mixture to achieve an average of 2−3 mol of biotin incorporated at lysyl residues/mol of protein.

The specific hydrolytic activities of the biotinylated lipase are also shown in Figure 1. Increasing the extent of biotinylation of *C. cylindracea* lipase caused increasing loss of the hydrolytic activity of the enzyme. To ensure that sufficient biotin was incorporated into each lipase molecule for later avidin binding, a molar ratio of biotin to lipase of 52, which gave an average of 2.26 mol of biotin/mol of lipase, was chosen to biotinylate the lipase for immobilization. This biotinylated lipase retained about 70% of the hydrolytic activity of native lipase (unbiotinylated).

Table 1. Amino Acid Analysis Values for Proline and Threonine in Samples of Avidin, Lipase, and a 1:1 (w/w) Mixture of Avidin and Lipase[a]

sample	proline (μmol/mg of protein)	threonine (μmol/mg of protein)
avidin	1.12	5.32
mixture (1:1, w/w)	1.45	1.97
lipase	2.28	0.78

[a] Least squares fit: % lipase = 45.23 × Pro (μmol/mg) − 10.47 × Thr (μmol/mg) + 5.04.

Table 2. Total Protein Values, Amino Acid Analysis Values for Proline and Threonine in Acid-Hydrolyzed Samples, and Calculated Lipase Contents of CPG-3000, CPG-2000, and Nonporous Beads

	CPG-3000	CPG-2000	nonporous
total protein[a] (mg/g of bead)	3.05	7.40	0.018
proline[b] (μmol/mg of protein)	1.41	1.29	1.49
threonine[b] (μmol/mg of protein)	3.41	4.04	2.80
total lipase (mg/g of bead)	1.01	1.55	0.008
% lipase in total protein (w/w)	33	21	43

[a] Determined from OPA analyses. [b] Determined from amino acid analyses.

The amount of lipase covalently immobilized on CPG-3000, determined by an OPA spectrophotometric assay, was 6.78 mg of enzyme/g of beads. To determine the amount of biotinylated lipase immobilized on the avidin−glass, an amino acid analysis of the immobilized protein was performed and a linear regression of the percent lipase in the sample on the concentration of an amino acid whose concentrations in lipase and avidin were substantially different was constructed. Values of threonine and proline obtained from amino acid analyses of avidin, lipase, and a 50:50 mixture of avidin and lipase are shown in Table 1. The results indicated that avidin contains substantially more threonine but less proline than does lipase; hence, these data were used to generate a linear regression equation for determination of the quantities of each protein in unknown mixtures. Total amounts of proteins immobilized on various glass beads, as determined by the OPA analyses, are shown in Table 2. The amounts of lipase immobilized, calculated from the proline and threonine contents of acid hydrolysates of the beads, are also given. By using molecular weights of 66 500 for *C. cylindracea* lipase, as determined by SDS−PAGE, and 67 000 for avidin (Melamed and Green, 1963), the data in Table 2 reveal that 1 mol of biotinylated *C. cylindracea* lipase is bound for every 2, 4, and 1 mol of avidin tetramer on CPG-3000, CPG-2000, and nonporous glass beads, respectively.

Hydrolytic and Interesterification Activities of Immobilized Lipases. The hydrolytic activities of lipases immobilized via either biospecific adsorption or covalent linkage were determined from the release of free fatty acids from an olive oil emulsion (Table 3). Protein contents of various preparations of immobilized lipase, as determined by amino acid analyses or OPA analyses, were used to calculate the hydrolytic specific activities given in Table 3. The hydrolytic activity of commercial soluble *C. cylindracea* lipase was 819 μmol of FFA h^{-1} (mg of solid)$^{-1}$. Since the commercial lipase

Table 3. Hydrolytic Activities of Lipase Immobilized either by Biospecific Adsorption or Covalent Linkage on Various Glass Beads[a]

	bioselective adsorption			covalent linkage
	CPG-3000	CPG-2000	nonporous	CPG-3000
hydrolytic activity [μmol of FFA h^{-1} (mg of beads)$^{-1}$]	5.75	4.47	0.05	0.09
specific activity [μmol of FFA h^{-1} (mg of lipase)$^{-1}$]	5710	2880	6250	13

[a] Average of duplicate measurements.

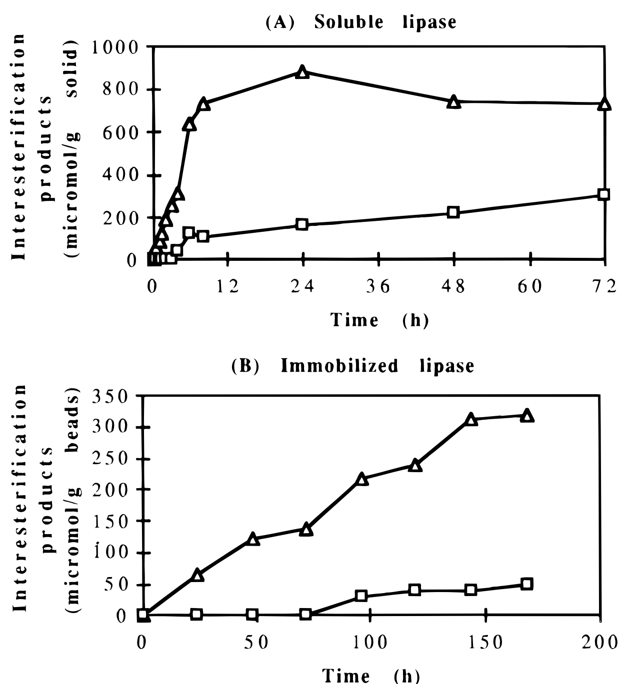

Figure 2. Reaction progress curves for interesterification catalyzed by 0.2 g of soluble *C. cylindracea* lipase (A) or 0.3 g of beads with lipase immobilized via biospecific adsorption on CPG-3000 (B). The reaction mixture contained 18.9 mM tricaprylin and 18.9 mM oleic acid dissolved in 60 mL of hexane. Both 32 C_{ECN} (△) and 40 C_{ECN} (□) products were determined.

contained about 10% protein as determined by the dye-binding method, the specific activity of the commercial *C. cylindracea* lipase preparation was calculated to be 8190 μmol of FFA h^{-1} (mg of protein)$^{-1}$.

The commercial *C. cylindracea* lipase contained ~2.3% water, and the $CaSO_4$-dried immobilized lipase contained ~3.5% water. Both commercial lipase and $CaSO_4$-dried immobilized lipase were used directly to catalyze interesterifications without further adjustment of the water contents. The interesterification activities of soluble and immobilized lipases were determined by measuring the interesterification products by reversed-phase HPLC. Amounts of 32 C_{ECN} and 40 C_{ECN} interesterification products formed at various times during a 72-h reaction period with soluble lipase and during a 196-h reaction period with immobilized lipase are shown in parts A and B of Figure 2, respectively. The 32 C_{ECN} products correspond to tricaprylins (trioctanoyl glycerol) in which one caprylic (octanoic) acid on the glycerol backbone was replaced by an oleic (9-octadecenoic) acid [1(2)-monooctadecenoyl-2,3(1,3)-dioctanoyl glycerol]. The 40 C_{ECN} products correspond to tricaprylins in which two caprylic acids on the glycerol backbone were replaced by oleic acid (2-monooctanoyl-1,3-dioctadecenoyl glycerol). Thus, production of the 32 C_{ECN} products preceded that of the 40 C_{ECN} products. Reversed-phase HPLC was not be able to detect any 40 C_{ECN} interesterification products until the soluble lipase-catalyzed interesterification reaction had proceeded for at least 4 h. It took even longer (after 96 h) to detect the presence of the 40 C_{ECN} products with immobilized lipase-catalyzed interesterification. At the 2.3% water content of soluble *C. cylindracea* lipase, 58% of the reaction products were produced by interesterification and 42% resulted from hydrolysis. However, only 26% interesterification products were found in the reaction catalyzed by immobilized lipase, which had a 3.5% water content. The reaction progress curves of the soluble lipase and lipase immobilized via biospecific adsorption at various oleic acid concentrations are shown in Figure 3. The initial rates of the interesterification reactions at various oleic acid concentrations were used to construct double-reciprocal plots (Figure 4) from which the kinetic parameters of the lipase-catalyzed interesterification were calculated (Table 4). Lipase-catalyzed interesterification in organic solvent follows a bi-bi-ping-pong reaction mechanism (Chulalaksananukul *et al.*, 1990; Miller *et al.*, 1991); hence, the data obtained from the double-reciprocal plots represent apparent kinetic parameters.

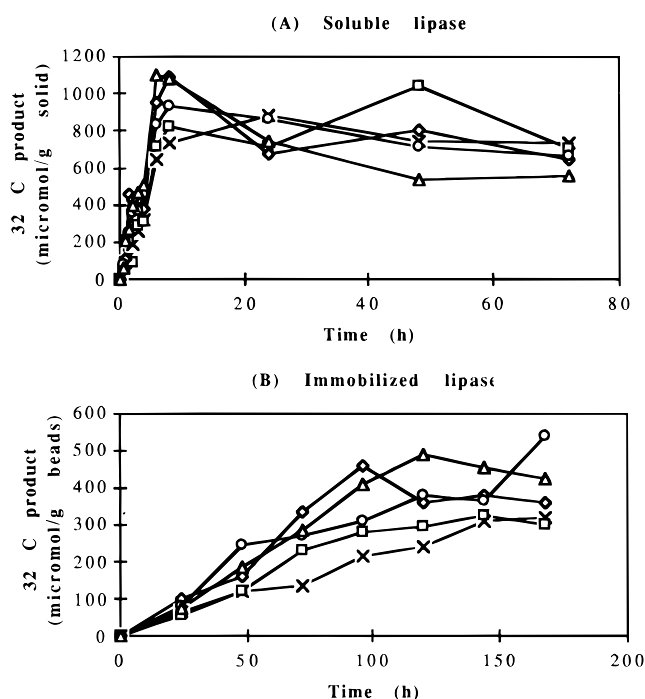

Figure 3. Reaction progress curves for interesterification catalyzed by either 0.2 g of soluble *C. cylindracea* lipase (A) or 0.3 g of beads with lipase immobilized via biospecific adsorption on CPG-3000 (B). The reaction mixture contained 18.9 mM tricaprylin and various concentrations of oleic acid dissolved in 60 mL of hexane. Oleic acid concentrations: (×) 18.9 mM; (□) 37.8 mM; (◇) 56.7 mM; (○) 75.6 mM; (△) 94.5 mM.

DISCUSSION

C. cylindracea lipase required a molar ratio of biotinylation reagent to lipase of 50−60 in the reaction mixture to achieve an average of 2−3 mol of biotin incorporated at lysyl residues/mol of protein. Although the actual amino acid sequence of the *C. cylindracea* lipase used in this study is not known, the cDNA sequences of *C. cylindracea* ATCC 14830 lipases have

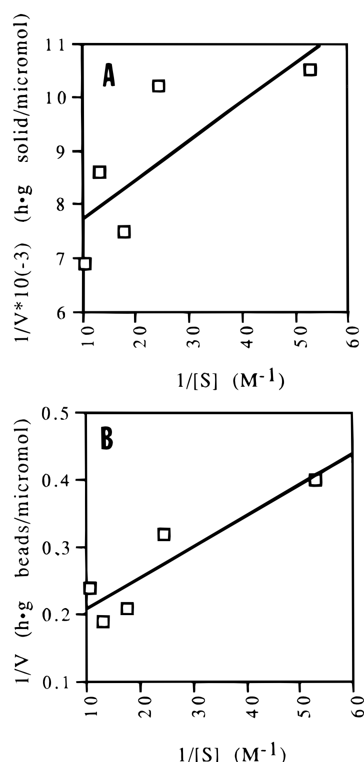

Figure 4. Lineweaver–Burk plots for interesterification catalyzed by either 0.2 g of soluble *C. cylindracea* lipase (A) or 0.3 g of beads with lipase immobilized by biospecific adsorption on CPG-3000 (B). The reaction mixture contained 18.9 mM tricaprylin and various concentrations of oleic acid dissolved in 60 mL of hexane. The initial rates of interesterifications were determined from the first 12-h data points for soluble lipase-catalyzed reactions and from the first 96-h data points for immobilized lipase-catalyzed reactions.

Table 4. Apparent Kinetic Parameters[a] for Interesterification Catalyzed by Soluble *C. cylindracea* Lipase and the Lipase Immobilized via Biospecific Adsorption on CPG-3000

parameter	soluble lipase	immobilized lipase
K_M (mM)	10.4	30.8
V_{max} [μmol of 32 C_{ECN} products h^{-1} (g of lipase)$^{-1}$]	1430	5960
k_{cat} (h^{-1})	95	400
k_{cat}/K_M (h^{-1} mM^{-1})	9.3	12.9

[a] Initial rates were measured using reversed-phase HPLC for determinations of the 32 C_{ECN} interesterification product. Apparent K_M and V_{max} values were calculated from double-reciprocal plots (Figure 4) of the average of duplicate sets of data points.

been obtained and the deduced primary structures reported (Longhi *et al.*, 1992; Lotti *et al.*, 1993). The lysine content of these lipases ranged from 18 to 22 residues of a total of 534 residues in each of the five cDNA clones. The molecular size of these lipases (58 000) is similar to that determined for the commercial lipase (66 500). Assuming that the lipase used in this study has a similar lysine content, the molar ratio of biotinylation reagent to lysyl residues was about 3.

Increasing biotinylation of lysyl residues caused increasing loss of enzyme activity; for example, 37% was lost when an average of 3 residues was modified. This result is consistent with that observed for other enzymes. Bayer *et al.* (1990) reported that the specific activity of biotinylated enzymes ranged from 60 to 80% of that of the unmodified forms. Huang *et al.* (1995) suggested that the loss of activity observed with biotinylated transglutaminase may have been caused by steric hindrance of the active site. The primary structure of *C. cylindracea* ATCC 14830 lipase indicated that a lysyl residue is 5 residues upstream from the proposed concensus sequence (G-X_1-S-X_2-G) in each of the five lipases (Longhi *et al.*, 1992; Lotti *et al.*, 1993). Although we have not determined which residues are modified, it is possible that a modified residue near the active site is affecting the activity.

The pore size of the glass beads affected the extents of matrix biotinylation as well as the amount of avidin and biotinylated lipase immobilized. The concentration of biotinyl groups in units of micromoles per gram was 5-fold greater for CPG-2000 as compared to that for CPG-3000. However, using the commercial specifications for surface area of CPG-3000 and CPG-2000, 9 and 11.5 m^2/g, respectively, gives biotinyl group concentrations of 0.022 and 0.087 $\mu mol/m^2$ for CPG-3000 and CPG-2000, respectively. Thus, some but not all of the difference is accounted for by the difference in surface area. Similar calculations for avidin gave 3.4 and 7.6 $nmol/m^2$ with CPG-3000 and CPG-2000, respectively. Values for lipase were similar with both matrices; *viz.* 1.7 and 2.0 $nmol/m^2$ for CPG-3000 and CPG-2000, respectively. Thus, although the surface concentration of avidin was higher with CPG-2000, fewer biotinylated lipase molecules were bound per tetrameric avidin than with CPG-3000. The smaller pore size of CPG-2000 (198 nm) may have limited access of biotinylated lipase to the avidin-binding sites. The effect of pore size on limiting the amount of protein immobilized due to limited access has been noted previously for the membrane enzyme sulfhydryl oxidase (Janolino and Swaisgood, 1978). In a study of *Rhizomucor miehei* lipase, Bosley and Clayton (1994) observed that the amount of enzyme immobilized by hydrophobic adsorption on derivatized CPG was similar at pore diameters above 150 nm. In our study, however, the effective pore diameter would have been reduced by about 14.4 nm [calculated from the $D_{20,w}$ for avidin reported by Green (1964)] due to the adsorbed avidin. Furthermore, the steric requirements for insertion of the biotinyl group into avidin subunits would be more severe than those for simple hydrophobic adsorption.

In the case of nonporous beads, access to pore surface area would not be a problem, and similar calculations using a surface area of 0.03 m^2/g indicate surface concentrations of 5.1 and 3.9 $nmol/m^2$ for avidin and lipase, respectively. Hence, nearly every molecule of tetrameric avidin had a molecule of lipase bound. Assuming a uniform distribution of protein on the surface, these calculations also indicate that each lipase molecule would occupy a square measuring 207 Å on each side.

The hydrolytic activity of the immobilized lipase will be affected by both access to the pore volume and mass transfer limitations within the pores. Comparison of the specific activities of the biotinylated enzyme with that immobilized on CPG-3000 (302-nm pore diameter) suggests that neither of these factors had much effect on the activity. However, the activity of the enzyme immobilized on CPG-2000 was reduced by about 50%. The lack of activity loss for lipase immobilized on CPG-3000 was unexpected because the average diameter of emulsified oil globules is larger than the pore diameter (Lieberman and Ollis, 1975; Garcia *et al.*, 1991). Two possible explanations of the high activity are (1) a broad distribution of globule size such that some would have access to all the pore volume and (2) immobilization of

most of the lipase nearer the surface of the beads. Obviously, substrate access was restricted in the CPG-2000 beads. Using olive oil as the substrate, Reetz *et al.* (1996) observed activities between 5 and 41% of the soluble enzyme with *R. miehei* lipase entrapped in various hydrophobic silica gels. Although the particle size and pore size of these gels were not determined, apparently substrate was able to penetrate the gel.

With the nonporous beads, neither substrate access nor pore mass transfer limitations would be factors; therefore, any change in activity must result from the immobilization *per se*. The hydrolytic activity with emulsified olive oil was not changed from that observed with the soluble biotinylated enzyme. However, very little hydrolytic activity was displayed by the enzyme attached directly to the surface of CPG-3000 by covalent reaction of lysyl residues with the succinamidopropyl chains. Thus, spacing of the enzyme from the surface through the biotinyl chains (22.4-Å maximum length) and avidin (70-Å diameter) appears to be important for its hydrolytic activity. Such spacing probably provides for more movement of the lipase molecules, allowing them to bind on the surface of oil globules. Studies of immobilized transglutaminase, which also acts on substrates of large molecular size, have shown that the presence of a protein spacer between the enzyme and the surface was required for measurable activity (Oh *et al.*, 1993).

The interesterification activity of the immobilized lipase was 4-fold greater than that for the soluble enzyme. However, the Michaelis constant was 3-fold larger, suggesting that mass transfer limitations may have existed in the CPG-3000 pore volume. The interesterification activity observed here was 1.5-fold larger than that observed by Wisdom *et al.* (1984) for incorporation of myristic acid into palm mid-fraction using an *Aspergillus* sp. lipase adsorbed on Hyflo Supercel. Examination of the esterification of lauric acid with 1-octanol in organic solvents using a variety of lipases entrapped in various hydrophobic silica gels yielded activities ranging from 1.1- to 88-fold greater than that with the corresponding soluble enzyme (Reetz *et al.*, 1996). Furthermore, the best activity yields were observed with the more hydrophobic gels. Bosley and Clayton (1993) also observed increased esterification activity when *R. miehei* lipase was adsorbed on CPG derivatized with longer alkyl chain lengths. Several reasons for the increased esterification activity of immobilized lipase have been suggested, including better dispersion of the enzyme in the hydrophobic gel and the “freezing” of the enzyme in the active conformation with the α-helical loop removed from the active site (Reetz *et al.*, 1996). As an alternative, we suggest that the fatty acid substrate may be partitioned into the matrix phase by hydrophobic interaction with the alkyl chains or perhaps by binding to avidin, which has 18% homology with fatty acid-binding protein (Green, 1990) and also has a β-barrel structure.

Water content of the interesterification reaction mixture will influence the direction of the reaction toward either hydrolysis or interesterification (Macrae, 1983; Lilly and Dunnill, 1987; Chulalaksananukul *et al.*, 1990; Miller *et al.*, 1991). A higher yield of products but a slower reaction rate was observed at low water contents (Yamane, 1987). The commercial lipase used in this study contained about 0.23 g of water/g of enzyme, whereas the lipase immobilized on CPG-3000 contained roughly 35 g of water/g of enzyme. Thus, with the soluble lipase-catalyzed interesterification, 58% of the products resulted from interesterification while 42% were derived from hydrolysis, whereas with the immobilized enzyme 26% of the products came from interesterification.

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