Affinity chromatography of lipase with hydrophobic ligands coupled to cyanogen bromide-activated agarose

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Abstract The behavior of lipase produced by *Pseudomonas mephitica* var. *lipolytica* toward hydrophobic residues coupled to spacer gels that were prepared by coupling a primary amine to CNBr-activated agarose, was studied. The lipase adsorbed on the ligand of a long unbranched aliphatic chain, a benzene ring, or deoxycholic acid was only slightly or not at all eluted at pH 5 or pH 11 by buffers containing 1 M NaCl. The lipase was eluted by liquid containing a surfactant or an organic solvent miscible with water, indicating greater involvement of hydrophobic forces. The adsorption of propane, cyclopentane, cyclohexane, cycloheptane, or chrysene appears to be achieved through electrostatic forces, inasmuch as desorption was caused by buffer containing 1 M NaCl at pH 11. The amount of lipase adsorbed on these hydrophobic ligands was about the same as that adsorbed on the ligands belonging to the first group. Since little lipase was adsorbed on cyclopropane, cyclooctane, pyridine, methane, *n*-pentane, or branched aliphatic chains, these ligands appear to impose steric hindrance on the adsorption of lipase, or they may be too small to fit into the hydrophobic sites of lipase.

Supplementary key words *Pseudomonas mephitica* var. *lipolytica* · sodium deoxycholate · deoxycholic acid · hydrophobic force · electrostatic force · steric hindrance · hydrophobic ligand size

In a previous paper (1) we reported that agarose derivatives containing linear aliphatic amines having an even number of carbon atoms and longer than *n*-nonylamine had a high affinity for lipase of *Pseudomonas mephitica* var. *lipolytica*. Brockerhoff (2) suggested in his study on substrate specificity of pancreatic lipase that lipase has a hydrophobic slit or pocket forming the binding site of lipase that accepts aliphatic chains and aromatic rings more easily than branched structures. It is of interest to study the behavior of lipase toward many kinds of hydrophobic compounds using affinity chromatography techniques. Chromatography with hydrophobic ligands has recently been used by many workers (3–12) for the fractionation of biological materials. However, the chromatography has not yet been used with ligands, such as aliphatic chains, aromatic, alicyclic, and heterocyclic compounds.

In this paper we describe affinity chromatography of lipase produced by *Pseudomonas mephitica* var. *lipolytica* with various hydrophobic compounds coupled to CNBr-activated Sepharose 4B.

MATERIALS AND METHODS

Materials

The lipase used was the crude enzyme prepared from extracellular lipase produced by *Pseudomonas mephitica* var. *lipolytica* (13). The extracellular lipase was precipitated at pH 4, extracted at pH 8, and was either dialyzed against tap water or filtered through Sephadex G-50 with Tris buffer at pH 8 unless otherwise stated; ionic strength was 0.02. The lipase preparation was stable between pH 5 and pH 11 at 4°C, and most activity still remained after the enzyme was kept at 50°C for 5 hr in Tris buffer. CNBr-activated Sepharose 4B, AH-Sepharose 4B (1,6-diaminohexane agarose) and CH-Sepharose 4B (6-aminohexanoic acid agarose) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Cyclopropylamine, cyclopentylamine, cyclooctylamine, dodecylamine, and 6-aminochrysene were purchased from Aldrich Chemical Co., Milwaukee, Wis.; deoxycholic acid and sodium deoxycholate were purchased from Difco Laboratories, Detroit, Mich.; 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo.; and Tris (hydroxymethyl)aminomethane was purchased from E. Merck, Darmstadt, Germany. Other chemicals were obtained from Tokyo Kasei Co. and Wako Pure Chemical Co.

Methods of affinity chromatography with hydrophobic ligands

Agarose derivatives were prepared by the following three methods. (*1*) Primary amine agarose was prepared by coupling a primary amine to CNBr-activated Sepharose 4B by the same method as described in a previous paper (1). (*2*) Agarose-6-aminohexanoyl primary amine was prepared as follows: 1 g of CH-Sepharose 4B was washed with 0.5 M NaCl and distilled water and the washed gel was suspended in 50% dioxane (aqueous) containing 1–2 mM of primary amine. The coupling reaction was carried out at room temperature with continuous shaking for 24 hr or more in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (400 mg) with the pH maintained at 4.5–6.0. The coupled gel was successively washed three times with buffers of pH 4, pH 8, and a 1:1 mixture of ethanol and buffer, pH 8; all solutions contained 0.5 M NaCl. (*3*) Deoxycholic acyl 1,6-diaminohexane-agarose was prepared by coupling deoxycholic acid to AH-Sepharose 4B in the same way as in the preparation of the CH-Sepharose 4B derivative.

The prepared agarose derivative was poured into a column 0.97 mm in diameter and equilibrated with starting buffer. Three ml aliquots of eluant were allowed to percolate through the column and 3 ml fractions were collected. The chromatography, unless otherwise stated, was carried out at 4°C.

Estimation of ligand concentration

The ligand concentration of the primary amine derivative was estimated as follows. Coupled gel was washed in the same way described in the preceding procedures and then was further washed successively with distilled water and absolute methanol. After the final rinse, the gel was dried in a vacuum desiccator, and the amount of nitrogen in the dry gel was measured with a nitrogen analyzer (Coleman model 29B). The nitrogen content of uncoupled gel was also measured in the same manner described above. The latter value was subtracted from the former and the ligand concentration was calculated.

Assay of lipase and protein

Lipolytic activity was assayed by a modification of Nord's method (14). The reaction mixture, composed of 5 ml of olive oil emulsion, 4 ml of 0.1 M phosphate buffer, pH 7, and 1 ml of enzyme, was incubated at 60°C for 20 min. The enzyme reaction was terminated by adding 20 ml of acetone–ethanol 1:1. The free fatty acid was titrated with 0.05 N NaOH solution. One unit of lipase was defined as the amount of enzyme that liberates 1 μmole of fatty acid per min at 60°C, pH 7. In order to obtain the specific activity, protein was assayed by the method of Lowry et al. (15) using bovine serum albumin as a standard; Tris buffer or eluant was used as the blank. Specific activity was expressed as the activity per mg of protein.

RESULTS

Effect of the arm length

We previously reported that lipase produced by *Pseudomonas mephitica* var. *lipolytica* was adsorbed on *n*-octylamine coupled to CNBr-activated Sepharose 4B and that the agarose derivative was the lowest *n*-aliphatic amine of all the derivatives on which lipase could be adsorbed (1). In the present paper, the adsorption of lipase on a phenylalkylamine series was studied and the results are shown in **Table 1**. It was found that lipase was adsorbed on phenylmethylamine-agarose. The length of phenylmethylamine was one-half as long as that of *n*-octylamine. This is illustrated in **Fig. 1**.

In the case of phenylpropylamine or phenylethylamine, once the column was washed with 6 ml of Tris buffer, substantially no elution of lipolytic activity was observed even when additional Tris buffer was added. In the case of phenylmethylamine, however, some elution of the activity (tailing) was still observed after washing with 16 ml of Tris buffer. The data indicated that two or more carbon

TABLE 1. Adsorption of lipase on phenylalkylamine-agarose

Ligand	Activity of Eluted Lipase	Activity of Adsorbed Lipase	Ligand Concentration[a]
	units		*μmoles/ml of gel*
Phenylamine (aniline)	170.0	0.0	0.2 (7.0[b])
Phenylmethylamine	46.4	113.6	1.2
Phenylethylamine	40.7	119.3	0.7
Phenylpropylamine	49.2	110.8	1.5

Each type of phenylalkylamine was covalently bound to CNBr-activated Sepharose 4B and the gel (1 ml) was packed in a column and 2 ml of lipase solution (160 units) was applied; elution was carried out with Tris buffer. The activity of eluted lipase was subtracted from that of applied lipase and the subtracted value was expressed as the activity of adsorbed lipase.

[a] In determining ligand concentration it was assumed that there was no loss of nitrogen during coupling, and that the coupling occurred in *N*-substituted isourea.

[b] The lipase was not adsorbed on gels with ligand concentrations of 0.2 or 7.0 μmoles/ml. In the latter case, the gel was prepared by reaction with 10 times the concentration of aniline as described in the previous paper (1).

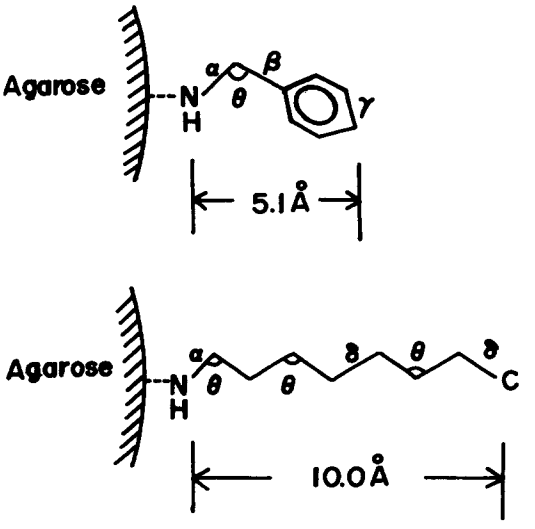

Fig. 1. The smallest compound of all the phenylalkylamines and aliphatic amines coupled to CNBr-activated Sepharose 4B on which lipase was adsorbed. The length was calculated from following bond distances (16) and bond angle, $\alpha = 1.47$ Å, $\beta = \delta = 1.54$ Å, $\gamma = 1.40$ Å, and $\theta = 109.5°$.

atoms gave a sufficient length to the spacer that served to connect Sepharose 4B to the benzene ring.

Adsorption of aliphatic chains coupled to CH-Sepharose 4B

Tables 2 and 3 show the behavior of the lipase adsorbed on various kinds of aliphatic amines coupled to a spacer gel, CH-Sepharose 4B. The lipase was not adsorbed on CH-Sepharose 4B itself and for the most part passed through the column. As reported in the previous paper (1), lipase was not adsorbed on *n*-butylamine agarose, but it was adsorbed on agarose-6-aminohexanoyl *n*-butylamine.

As shown in **Table 2**, the lipase adsorbed on the ligands of *n*-butylamine and 2-ethylhexylamine, which have sufficiently long unbranched chains, was only slightly eluted at pH 5 or pH 11 with buffers containing 1 M NaCl or with Tris buffer, but was eluted with Tris buffer containing sodium deoxycholate. The lipase adsorbed on the ligand of a branched aliphatic chain having no long unbranched chain was almost completely eluted at pH 5 or pH 11 with buffers containing 1 M NaCl. The amount of lipase adsorbed on the branched aliphatic ligand was less than that adsorbed on agarose-6-aminohexanoyl *n*-butylamine. Among the branched aliphatic amines, lipase was adsorbed to a greater degree on the large hydrophobic ligand, *tert*-octylamine (1,1,3,3-tetramethylbutylamine), than on the small one, *tert*-butylamine.

As shown in **Table 3**, little lipase was adsorbed on the ligands of methylamine or *n*-pentylamine coupled to CH-Sepharose 4B, and the adsorbed lipase was released at pH 5 or pH 11 with buffers containing 1 M NaCl. The amount of lipase adsorbed on agarose-6-aminohexanoyl *n*-propylamine was nearly the same as that adsorbed on *n*-butylamine, *n*-hexylamine or *n*-heptylamine coupled to CH-Sepharose 4B, but the lipase adsorbed on the former ligand was almost completely eluted at pH 11 with buffer containing 1 M NaCl. Agarose-6-aminohexanoyl ethylamine was an exception. The gel was prepared two times, but the ligand concentrations of the gels were zero in both cases.

Adsorption on hydrophobic rings coupled to CH-Sepharose 4B

The results for aromatic, alicyclic, and heterocyclic compounds coupled to CH-Sepharose 4B, are

TABLE 2. Adsorption of lipase on agarose-6-aminohexanoyl aliphatic amines

Ligand	Lipase Activity in Eluate A → B → C → D → E					Ligand Concentration
	units					*μmoles/ml of gel*
None	105.8	0.0	0.0	0.0	0.0	0.0
n-Butylamine	17.8	0.0	0.0	2.7	96.5 (136.9)	4.0
iso-Butylamine	55.1	19.1	33.3	0.0	0.0	11.0
tert-Butylamine	56.5	19.8	25.7	2.1	0.0	17.8
sec-Butylamine	86.7	14.1	18.7	0.0	0.0	4.2
tert-Octylamine	37.1	24.3	37.8	0.0	1.0	4.9
2-Ethylhexylamine	10.0	1.5	0.0	0.0	111.0 (132.3)	3.2

The aliphatic amine was covalently bound to CH-Sepharose 4B. Each type of agarose derivative (1 ml) was packed in a column, 3 ml of lipase solution (144.9 units, sp act 22.0) was applied, and elution was attempted by successively varying the conditions. A and D are the eluates of Tris buffer; B, the eluate of 0.05 M acetate buffer containing 1 M NaCl at pH 5; C, the eluate of 0.05 M Na_2CO_3–$NaHCO_3$ buffer at pH 11 containing 1 M NaCl; and E, the eluate of Tris buffer containing sodium deoxycholate. "None" refers to use of CH-Sepharose 4B itself as the adsorbent. Figures in parentheses express specific activity.

TABLE 3. Adsorption of lipase on agarose-6-aminohexanoyl *n*-aliphatic amines

Ligand	Lipase Activity in Eluate A → B → C → D → E	Ligand Concentration
	units	*μmoles/ml of gel*
Methylamine	172.7 (30.5) 70.0 (60.8) 30.9 (80.3) 0.0 0.9	17.6
Ethylamine	284.4 0.0 2.7 0.0 1.4	0.0
n-Propylamine	40.1 59.2 157.9 (122.8) 0.0 3.7	7.4
n-Butylamine	37.1 0.0 25.0 3.6 127.9 (195.8)	4.0
n-Pentylamine	130.5 30.3 109.7 (235.1) 0.0 8.0	3.8
n-Hexylamine	29.4 0.0 18.9 5.2 129.4 (186.2)	7.2
n-Heptylamine	44.7 0.0 23.8 3.4 119.3 (254.5)	7.8

The *n*-aliphatic amine was covalently bound to CH-Sepharose 4B. Each type of agarose derivative (1 ml) was packed in a column and 3.5 ml of lipase solution (329.2 units, sp act 40.4) was applied. Elution was carried out by the same procedure as in Table 2. Figures in parentheses express specific activity.

shown in **Table 4**. Although lipase was not adsorbed on aniline-agarose as shown in Table 1, it was adsorbed on agarose-6-aminohexanoyl aniline. The lipase adsorbed on the agarose-6-aminohexanoyl aniline was only slightly eluted at pH 5 or pH 11 with buffers containing 1 M NaCl, or with Tris buffer, but was eluted with Tris buffer containing sodium deoxycholate. The lipase adsorbed on the ligands of 6-aminochrysene, 2-aminopyridine or alicyclic rings was almost completely eluted at pH 5 or pH 11 with buffers containing 1 M NaCl. Lipase could be adsorbed on the ligands of aniline, 6-aminochrysene, cyclopentylamine, cyclohexylamine or cycloheptylamine better than on those of 2-aminopyridine, cyclopropylamine, or cyclooctylamine. The amount of lipase adsorbed on the former ligands was nearly the same as that adsorbed on agarose-6-aminohexanoyl *n*-butylamine in Table 2.

Adsorption on deoxycholic acid coupled to AH-Sepharose 4B

Table 5 shows that lipase was adsorbed on AH-Sepharose 4B and on deoxycholic acyl 1,6-diaminohexane agarose. The greater part (63.9%) of the lipase adsorbed on AH-Sepharose 4B was eluted with Tris buffer containing 1 M NaCl. The lipase adsorbed on the ligand of deoxycholic acid, however, was not eluted with the buffer but was eluted by Tris buffer containing sodium deoxycholate. The recovery ratio at this time was 100.4% and the specific activity increased to 3.6 times the original level.

Desorption of lipase adsorbed on hydrophobic compounds

Table 6 shows a comparison of eluants with which lipase adsorbed on various kinds of hydrophobic ligands could be eluted. As reported in a previous paper (1), anionic and nonionic surfactants and protein denaturing agents brought about desorption. In the case of 3 M guanidine hydrochloride, the elution was done rapidly and the eluate was dialyzed immediately against Tris buffer at 4°C to protect against inactivation of lipase and destruction of the agarose gel (17). Desorption was also caused by a mixture of buffer and organic solvent miscible

TABLE 4. Adsorption of lipase on hydrophobic rings coupled to CH-Sepharose 4B

Ligand	Lipase Activity in Eluate A → B → C → D → E	Ligand Concentration
	units	*μmoles/ml of gel*
Aniline	9.3 0.0 3.5 0.0 83.3 (191.9)	1.9
6-Aminochrysene	9.6 5.6 73.8 (125.7) 0.0 20.6	14.0
2-Aminopyridine	20.8 41.8 44.4 0.0 1.7	4.2
Cyclopropylamine	52.9 26.9 26.8 (47.6) 1.0 0.0	0.6
Cyclopentylamine	7.9 6.2 88.1 (75.5) 0.0 0.0	17.4
Cyclohexylamine	11.3 0.0 87.3 (44.8) 0.0 5.1	18.2
Cycloheptylamine	10.1 1.8 111.3 (94.1) 0.0 0.0	8.9
Cyclooctylamine	45.2 0.0 44.9 (78.5) 0.0 12.5	7.6

Each type of agarose-6-aminohexanoyl primary amine (1 ml) was packed in a column and 3 ml of lipase solution (144.9 units, sp act 22.0) was applied. The elution was carried out by the same procedure as in Table 2. Figures in parentheses express specific activity.

TABLE 5. Adsorption of lipase on AH-Sepharose 4B and deoxycholic-acyl-1,6-diaminohexane-agarose

Eluant	Lipase Activity in Eluate: Deoxycholic-acyl-1,6-diamino-hexane-agarose	Lipase Activity in Eluate: AH-Sepharose 4B
	units	
Tris buffer	22.6	20.2
↓		
0.1 M Phosphate buffer at pH 6	0.0	0.0
↓		
Above buffer with addition of 1 M NaCl	0.0	0.0
↓		
Tris buffer with addition of 1 M NaCl	0.0	87.9
↓		
0.1 M Na_2HPO_4–NaOH buffer at pH 11 with addition of 1.5 m KCl	0.0	
↓		
Tris buffer with addition of 0.5% sodium deoxycholate	137.9 (157.9)	61.6

Each type of agarose derivative (1 ml) was packed in a column and 2 ml of lipase solution (sp act 43.7) was applied; elution was carried out by varying the eluant successively. The figure in parentheses expresses specific activity.

with water. The recovery ratio was increased when the eluant of organic solvent contained 0.5 M NaCl. The elution with organic solvent provided lipase which had relatively high specific activity.

It was reported that the hydrophobic affinity chromatogram was developed by decreasing the ionic strength or the temperature or by increasing the pH (7, 8, 12). The reported effects were experimentally examined, and the results obtained are listed in **Table 7**. Little lipase elution was caused by decreasing the ionic strength or by increasing the pH. No lipase elution was caused by decreasing the temperature.

DISCUSSION

The mode of adsorption of lipase on hydrophobic compounds coupled to spacer gels that are prepared by coupling a primary amine to CNBr-activated agarose is divided into three groups in accordance with the structure of the hydrophobic compounds.

The first group included a long unbranched aliphatic chain, a benzene ring, and deoxycholic acid. The lipase adsorbed on these hydrophobic ligands was only slightly or not at all eluted at pH 5 or pH 11 by buffers containing 1 M NaCl but was eluted by liquid containing a surfactant or an organic solvent miscible with water, indicating a greater involvement of hydrophobic forces. Hydrophobic sites or "pockets" of lipase may be assumed to play an important role in this binding. Little elution of lipase was caused by decreasing the ionic strength or increasing the pH and no elution was caused by decreasing the temperature. Desorption with organic solvent was incomplete in the absence of 0.5 M NaCl. As compared with elution conditions of the hydrophobic chromatogram previously reported (6, 7, 12), these results suggest that the binding also involves electrostatic forces. It is also known that primary amine coupled to the cyanogen bromide reaction gives rise to a positively charged matrix (4, 10).

The second group included *n*-propylamine, cyclopentylamine, cyclohexylamine, cycloheptylamine, and 6-aminochrysene. The adsorption on these ligands appears to be achieved mainly through electrostatic forces, because desorption was caused at pH 11 by buffer containing 1 M NaCl. The amount of lipase adsorbed on hydrophobic ligands belonging to the second group was about the same as that adsorbed on those belonging to the first group. This result does not mean that the hydrophobic ligands

TABLE 6. Elution of lipase with surfactants, denaturing agents, and organic solvents

Eluant	Recovery	Specific Activity	Agarose Derivative
	%		
0.5% Sodium deoxycholate in Tris buffer	95.9	71.3	Agarose-6-aminohexanoyl cyclohexylamine
0.5% Triton X-100 in Tris buffer	66.5	94.1	*n*-Dodecylamine-agarose
0.5% Sodium dodecyl sulfate in Tris buffer	96.3	54.9	Agarose-6-aminohexanoyl cyclohexylamine
3 M Guanidine hydrochoride in Tris buffer	64.6	54.1	Agarose-6-aminohexanoyl 2-aminopyridine
Dioxane–water mixture (1:1)	38.0		Phenylmethylamine-agarose
Ethanol–Tris buffer mixture (1:1)	62.9	89.1	*n*-Undecylamine-agarose
Ethanol–0.1 M $NaHCO_3$ solution containing 0.5 M NaCl mixture (1:1)	95.9	90.8	Agarose-6-aminohexanoyl cyclohexylamine
Acetone–0.1 M $NaHCO_3$ solution containing 0.5 M NaCl mixture (1:1)	101.5	163.0	*n*-Dodecylamine-agarose

Each type of agarose derivative (1 ml) was packed in a column and 2 ml of lipase solution (specific activity 43.7) was added. The column was washed with 16 ml of Tris buffer. Thereafter, the adsorbed lipase (about 130 units) was eluted by a different eluant. In the case of an eluant which affected the activity of lipase, the obtained data were adjusted to compensate for the influence of the eluate.

TABLE 7. Elution of lipase with decreasing the ionic strength or the temperature or increasing the pH

Agarose Derivative	Lipase Activity in Eluate (units)		
	Starting conditions		
Agarose-6-aminohexanoyl aniline	μ = 0.1, pH 9[a] 14.3	→ μ = 0.002, pH 9[a] 2.5 (36.2)	
Agarose-6-aminohexanoyl aniline	μ = 0.1, pH 9[a] 19.0	→ μ = 0.002, pH 11[a] 18.4 (75.5)	→ 0.5% Bile salt[b] 296.8
n-Dodecylamine-agarose	23.8	43.6 (57.7)	322.4
Agarose-6-aminohexanoyl aniline	μ = 0.1, pH 9[a] 7.8	→ μ = 0.1, pH 11[a] 1.1	→ 0.5% Bile salt[b] 297.5
Agarose-6-aminohexanoyl aniline	μ = 0.1, pH 9[a] 14.2	→ μ = 1.1, pH 9[c] 0.0	→ 0.5% Bile salt[b] 305.5
Agarose-6-aminohexanoyl aniline	Tris buffer at 50°C 157.2	→ Tris buffer at 4°C 0.0	
n Dodecylamine-agarose	51.3	0.0	→ 0.5% Bile salt[b] 264.0

Each type of agarose derivative (1 ml) was packed in a column and 3 ml of lipase solution (313.3 units, sp act 33.1), equilibrated with the starting eluant, was applied to a column. Elution was carried out by varying the conditions successively. Figures in parentheses express specific activity.

[a] $NaHCO_3$–Na_2CO_3 buffer.

[b] Sodium deoxycholate dissolved in Tris buffer.

[c] One M NaCl dissolved in 1 liter of $NaHCO_3$–Na_2CO_3 buffer at pH 9 (ionic strength μ = 0.1).

of the second group impose a steric hindrance on lipase adsorption. Jost, Miron, and Wilchek (10) recently proposed that "detergent-like action" is a possible explanation in adsorption similar to this.

The third group included cyclopropylamine, cyclooctylamine, 2-aminopyridine, methylamine, *n*-pentylamine, and branched aliphatic amines that have no long unbranched chain. The hydrophobic ligands of the third group may be too small to fit into the hydrophobic sites of lipase, or they may impose steric hindrance on the adsorption of the enzyme, since little lipase was adsorbed on these ligands. The retention is achieved mainly through electrostatic forces because desorption was caused by buffer containing 1 M NaCl at pH 5 or pH 11. However the hydrophobic interactions apparently contribute to the adsorption since lipase was adsorbed to a greater degree on the large hydrophobic ligand, *tert*-octylamine, than on the small one, *tert*-butylamine.

n-Butylamine was the shortest *n*-aliphatic amine of all the derivatives coupled to CH-Sepharose 4B that may fit in the hydrophobic sites of lipase, because the mode of adsorption on agarose-6-aminohexanoyl *n*-butylamine belongs to the first group. The mode of adsorption on agarose-6-aminohexanoyl *n*-pentylamine, however, belongs to the third group. *n*-Pentylamine has an odd number of carbon atoms and does not have such a long carbon chain as *n*-heptylamine. It seems to be difficult for the ligand of *n*-pentylamine to form a conformation similar to that of *n*-butylamine. In the previous experiment we also observed that *n*-aliphatic amine agarose of an *n*-aliphatic amine with an even number of carbon atoms had a higher affinity for *Pseudomonas* lipase than an amine with an odd number (1).

The spacer gels used in this study were CH-Sepharose 4B and AH-Sepharose 4B. CH-Sepharose 4B was prepared by coupling 6-aminohexanoic acid to CNBr-activated Sepharose 4B, and is considered as a cation exchanger that has a free carboxyl group at the end of the ligand. AH-Sepharose 4B was prepared by coupling 1,6-diaminohexane to CNBr-activated Sepharose 4B and is a kind of anion exchanger that has a free amino group at the end of the ligand. Lipase was not adsorbed on CH-Sepharose 4B but was adsorbed on AH-Sepharose 4B. This result implies that lipase is an acid protein that is adsorbed on an anion exchanger.

The lipase adsorbed on AH-Sepharose 4B was eluted in two fractions. One fraction was eluted by Tris buffer containing 1 M NaCl while the other fraction remained bound even in Tris buffer containing 1 M NaCl but could be eluted with Tris buffer containing 0.5% sodium deoxycholate. The retention of the latter fraction was mainly due to the interactions of hydrocarbon extensions (arms). The function of the arms can be explained by relief of steric restrictions imposed by the matrix backbone, and perhaps by the increased flexibility and mobility of the ligand when it protrudes fur-

ther into the solvent (17). The arms may also keep the positively charged tops of the ligands apart from the matrix backbone, which may be negatively polarized, and the acid protein may more readily approach these ligands. The other significant function of the hydrocarbon extensions is that they may themselves contribute to the binding of lipase through hydrophobic interactions (3).

The smallest compounds of all the phenylalkylamines and aliphatic amines coupled to CNBr-activated agarose on which lipase was adsorbed were *n*-octylamine agarose and phenylmethylamine agarose. The length of *n*-octylamine was twice that of phenylmethylamine, which has a greater hydrophobic residue (a phenyl group). According to Hofstee (18), the hydrophobicity of a phenyl group corresponds to that of 3–4 straight chain hydrocarbons. Granting that the corresponding length of the hydrophobicity was subtracted, the length of *n*-octylamine was longer than phenylmethylamine.

The high affinity of lipase for hydrophobic interface has been reported for a long time. Attempts to use this property in the purification of lipase have been tried by many workers (1, 9, 11, 19–22) since Willstätter, Waldschmidt-Leitz, and Memmen (19) pointed out that an appreciable purification of the enzyme could be achieved by adsorption of powdered stearic acid. As reported in the present paper, however, the adsorption of lipase on hydrophobic residues did not always occur through biospecific affinity, a complementary stereostructural agreement such as the lock-and-key relationship. Therefore, many factors must be considered when affinity chromatography is used for the purification of lipase.

Manuscript received 28 May 1975 and in revised form 15 December 1975; accepted 18 February 1976.

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