The Enzymatic Hydrolysis of Triglyceride–Phospholipid Mixtures in an Organic Solvent

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ABSTRACT: The abilities of four commercially available lipolytic enzymes [three immobilized lipases—Lipozyme IM-20, SP-435 (Novo Biolabs, Danbury, CT), and AY-30/Celite (Amano Enzyme Co., Ltd., Troy, VA)—and a nonimmobilized Amano phospholipase B preparation] to hydrolyze mixtures of triacylglycerols (TG) and phospholipids (PL) were determined. All of the lipases hydrolyzed both types of substrates in water, with maximum rates of TG hydrolysis exceeding those of PL hydrolysis by between 20- and 200-fold. The phospholipase B preparation was inactive against both TG and PL in water. All the enzymes showed some activity against lipids in hexane. The amount of activity was sharply dependent on the amount of water added to the reaction. Lipozyme IM-20 and AY-30/Celite hydrolyzed both TG and PL in hexane. Their estimated initial activities were between 10- and 100-fold lower than those in water. Complete hydrolysis of the TG (measured as the hydrolysis of at least one ester bond in each molecule) was achieved, whereas only 40–60% of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were hydrolyzed. Lipase SP-435 was inactive against TG in hexane but hydrolyzed PC at a rate comparable to that seen in water, and it achieved complete hydrolysis of this substrate. Amano phospholipase B was inactive against TG in hexane but completely hydrolyzed the PC. The abilities of the enzymes to hydrolyze the TG, PC, and PE components of soybean soapstock, a by-product of edible oil production, were also examined. Lipozyme IM-20 hydrolyzed all the TG and a fraction of the PL in soapstock. SP-435 and AY-30/Celite were active only on soapstock that had been acidified prior to being dissolved in hexane. SP-435 displayed significant activity only toward PE under these conditions, whereas AY-30/Celite was active only toward TG. Phospholipase B was inactive against soapstock in hexane. The identity of the acid used in the acidification of soapstock affected the degree of hydrolysis by AY-30/Celite, with nitric and hydrochloric acids giving the best activity.

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The high substrate and product specificities of enzymes, coupled with their abilities to operate at or near ambient temperature and pressure, make them attractive candidates for the conduct of chemical reactions. Provided that the water activity is sufficiently high, lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) hydrolyze the carboxylic acid ester bonds of water-insoluble substrates, most prominently triacylglycerols (TG). At reduced water activities, these enzymes catalyze ester synthesis and transesterification. Because lipases play important physiological roles, and because many industrially and medically useful substances are esters, there has been substantial effort in the characterization of these enzymes and their development as applied catalysts (1–3). As a result, numerous lipases with a variety of biochemical properties and catalytic specificities have been described. These efforts increased greatly after it was demonstrated that lipases and other enzymes are active and stable in organic solvents (4–6).

Lipases are catalytically versatile, and they are not limited solely to TG as substrates. Several of these enzymes are active against phospholipids (PL) (7–14). This capability has been employed for the hydrolytic removal of PL contaminants from preparations of other materials (15,16). It has also been used in the preparation of lysophospholipids, for use as fungicides and emulsifiers, through either the hydrolysis (17) or alcoholysis (18) of PL. Lipases have also proven useful for the exchange of free fatty acids into PL (19–21), a reaction which has been exploited for radioactive labelling of PL (22) and for their enrichment with n-3 polyunsaturated fatty acids by using either free acids (23,24) or TG (25) as substrates.

It is likely that phospholipases, not lipases, are largely responsible for PL hydrolysis in nature. Nonetheless, lipases are reasonable choices for PL hydrolysis *in vitro* because they are relatively small enzymes that require neither cofactors nor ionic activators for activity. In addition, they have been more thoroughly developed as applied catalysts, in terms of commercial availability and the accumulation of basic information regarding their use, than have the phospholipases.

The original examinations of PL hydrolysis by lipases were conducted in water. We have recently extended these studies to organic solvents (26,27), where PL exhibit increased solubility and reduced viscosity compared to aqueous systems. This work demonstrated that lipases are hydrolytic-

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ally active against pure phosphatidylcholine (PC) in a number of organic solvents and examined the water requirements and the effects of reaction conditions on enzyme activity.

Because PL and TG are sometimes encountered as natural mixtures, we have now expanded these studies to determine the abilities of lipases to hydrolyze the components of such mixtures. This work includes examinations of the enzymatic hydrolysis both of artificial mixtures of TG and PL, and of soybean oil soapstock, a heterogenous by-product of edible oil production that contains TG, PL, and other components (28). Successful development of a method for the hydrolysis of such mixtures may facilitate the recovery and use of the fatty acids that are contained in their lipid components.

EXPERIMENTAL PROCEDURES

Chemicals. Olive oil, semi-purified soybean L-α-PC [containing 20% PC and an approximately equal amount of phosphatidylethanolamine (PE)], 1-monoolein, 1,3-diolein, L-α-PE (soybean), and oleic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Pure L-α-PC (>99%, from soybeans) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). *Lyso*-lecithin (plant) was obtained from Matreya, Inc. (Pleasant Gap, PA). Food-grade soybean oil, obtained locally, was used directly as a source of soy TG. The immobilized lipase preparations Lipozyme IM20 and SP-435 were obtained from Novo Biolabs (Danbury, CT). Lipase AY-30 and phospholipase B were the generous gifts of Amano Enzyme U.S.A. Co., Ltd. (Troy, VA). Celite Analytical Filter Aid (Cat. no. C211) was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Burdick and Jackson Brand hexane and isopropanol were purchased from Baxter (Muskegon, MI). Soybean soapstock was generously provided by Cargill (Minneapolis, MN) and stored under nitrogen at −20°C. The supplier indicated that the soapstock contained 45% water, 12% neutral oil, 32% phospholipids, and 10% fatty acids, and had a pH of 10.3.

Enzyme immobilization. Sufficient water was added to Celite (1 g) to just moisten it. Lipase AY-30 (0.5 g) dissolved in 3 mL of 20 mM sodium phosphate buffer, pH 7.5, was thoroughly mixed into the Celite, and the liquid was evaporated under a stream of nitrogen (17–20 h) at room temperature. The residual material, which appeared dry, was stored under refrigeration.

Determination of hydrolytic activity. The lipolytic and phospholipolytic activities of each enzyme were determined by means of a pH-stat method with a continuous titrating pH meter (Radiometer, Copenhagen, Denmark) (29). Incubations were conducted at 37°C. Each enzyme was assayed at its pH optimum, determined in preliminary experiments. Activity against triglycerides was measured with an emulsion of olive oil (18% wt/vol, approximately 200 mM) and gum arabic (4.2% wt/vol) in water. Pure PC (13.9% wt/vol in water, approximately 185 mM) was the substrate for the measurement of phospholipase activity. All assays were conducted in the presence of 15 mM added calcium chloride. A unit of activity is defined as that amount of enzyme catalyzing the release of one μmole of fatty acid per minute.

Three substrate mixtures were employed for the determination of hydrolytic activities in organic solvent. Hexane (8 mL) was the solvent in all cases, water-saturated in the case of defined and semi-pure substrates, and neat for soapstocks. The substrate mixtures were: (i) a fully defined solution containing pure PC (0.13 g, 22 mM final concentration) and soy triglycerides (0.17 mL, 22 mM); (ii) semi-pure PC (0.8 g, which provided a source of PC and PE at a concentration of approximately 25 mM each), and soy triglycerides (0.17 mL, 22 mM); and (iii) soybean soapstock (0.5 g/reaction). These substrates were dissolved in hexane prior to addition of the enzymes. Additional water was included in reactions containing substrates (i) and (ii), in amounts required to support maximum enzyme activity. No additional water was required due to the high moisture content of soapstock. For each enzyme, the amount of catalyst employed was chosen so as to obtain substantial hydrolysis of at least one component of the reaction mixture within a reasonable time span. Reactions were conducted at 42°C in 20 × 150 mm screw-cap tubes, shaken at 300 rpm in an orbital shaker. Following incubation, residual TG, PC, and PE levels were determined by high-performance liquid chromatography (HPLC) with a Hewlett-Packard (Valley Forge, PA) 1050 Chromatography System equipped with a 3 × 100 mm LiChrosorb SI 60-5 column (Chrompack Inc., Raritan, NJ). Samples containing soapstock were filtered over Millipore Brand Millex FX_{13} membranes (0.5 μm, Sigma Chemical Co.) prior to HPLC analysis. The mobile phase programs for HPLC involved gradients of isopropanol and water in hexane/0.6% glacial acetic acid (Table 1) and were developed on the basis of the earlier work

TABLE 1
Solvent Gradients Used in High-Performance Liquid Chromatography Analysis of Lipid Mixtures[a]

Time (min)	Hexane	Acetic acid	Isopropanol	Water
For the analysis of defined pure and semi-pure mixtures[b]				
0.00	98.4	0.6	1.0	0.0
3.53	39.4	0.6	60.0	0.0
7.06	39.4	0.6	51.0	9.0
12.94	39.4	0.6	51.0	9.0
16.46	39.4	0.6	60.0	0.0
19.92	98.4	0.6	1.0	0.0
29.40	98.4	0.6	1.0	0.0
For the analysis of complex lipid mixtures (soapstock)[c]				
0.00	99.3	0.6	0.1	0.0
6.00	93.4	0.6	6.0	0.0
10.00	39.4	0.6	60.0	0.0
20.00	39.4	0.6	51.0	9.0
30.00	39.4	0.6	51.0	9.0
35.00	39.4	0.6	60.0	0.0
40.00	99.3	0.6	0.1	0.0
72.00	99.3	0.6	0.1	0.0

[a]Values expressed as percent (vol/vol) of eluting solution.
[b]Flow rate: 0.85 mL/min.
[c]Flow rate: 0.5 mL/min.

of Moreau *et al.* (30). Baseline resolution of analyte peaks was achieved. Peaks were detected by means of a mass-based detector (ELSD IIA; Varex, Burtonsville, MD), which was operated with a nebulizer temperature of 60°C and a nitrogen flow rate of 3.5 L/min. Peaks were identified by comparison of their retention times to those of known standards, and quantitated by reference to response curves generated with pure compounds. The degree of hydrolysis of each material was calculated as shown in Equation 1 for PC:

$$\% \text{ hydrolysis} = (\text{original PC} - \text{remaining PC})/(\text{original PC}) \times 100 \quad [1]$$

where "original PC" was the amount of PC detected in an enzyme-free reaction. Note that in the use of such an equation one does not differentiate between the hydrolysis of one, two, or three ester bonds in the substrate. All data are the averages of duplicate determinations. The standard variation between replicate determination was less than 6%.

RESULTS AND DISCUSSION

The purpose of this work was to determine the hydrolytic activities of four commercially available enzymes against mixtures of TG and PL. The enzymes used were chosen to represent a range of substrate and positional specificities (Table 2) and consisted of three lipases and a phospholipase B. Immobilized enzymes were primarily used in this work because they are more likely to be suitable for large-scale continuous applications at a later date. Also for this reason, the AY-30 lipase, which was received as a powder, was immobilized on Celite prior to use. This carrier had been previously shown to result in a high-activity immobilized enzyme (27). In addition, a newly available phospholipase B preparation was examined to obtain information regarding its activity and substrate range in aqueous and nonaqueous environments. This enzyme was received as a loose powder and used without further modification.

Hydrolytic activities in aqueous solution. Each of the three lipases examined hydrolyzed both TG and pure PC in aqueous solution. Activities were considerably greater toward triglycerides (Table 3). Lipozyme IM-20, which contains the lipase from *Rhizomucor miehei,* and SP-435, which contains *Candida antarctica* lipase B, displayed roughly comparable activities and were about 25-fold more active on TG than on PC (Table 3). In contrast, AY-30 lipase, produced by *C. rugosa* and immobilized on Celite, had a much higher relative activity toward TG and a TG/PC activity ratio in excess of 400 (Table 3). Differences in activity ratios are not unexpected because the structures of these three lipases show little homology (31–33). Despite numerous attempts at pH values between 3.5 and 9, and the use of as much as 100 mg of enzyme preparation, no activity was demonstrated by Amano phospholipase B toward either TG or PC in this assay, which has a practical lower detection limit of 2 Units (U) of lipolytic activity.

Hydrolytic activities toward TG and PL in organic solvents. Figure 1A presents the time course of hydrolysis obtained when 35 mg of Lipozyme IM-20 was incubated with soy oil and semi-pure PC under the conditions described in the *Determination of hydrolytic activity* section. The reaction contained 60 μL of added water, the amount conferring maximum enzyme activity in this system (data not shown). Because the PC used also contained PE, the hydrolysis of both these phospholipids could be monitored. TG was rapidly and completely hydrolyzed, full hydrolysis being achieved within 10–20 h of incubation. PC and PE were hydrolyzed at essentially equal rates, much lower than that of TG. Lipozyme was unable to completely hydrolyze the phospholipid substrates, with the reaction stopping at about 60% completion after 40–50 h of incubation.

Although these reactions were not monitored in a continuous mode, it is possible to estimate initial reaction rates from Figure 1A. This yields an estimated inital activity toward TG and PC of 4×10^{-2} and 1.6×10^{-3} U/mg of enzyme, respectively. Compared to the corresponding values in water (Table 3), this indicates a roughly tenfold reduction in activity. The ratio of the estimated activities toward TG and PC in hexane is 25, which agrees well with the corresponding value calculated for the aqueous reaction (Table 3).

The termination of PC and PE hydrolysis by Lipozyme IM-20 at 60% completion is in agreement with previous observations (26), and suggests that there may be a subpopulation of PL, perhaps with a particular type or family of fatty acids at the *sn*-1 position (where Lipozyme preferentially acts) which cannot be hydrolyzed by the enzyme. The possibility of using such a hydrolytic selectivity to fractionate PL mixtures, producing subpopulations with uniform fatty acid content, remains to be investigated.

The hydrolysis of TG–PC/PE mixtures by Celite-immobilized AY-30 lipase was investigated by using reactions that contained 45 mg of enzyme and 80 μL water, the optimum for the conditions employed (data not shown). The progress curve for hydrolysis (Fig. 1B) was similar to that for

TABLE 2
Enzymes Investigated

Name	Supplier[a]	Source	Nominal specificity	Form
Lipozyme IM20	Novo	*Rhizomucor miehei*	Acylglycerols 1,3-	Immobilized on Duolite
Novozyme 435	Novo	*Candida antarctica* Lipase B	Acylglycerols nonspecific	Immobilized on Acrylic
AY-30	Amano	*C. rugosa*	Acylglycerols nonspecific	Immobilized on Celite C211
Phospholipase B	Amano	*Corticium* sp.	Phosphoglycerols nonspecific	Powder

[a]Novo (Danbury, CT); Amano (Troy, VA).

TABLE 3
Aqueous Hydrolytic Activity of Selected Lipases

Enzyme[b]	pH	Activity (U/mg)[a] Olive oil	Activity (U/mg)[a] Phosphatidylcholine	[lipolytic/phospholipolytic] Ratio
Lipozyme IM20	8	5.2×10^{-1}	2.3×10^{-2}	23
Novozyme 435	9	2.7×10^{-1}	9.8×10^{-3}	28
AY-30/Celite	9	2.1	4.6×10^{-2}	456
Phospholipase B	Various	N.D.[c]	N.D.	—

[a]U = μmole fatty acid released per minute.
[b]Lipozyme IM20 and Novozyme 435 from Novo Biolabs; AY-30/Celite and phospholipase B from Amano (source locations as in Table 2).
[c]N.D. = none detected.

Lipozyme (Fig. 1A). Complete TG hydrolysis was achieved within 20 h, while hydrolysis of the PL was incomplete after even 80 h. Based on the data of Figure 1B, the estimated initial activity toward TG was 1.5×10^{-2} U/mg enzyme, roughly

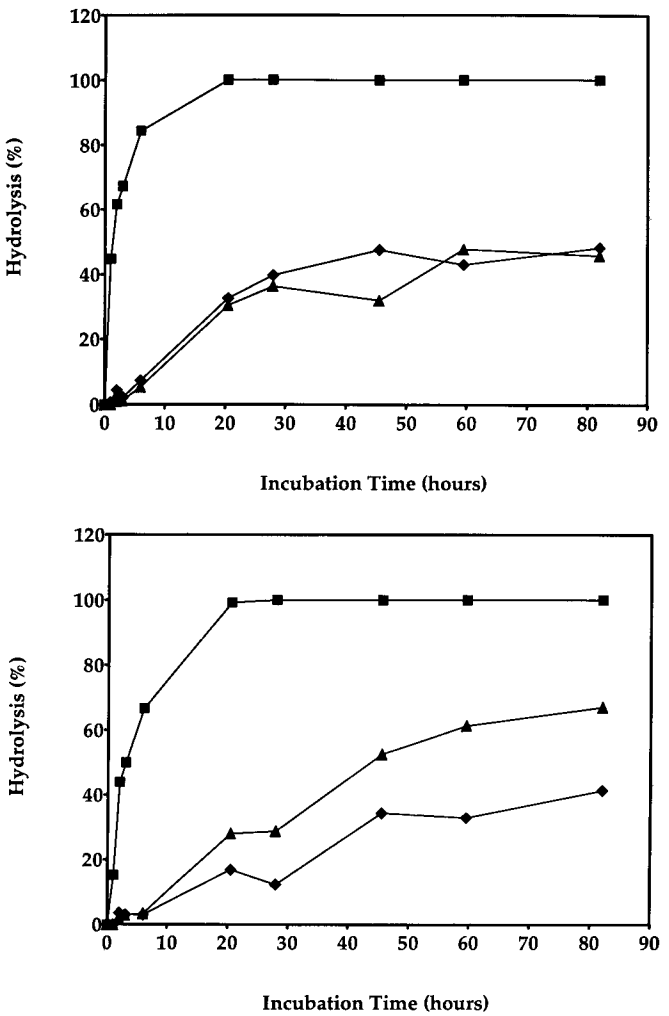

FIG. 1. Time course of the hydrolysis of a mixture of soybean triacylglycerols, phosphatidylcholine, and phosphatidylethanolamine in hexane by the immobilized lipases (A) Lipozyme IM-20 (35 mg; Novo Biolabs, Danbury, CT) and (B) AY-30/Celite (45 mg; Amano Enzyme U.S.A. Co., Ltd., Troy, VA). The reaction conditions and methods used to assess the degrees of hydrolysis are described in the text. Triacylglycerol, ■; phosphatidylcholine, ▲; phosphatidylethanolamine, ◆.

100-fold lower than the corresponding activity in water (Table 3). The estimated hydrolytic activity toward PC in hexane was 1.2×10^{-3} U/mg enzyme, approximately 40-fold lower than the corresponding activity in water. The ratio of the activities toward TG and PC in hexane was 13, comparable to the ratio calculated for Lipozyme. The corresponding ratio for the AY-30 lipase in water was 456 (Table 3). The reduction in this ratio in hexane is due to greatly reduced activity toward TG in hexane as compared to TG in water.

Both SP-435 and Amano phospholipase B were inactive toward semi-pure PC in hexane. However, they did show activity toward pure PC, which was therefore used as the PL source for these enzymes. The time course of PC hydrolysis by 20 mg of lipase SP-435 in hexane solutions containing various amounts of water is shown in Figure 2A. There was moderate PC hydrolysis in the absence of added water. Hydrolytic activity was greatest in the presence of small amounts of added water, between 5 and 15 μL per 8 mL reaction, and greatly reduced at higher water levels. Under the optimal conditions, maximum hydrolysis, exceeding 90%, was obtained. Based on the data in Figure 2A, the initial activity of SP-435 toward PC in hexane at the optimal water levels is estimated to be approximately 2×10^{-2} U/mg. This is similar to the value for the comparable reaction in water (Table 3).

Surprisingly, despite its significant activity toward TG in water (Table 3), lipase SP-435 was completely unable to hydrolyze this substrate in hexane, regardless of the amount of added water. The cause of this behavior is not known. Because PL is still hydrolyzed, it is clear that the active site geometry and, probably, the overall three-dimensional structure of the enzyme are relatively intact. In aqueous solution, lipases are inactive against dissolved substrates but active against water-insoluble ones. This is thought to result from the fact that under these conditions the active site is covered by a portion of the enzyme, known as the lid (31). Lids have been found in virtually all lipases examined to date, including the enzyme in SP-435 (33). The lid is believed to move in the presence of an oil–water interface, exposing the active site and some hydrophobic surface residues to substrate (34,35). We have determined that Lipozyme IM-20 and AY-30/Celite are capable of hydrolyzing TG in hexane, regardless of the presence of PL (data shown here, and Haas, M.J., D.J. Cichowicz, W. Jun and K. Scott, unpublished work). In such

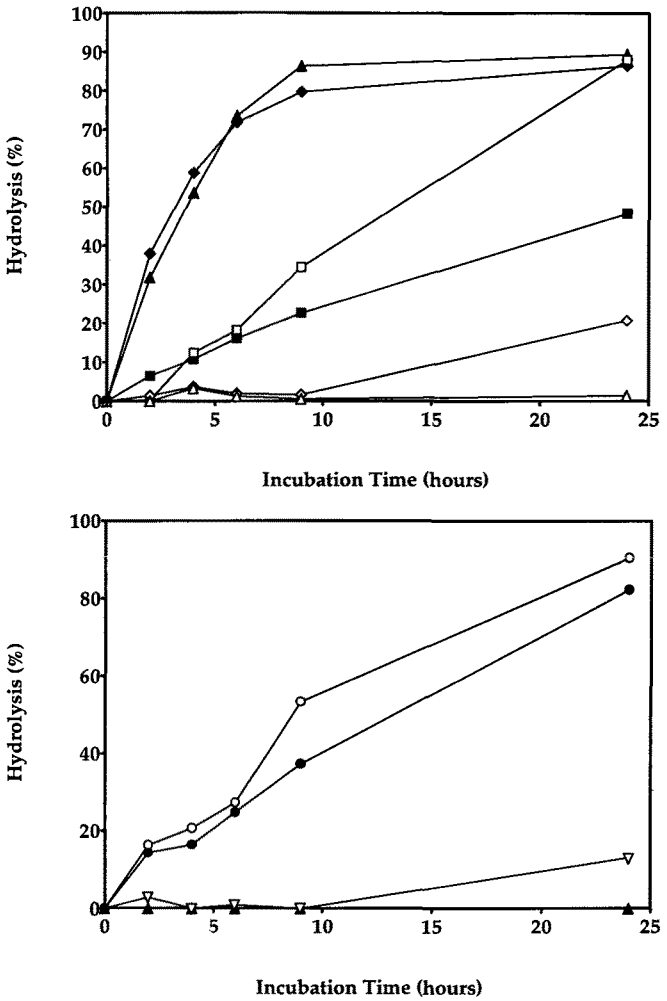

FIG. 2. Time course, and dependence on added water, of the hydrolysis of soybean phosphatidylcholine (PC) in a hexane solution containing both triacylglycerol and PC. (A) Lipase SP-435 (20 mg; Novo Biolabs), (B) phospholipase B (20 mg; Amano). Company sources as in Figure 1. Reaction conditions and methods of determination of degrees of hydrolysis are described in the text. Amounts of added water (μL): 0, ■; 5, ◆; 10, ▲; 15, □; 20, ◇; 25, △; 30, ○; 50, ●; 70, ▽.

a solvent, the TG is completely soluble, and no substrate–solvent interface exists. The fact that TG are hydrolyzed by these lipases under these conditions indicates that their active sites are exposed to substrate. Perhaps the hydrophobicity of the solvent is sufficient to cause movement of the lid, or the lid is spontaneously in the "open" configuration some portion of the time or in some fraction of the enzyme population. The inactivity of the SP-435 lipase against TG in hexane may indicate that for this enzyme a hydrophobic solvent is not in itself sufficient to expose the active site. Presumably some micelles are formed when PL and water are present in the hexane, providing the hydrophobic surfaces necessary for activation of SP-435. The failure of SP-435 to hydrolyze TG suggests that this enzyme may be suitable for the hydrolytic removal of PL from mixtures of PL and TG, without affecting the latter component.

As observed in aqueous reactions (Table 3), Amano phospholipase B did not hydrolyze TG in hexane. However, this enzyme was active against PC in this solvent. Figure 2B depicts the hydrolysis of PC in a mixture of TG and pure PC by 20 mg of phospholipase B in hexane. As with all the enzymes studied here, activity was sharply water-dependent, with the greatest rate and extent of hydrolysis occurring in reactions containing 30–50 μL of water over and above that required to saturate the 8 mL reaction volume. Previous work has shown that the water requirements in these reactions are unique to each enzyme, and vary in proportion to the substrate concentration (27). At the optimal water concentration of 30 μL, virtually complete PC hydrolysis was probably obtained within 15–20 h (Fig. 2B). This is comparable to the time required by SP-435 (Fig. 2A), whose phospholipolytic activity previously has not been appreciated. It must be kept in mind, however, that these two enzyme preparations differ in form (immobilized vs. powder) and probably also in protein content. Therefore, their activities are not strictly comparable on a mass basis.

Hydrolysis of the lipid components of soybean soapstock. Soybean soapstock is a heterogenous mixture of several components. With the HPLC techniques described in the Materials and Methods section, it was possible to separate, identify, and quantitate the TG, PC, and PE components of this material (Fig. 3).

No hydrolysis occurred when hexane solutions of soapstock were initially incubated with lipases SP-435 and AY-30/Celite. However, when this alkaline material (pH 10.3) was neutralized with HCl before being dissolved in hexane, hydrolytic activity was detected. Therefore, the effects of pH upon the susceptibility of soapstock to enzymatic digestion were examined further. Aliquots of soapstock were adjusted to pH levels between 3.5 and 9, dissolved in hexane (0.5 g/8

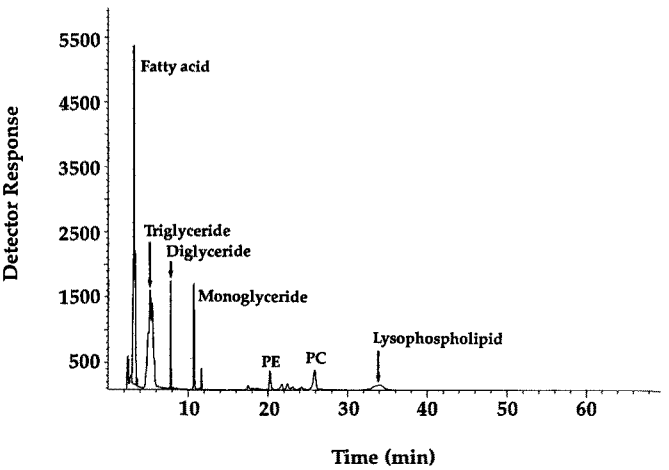

FIG. 3. Fractionation of soybean soapstock (1.25 mg in 20 μL hexane) by high-performance liquid chromatography. The chromatographic system was as described in the text, with elution of the column according to the solvent timetable described in Table 3. The identities of the peaks were established by reference of their retention times to those of pure compounds. PC: phosphatidylcholine; PE: phosphatidylethanolamine.

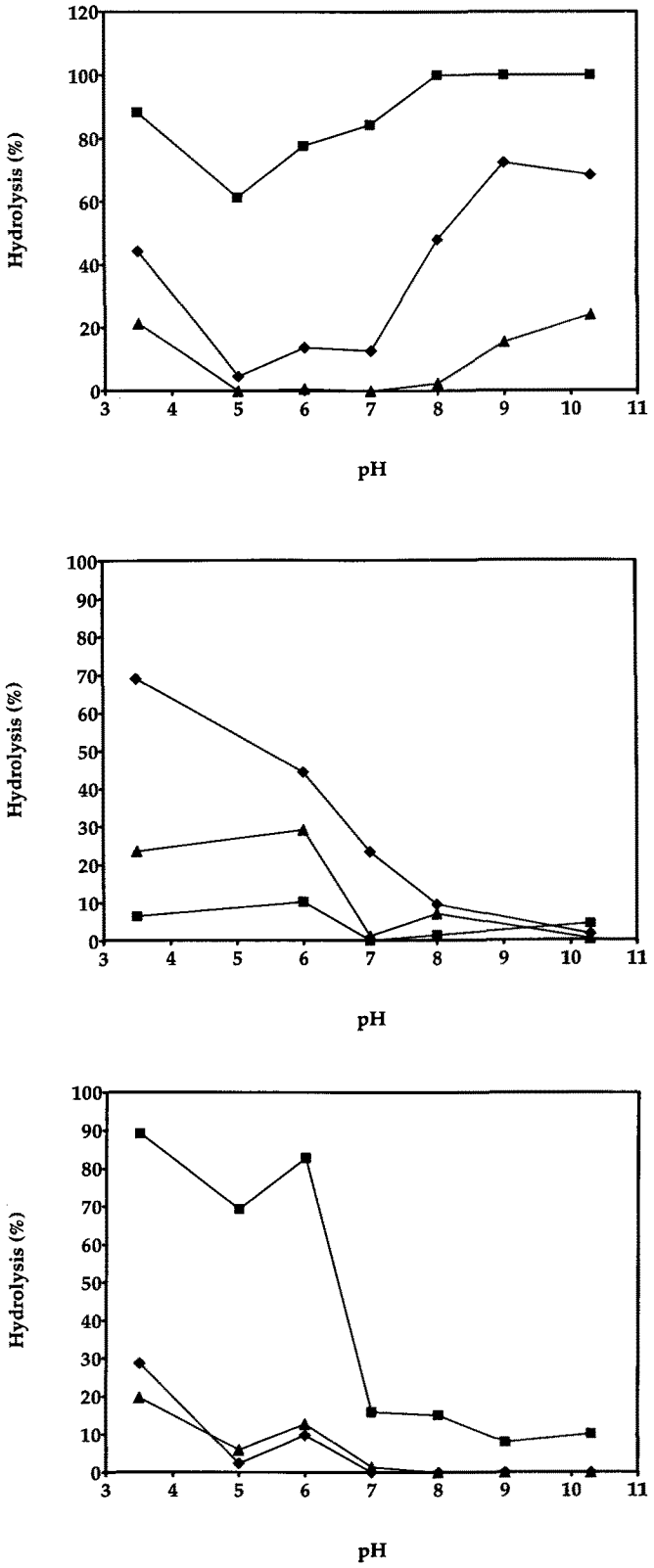

FIG. 4. pH-Dependence of the hydrolysis of the triacylglycerol (■), phosphatidylcholine (▲), and phosphatidylethanolamine (◆) components of soybean soapstock (0.5 g) in hexane (8 mL) by (A) Lipozyme IM-20 (50 mg, 20 h), (B) SP-435 (100 mg, 20 h), and (C) AY-30/Celite (150 mg, 18 h). Details of the reaction conditions and determination of the extents of hydrolysis are described in the text. Company sources and locations as in Figures 1 and 2.

mL reaction), and incubated with the enzymes (Fig. 4). Lipozyme IM-20 (50 mg) was most active against unneutralized soapstock (Fig. 4A), hydrolyzing all of the triglyceride, 70% of the PE, and about 20% of the PC in 20 h. This behavior is analogous to that seen in aqueous solution, where optimal activity was seen at pH 9. This enzyme displayed good activity against TG at all pH values tested, and it also hydrolyzed PE moderately well at pH 3.5 (Fig. 4A). (Nonenzymatic TG and PL hydrolysis was not detected at any pH value.) The pattern of lower activity against PC, compared with PE, is similar to that reported by Slotboom *et al.* (9), who found that anionic phospholipids were digested more rapidly by lipase than were zwitterionic ones.

In contrast to Lipozyme IM-20, and to its own behavior in aqueous solutions (Table 3), SP-435 lipase (100 mg, 20 h) was inactive toward the TG and PL components of soapstock at alkaline pH (Fig. 4B). The inactivity toward TG extended to all pH values examined and is consistent with the observation, with more defined substrates, that SP-435 does not hydrolyze TG in hexane. Activity was demonstrated toward PL in acidified soapstock (Fig. 4B), increasing steadily as the acidity of the substrate was reduced. Seventy percent of the PE was hydrolyzed at pH 3.5, the lowest pH examined, whereas PC hydrolysis never exceeded 30%. It is a notable feature of this enzyme that both its substrate range and pH-activity profile vary greatly between aqueous and organic solvents.

As with SP-435, lipase AY-30/Celite (150 mg, 18 h) was virtually inactive on alkaline soapstock (Fig. 4C). Unlike SP-435, however, at acidic pHs, this enzyme was quite active against TG and only slightly active on PL. The data suggest that more complete PL hydrolysis might be achieved by this enzyme at pH values even lower than those examined here.

Regardless of pH, neither the TG nor the PL components of soapstock were hydrolyzed by Amano phospholipase B (150 mg, 20 h) in hexane. The catalyst was apparently hygroscopic, and formed a liquid drop rapidly in the bottom of the reaction tube. This resulted in poor mixing of the enzyme with the hexane phase that contained the substrates, and it also may have inactivated the enzyme. For water-rich substrates, such as soapstocks, the successful use of Amano phospholipase B as a catalyst may rely on reducing the hygroscopic nature of the enzyme preparation.

TABLE 4
Effect of Neutralizing Acid upon Soapstock Hydrolysis by Immobilized *Candida rugosa* Lipase

Neutralizing acid	% Hydrolyzed[a]		
	TG	PE	PC
Hydrochloric	95	28	17
Sulfuric	87	10	1
Acetic	72	18	5
Nitric	95	22	14
Phosphoric	94	20	3

[a]TG, triacylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

To investigate the role of the identity of the neutralizing acid upon enzymatic hydrolysis, portions of soapstock were adjusted to pH 7 with various inorganic acids, and aliquots (0.2 g) were incubated with immobilized AY-30 lipase (0.2 g) in 8 mL hexane for 20 h. The choice of neutralizing acid had some effect on the efficiency of hydrolysis (Table 4). This effect was not large for TG—more than 90% hydrolysis was achieved in samples neutralized with nitric, hydrochloric, or phosphoric acids. The degree of hydrolysis was reduced slightly when either sulfuric or acetic acid was used in neutralization, but even then hydrolysis exceeded 70%. PL hydrolysis was best in samples neutralized with nitric or hydrochloric acid and poorest when sulfuric acid was employed. As the degree of activity reduction toward PL is much greater than that toward TG, it is unlikely that the acid effect is due to a nonspecific interaction of the acid with the enzyme. (Such an interaction would be expected to result in comparable reductions in activity toward all substrates.) Rather, the data suggest that the acid effect is due to some interaction between the acid and the substrate. This interaction particularly affects enzyme activity in the case of phospholipids. The data indicate that the best overall hydrolysis by AY-30 lipase would occur when either nitric or hydrochloric acid is used as neutralizing agent. Of all the conditions examined here, the most complete hydrolysis of the TG and PL components of soapstock was achieved by the use of Lipozyme IM-20 without pH adjustment of the substrate.

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