The Hydrolysis of Phosphatidylcholine by an Immobilized Lipase: Optimization of Hydrolysis in Organic Solvents

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The ability of a commercial immobilized lipase preparation (Lipozyme) to hydrolyze the fatty acyl ester bonds of soybean phosphatidylcholine in organic media was investigated. Response surface methodology, based on a Modified Central Composite design, was employed to examine the effects on hydrolysis of solvent polarity, water, pH, duration and temperature of incubation, and the amounts of substrate and catalyst. A second-order regression model was developed, which allows evaluation of the effects of these parameters, alone or in combination. Hydrolysis increased in a relatively straightforward manner in response to increases in incubation time and the amount of catalyst and was approximately constant over the range of substrate amounts studied. Solvent polarity had a profound effect on the degree of hydrolysis, and the qualitative and quantitative degrees of this effect were dependent upon the values of the other parameters studied. Conditions were identified where enzyme activity was strong in either nonpolar or polar solvents, with activity increasing as the polarity of the medium increased. Enzyme activity was minimum at about 37°C, increasing below and above this temperature. Activity was not affected by the presence of acid or base in the reactions. Increasing amounts of water stimulated enzyme activity in solvents more polar than hexane, while in less polar solvents water inhibited activity.

KEY WORDS: 2-Butanone, dodecane, hexane, hydrolysis, isooctane, lipase, Lipozyme, 2-octanone, phosphatidylcholine, phospholipid.

Phospholipids (PL) comprise up to 80% of the mass of biological membranes. As such, they are plentiful constituents of all living organisms and play vital roles in membrane integrity, function and cell viability. Due to their superior emulsification properties, PLs and their partial hydrolysis products, lysophospholipids (LPL), find numerous applications in the food, pharmaceutical, personal care and other industries. There is interest in the hydrolysis of PLs: to generate desirable LPLs, to modify the fatty acid composition of PLs through re-esterification, to remove PLs from preparations of other biological materials and to recover their fatty acid constituents for other applications.

Conventional methods of fat hydrolysis are inappropriate for phospholipids, which foul the reactors used in high-temperature, high-pressure fat splitting. Enzymatic catalysis offers the potential of a nondestructive, energy-efficient route for PL hydrolysis. Numerous phospholipases of the A_1 and A_2 types have been identified, and their abilities to hydrolyze the fatty acyl ester bonds of PLs have been characterized. However, these enzymes are not readily or economically available in the amounts required for the conduct of large-scale reactions.

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Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) also hydrolyze the fatty acyl ester bonds of PLs. In 1964, Laboureur and Labrousse first demonstrated this capability for *Rhizopus arrhizus* lipase (1). Phospholipolytic activity has since been demonstrated in several purified lipases, including those from porcine pancrease (2,3), human liver (4) and *Staphylococcus hyicus* (5). This capability has been employed for the preparation of PLs and LPLs of defined structure (6,7) and for the removal of PL contaminants from preparations of plasmalogens (8,9).

The studies cited above were all conducted in aqueous media. The limited water solubilities of PLs restrict the utility of such systems for PL hydrolysis. PLs are substantially more soluble in organic solvents, and it has been shown that lipases are active against them in these solvents. For example, using polyethylene-glycol-modified *Candida cylindracea* lipase in benzene, Yoshimoto *et al.* (10) reported the interesterification of PLs. The incorporation of free fatty acids into phosphatidylcholine (PC) by lipases was observed by Yagi *et al.* (12) with 1:1 water/hexane as the solvent and by Svensson *et al.* (11) with water-saturated toluene. Totani and Hara (13) investigated the transesterification of sardine oil fatty acids into soy PL in an equivolume mixture of water and hexane. In these studies, hydrolysis of the fatty ester bonds of the substrate was noted. However, because it was considered an undesirable competing reaction, steps were taken to minimize hydrolysis.

We are unaware of any published report of the ability of a lipase to hydrolyze PLs in organic solvents. Since PLs are plentiful constituents of biological materials and because they and their hydrolytic breakdown products can have profound physiologic roles and industrial applications, we have investigated the ability of a commercially available immobilized lipase to hydrolyze PC in organic media. A statistical experimental design was employed to coordinately examine the effects of several reaction parameters upon hydrolysis.

EXPERIMENTAL PROCEDURES

Materials. L-α-Phosphatidylcholine, (>99%, from soybeans) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Pure soybean PC for use as an analytical standard was obtained from Matreya, Inc. (Pleasant Gap, PA). Lipozyme IM20, consisting of *Rhizomucor miehei* lipase immobilized on the medium-base anion exchange resin Duolite 568 (14–16), was obtained from NOVO Biolabs (Danbury, CT). 2-Butanone, 2-octanone, 2,2,4-trimethylpentane (isooctane) and dodecane were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Burdick and Jackson Brand hexane and isopropanol were purchased from Baxter (Muskegon, MI). Solvents used in hydrolysis reactions were saturated at room temperature with distilled, deionized water prior to use.

Experimental design. The effects of seven factors upon the enzymatic hydrolysis of PC were examined. These were

solvent polarity, pH, incubation time and temperature and the concentrations of substrate, catalyst and water. The concept of pH is not a meaningful one in organic solvents. However, because the reactions involved an ionizable substrate and catalyst and because a distinct aqueous phase was present, the effects of acid or base upon hydrolysis were investigated. Solvent polarities were expressed and ranked in terms of the log *P* value as discussed by Laane *et al.* (17). The greater the log *P*, the less polar the solvent.

A Modified Central Composite design (18) consisting of 143 experimental points was used to investigate the effects of the seven experimental factors on hydrolysis and to yield a response surface regression equation. With 24 of the factorial settings duplicated and with repeated runs at the 'midpoint' to increase rotatability (18), a total of 189 experiments were conducted.

The experimental settings of the seven variables for the design are shown in Table 1. The minimum, maximum and centerpoint settings of these variables represent the "star" portion of the design, and the remaining settings comprise the factorial part. The organic solvents employed, corresponding to the log *P* values listed in Table 1, were: 2-butanone (smallest log *P*), 2-octanone, hexane, isooctane and dodecane. The centerpoint values, identified in a series of preliminary experiments, were those under which Lipozyme exhibited moderate activity in hexane.

Hydrolytic reactions. Preweighed PC and 8.00 mL of water-saturated solvent were placed in 20 × 150 mm screw-cap test tubes. These were capped and the PC was dissolved by shaking at 37°C. After returning to room temperature, the "pH" was adjusted by adding sufficient HCl or NaOH from stock solutions to adjust the reaction, had it been aqueous, to the desired pH. Water was added to achieve the desired final amount in consideration of that added during pH adjustment. Lipozyme was then added, tubes were capped, sealed with Teflon tape and shaken at 300 rpm in a constant-temperature incubator. Under the conditions employed here, nonenzymatic hydrolysis and binding of PC by Lipozyme were undetectable.

Analysis of hydrolysates. After incubation, shaking was stopped and the samples were allowed to reach room temperature. Twelve mL of isopropanol was added to each. One-hundred μL of the mixture was added to 2.90 mL of hexane/isopropanol (2:3, vol/vol), mixed and filtered through a Pasteur pipet containing a glass wool plug. Samples were stored at −20°C until analysis.

Residual PC was determined by normal-phase high-performance liquid chromatography (HPLC) on a 3 × 100 mm LiChrosorb DIOL column (Chrompack Inc., Raritan, NJ) eluted with a mobile phase consisting of hexane/isopropanol/water (40:56.25:3.75, vol/vol/vol) at a flow rate of 0.5 mL/min. The chromatography system consisted of a Shimadzu SCL-6A System Controller (Shimadzu Corp., Columbia, MD), two Shimadzu LC-6A pumps, a Rheodyne 7125 manual injector with a 20-μL loop (Rheodyne, Cotati, CA), a Waters Model 450 variable wavelength detector operating at 250 nm (Waters Associates, Bedford, MA), and a Spectra Physics SP4270 integrator (Spectra Physics Corp., San Jose, CA). In this system PC is baseline-resolved from lysophosphatidylcholine (LPC) and free fatty acids. The detection of analyte is based upon the absorption of 250 nm radiation by the double bonds of fatty acids. Reanalysis of selected samples with a mass-based detector (ELSD IIA Varex, Burtonsville, MD) confirmed that spectral detection was an appropriate method of assessing the degree of hydrolysis.

The hydrolysis of PC was calculated according to the equation:

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

RESULTS AND DISCUSSION

An immobilized lipase preparation was chosen to examine the hydrolysis of PC in organic media because it is readily available and also because the deposition of an enzyme on the surface of a granular carrier is an efficient manner of presenting the catalyst to the substrate. *R. miehei* lipase, the lipase in Lipozyme, hydrolyzes only the primary ester bonds of triglycerides (19) in aqueous solution. The enzyme also demonstrates this specificity in the interesterification of PL in organic solvents (11,12). LPC was generated from PC by Lipozyme. 1-LPC and 2-LPC were separated and resolved in the HPLC system employed here. Through the use of appropriate standards, it was determined that LPC produced from PC by Lipozyme was exclusively 2-substituted. Thus the enzyme retained its characteristic specificity in hydrolyzing PC.

To adequately and efficiently cover the seven-factor variable space of interest, a response surface experimental design was employed. Five values of each factor were studied, with the composition of each reaction dictated by a Modified Central Composite design (18). The ranges of enzyme amount, water and incubation time were chosen *via* preliminary studies, which identified conditions

TABLE 1

Settings for the Seven Experimental Factors Examined

Factor	Minimum		Centerpoint		Maximum
pH	3.5	6.0	7.0	8.0	10.5
Water (μL)	0	52.50	75.00	97.50	150.00
Polarity (log *P*)	6.6	4.5	3.5	2.4	0.29
(solvent)	(dodecane)	(isooctane)	(hexane)	(2-octanone)	(2-butanone)
Temperature (°C)	25.00	37.25	42.50	47.75	60.0
PC (g)	0.100	0.262	0.331	0.400	0.562
Time (h)	1.00	8.00	11.00	14.00	21.00
Enzyme (g)	0.013	0.06	0.08	0.10	0.147

resulting in 5 to 80% hydrolysis. These preliminary experiments were conducted in hexane, a solvent frequently used in the fats and oils industry. Its polarity was also selected as the centerpoint of the solvent polarity range. Incubation temperatures were chosen below the boiling points of the solvents examined.

In aqueous media, many lipases are activated by calcium (20,21). However, preliminary experiments indicated that it did not affect the hydrolysis of PC by Lipozyme in organic media. Therefore, calcium was not included in this study.

The estimated regression model resulting from the execution of the Modified Central Composite experimental design is shown in Equation 2:

$$\begin{aligned}\text{predicted \% hydrolysis} = {} & 250.6 - 7.008\,A + 1.704\,B - 4.088\,C \\ & - 9.391\,D - 432.6\,E + 0.7188\,F - 343.5\,G + 0.8465\,A^2 \\ & + 0.0044\,AB + 0.0007\,B^2 - 0.2836\,AC - 0.1897\,BC \\ & + 1.025\,C^2 - 0.0198\,AD - 0.0192\,BD - 0.1436\,CD \\ & + 0.1176\,D^2 - 3.494\,AE + 0.0212\,BE + 62.95\,CE + 1.672\,DE \\ & + 307.8\,E^2 - 0.1089\,AF - 0.0168\,BF - 0.8342\,CF \\ & + 0.0682\,DF - 3.585\,EF + 0.0897\,F^2 - 12.37\,AG - 3.671\,BG \\ & - 39.66\,CG + 14.75\,DG - 418.3\,EG + 28.23\,FG + 2452\,G^2 \end{aligned} \quad [2]$$

where A = pH, B = water (μL), C = solvent polarity (log *P*), D = temperature (°C), E = substrate (g), F = incubation time (h), G = enzyme (g), n = number of experiments (189), R^2 = coefficient of determination (0.8088), and s^2 = the estimated variance due to residuals (86.8). The regression equation was significant ($P < 0.0001$) and accounted for over 80% of the total variability in the response.

A canonical analysis of Equation 2 indicated that the most important factors affecting hydrolysis were solvent polarity, incubation temperature, the cross product of polarity and substrate concentration, and the cross-product of polarity and water concentration. The effects on hydrolysis of these and other factors are discussed below.

When calculated by Equation 2, the degree of hydrolysis was predicted to increase in a smooth fashion in response to increases in the length and temperature of incubation and the amount of catalyst employed. Figure 1 illustrates a typical response of this type, depicting the degree of hydrolysis as a function of the length of incubation and the amount of Lipozyme. To analyze the results graphically, it was necessary to fix the values of five of the variables in order to examine the dependence of hydrolysis upon the remaining two. In Figure 1 and in most of the figures presented here, these nonvariant parameters were set to the midpoints of their ranges. The height of the response surface changed as other values were chosen, but the general shape of the surface was unaltered. This phenomenon was observed throughout the data set. In the figures, the reader will note an absence of uniformity in the direction that indicates an increase on the x or z axes. The direction of these axes were chosen in each case so as to maximize the readers' view of the response surface. Predictive data gathered with a Central Composite

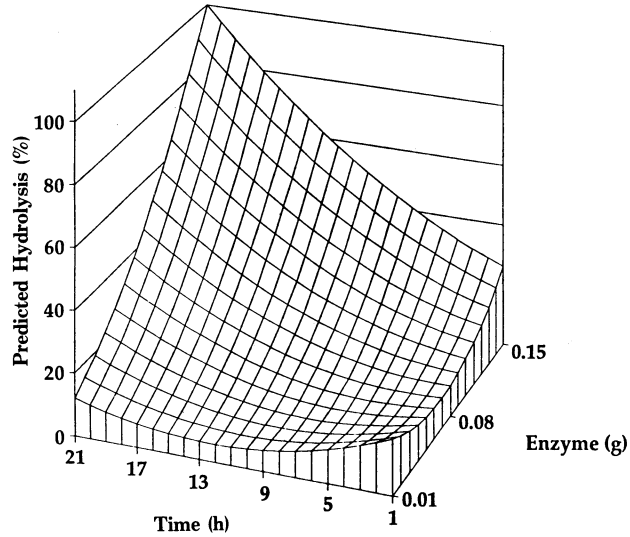

FIG. 1. The effects of the amount of Lipozyme and the length of incubation on the predicted degree of phosphatidylcholine (PC) hydrolysis, calculated according to Equation 2. The values of incubation temperature, pH, added water, media polarity and amount of PC were the centerpoints of the ranges examined in this experimental series, as indicated in Table 1. Reaction volume: 8 mL.

experimental design are most reliable in the interior ranges of the variables studied and less reliable near the edges of the variable space.

The hydrolysis reaction was not first-order dependent on the amount of catalyst or the incubation time when both of these values were small (Fig. 1). This may be correlated with the fact that the reaction mixtures became viscous during the initial phases of incubation and prevented efficient mixing of catalyst and substrate. After the initial viscous period, the reactions became more fluid, even in the absence of catalyst. The length of the viscous period was diminished in tubes where the PC underwent hydrolysis. At short incubation times and in tubes with small amounts of catalyst, however, this period lasted as long as 12 to 17 h. This severe viscosity and the concomitant reduction in the access of catalyst to substrate probably account for the low degree of hydrolysis under conditions of low catalyst and incubation time. After the viscosity fell, hydrolytic rates increased approximately twofold with doublings in reaction time (Fig. 1).

Figure 2 displays the predicted hydrolysis as a function of the amount of substrate present and the incubation temperature. The degree of hydrolysis was highest in reactions containing small amounts of PC and fell off at higher substrate loadings. However, when the amount, rather than the degree, of PC hydrolysis is considered under the conditions of Figure 2, the model predicts net hydrolysis to increase continuously as the substrate concentration increases (Fig. 2). Thus, substrate saturation of the enzyme was not observed in the range of PC concentrations and reaction conditions studied here.

Figure 2 also indicates that hydrolysis was minimum at about 37°C. Below this temperature the degree of hydrolysis was slightly higher. Above 37°C it was significantly increased. We are unaware of other reports of a minimum reactivity of a lipase in organic solvent at 37°C and suspect that it may not represent a significant

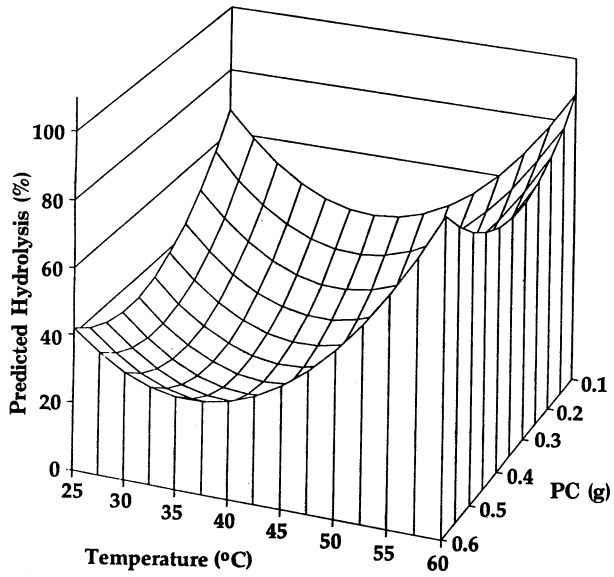

FIG. 2. The effects of the amount of substrate and the incubation temperature on the predicted degree of phosphatidylcholine (PC) hydrolysis by Lipozyme, calculated according to Equation 2. The values of incubation time, pH, added water, media polarity and amount of Lipozyme were the centerpoints of the ranges examined in this experimental series, as indicated in Table 1. Reaction volume: 8 mL.

difference from the level of activity at 25°C. Above about 37°C, hydrolysis increased as a smooth function of temperature at all values of other variables, as is shown for one combination of variables in Figure 2. The enzyme was active up to at least 60°C. Because hexane boils at 69°C, no higher temperatures were examined.

The predicted hydrolysis response surface indicated a distinct interaction between solvent polarity and substrate concentration (Fig. 3). At low substrate levels the degree of hydrolysis was greatly affected by the polarity of the media, ranging from essentially 0 to greater than 80% over the range of polarities examined. At higher substrate concentrations the polarity of the media had less of an impact on hydrolysis. The effect of polarity upon hydrolysis is further discussed below. Figure 3 also indicates that in solvents more polar than hexane (*i.e.*, log $P < 3.5$) the degree of hydrolysis falls coordinately with increases in substrate amount, with approximately the same amount of PC hydrolysis predicted to occur regardless of the initial PC concentrations. This indicates that the reaction may be substrate-saturated in these more polar solvents.

Water is a co-substrate in hydrolysis reactions, with one water molecule being consumed for each ester bond hydrolyzed. Water is also essential to the retention of enzyme activity in organic solvents (22,23). In studies of the lipase-mediated transesterification of phospholipids, it was noted that water could facilitate hydrolysis of the substrate (11–13). Our preliminary experiments indicated that water was required for PL hydrolysis, but that excessive amounts retarded enzyme activity. The effects of water on hydrolysis were therefore more fully examined. Measurable hydrolysis occurred in saturated solvents without additional water. Upon the addition of water, the

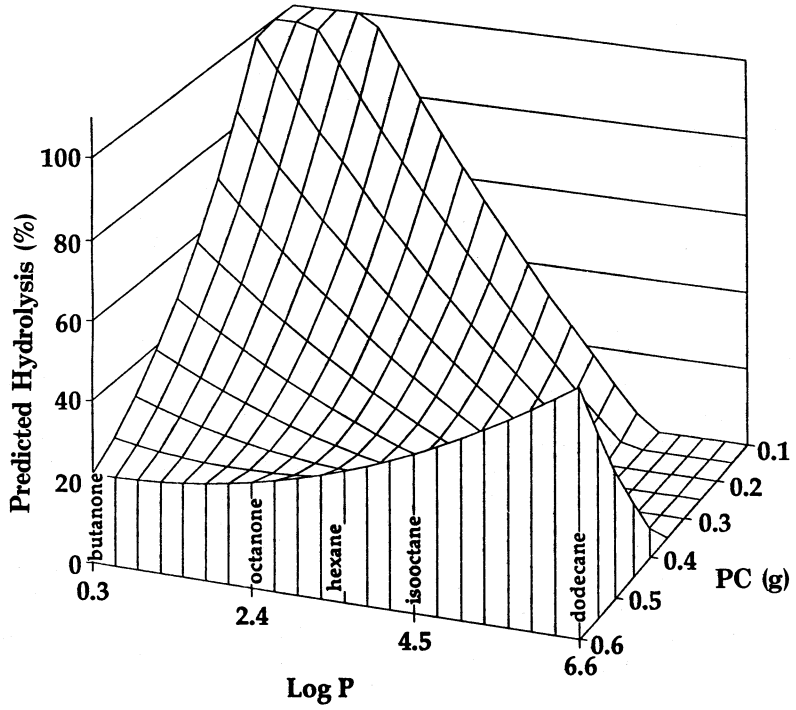

FIG. 3. The effects of the amount of substrate and the polarity of the solvent on the predicted degree of phosphatidylcholine (PC) hydrolysis by Lipozyme, calculated according to Equation 2. The values of incubation time, pH, added water, reaction temperature and amount of Lipozyme were the centerpoints of those examined in this experimental series, as indicated in Table 1. The solvents in which actual data were gathered were: 2-butanone (log P = .29), 2-octanone (log P = 2.4), hexane (log P = 3.5), isooctane (log P = 4.5) and dodecane (log P = 6.6). Reaction volume: 8 mL.

degree of hydrolysis varied depending on the amounts of water and of catalyst (Fig. 4). At low and moderate enzyme levels, the addition of water had little impact upon predicted hydrolysis in the hexane media shown in Figure 4. At higher enzyme levels hydrolysis was retarded by increasing amounts of water (Fig. 4). Thus, the impact of water levels upon hydrolysis is dependent upon the amount of catalyst present. Water levels also interact strongly with solvent polarity in their impact upon hydrolysis (see below).

Because PC contains ionizable groups, it was postulated that the addition of acid or base to the reaction might affect the suitability of PC as a lipase substrate. Na *et al.* (24) reported the hydrolysis of PC by phospholipase A_2 in aqueous-organic microemulsions to be sharply pH-dependent. However, in the systems employed here, the addition of acid or base did not affect the hydrolytic rate (data not shown). Regardless of the values of other variables, no more than a 10% variation in the degree of hydrolysis predicted was noted between pH values of 3.5 and 10. Some of this effect is probably due to the buffering action of the Duolite anion exchange resin in Lipozyme. It is also consistent with previous reports that the activity of an enzyme in an organic solvent is dependent upon the pH of its prior aqueous environment and is relatively insensitive to acid or base in the organic solvent (25,26). These data suggest that if one were attempting the hydrolysis of samples of PC in organic media there would be no need to monitor or adjust the pH of the substrate.

The most important single factor affecting the hydrolytic reaction was solvent polarity. In most cases, predicted and measured, activity increased as the polarity of the solvent was increased. Figure 5 presents an example of this behavior, displaying the effects of changes in polarity and incubation time upon the predicted extent of hydrolysis when other variables were at their mid-range

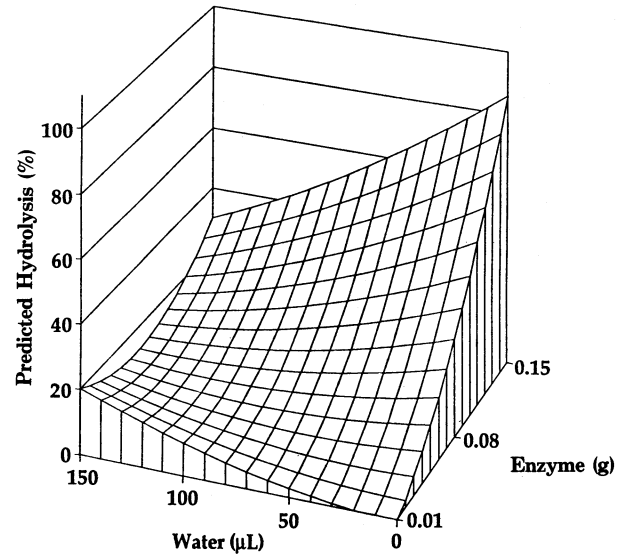

FIG. 4. The effects of added water and enzyme level on the predicted degree of phosphatidylcholine (PC) hydrolysis by Lipozyme, calculated according to Equation 2. The values of incubation time, pH, PC, reaction temperature and solvent polarity were the centerpoints of the ranges examined in this experimental series, as indicated in Table 1. Reaction volume: 8 mL.

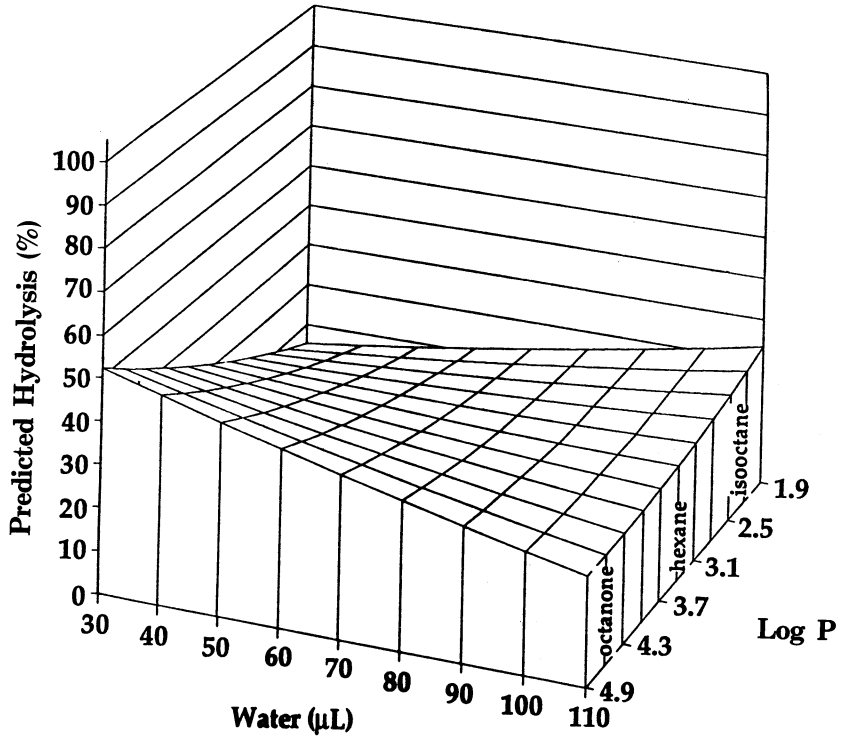

FIG. 5. The effects of added water and media polarity on the predicted degree of phosphatidylcholine (PC) hydrolysis by Lipozyme, calculated according to Equation 2. The values of incubation time, pH, PC, reaction temperature and the amount of catalyst were the centerpoints of the ranges examined in this experimental series, as indicated in Table 1. Reaction volume: 8 mL.

values. Thus, solvents such as 2-butanone and 2-octanone caused better hydrolysis than hexane and isooctane. To our knowledge this is the first time that such a relationship between polarity and enzyme activity has been observed. It is far more common for enzyme activity to increase as solvent polarity is reduced. For most enzyme reactions studied to date, catalytic activity was optimum in solvents less polar than hexane (*i.e.*, log $P > 3.5$) (23). The unique behavior reported here may be due to the relatively polar nature of the substrate under investigation, and it suggests a need to match the polarity of the solvent with that of the substrate.

There was little interaction between solvent polarity and the individual variables pH, enzyme amount, incubation temperature and incubation time. Regardless of the values of these factors, decreasing polarity caused a proportionate reduction in hydrolysis. The effect of polarity on hydrolysis was, however, modulated by the substrate concentration. At low PC concentrations, decreases in solvent polarity markedly reduced hydrolysis, whereas at higher substrate levels the effect was reduced or reversed (Fig. 3). The effect of polarity on hydrolysis was also dependent on the amount of added water. In nonpolar media, increases in the amount of water reduced hydrolysis, whereas similar amounts of water slightly enhanced hydrolysis in the most polar solvents examined (Fig. 5). This may indicate that in solvents of reduced polarity the enzyme is more susceptible to inactivation at the water-solvent interface.

Nonetheless, by using Equation 2 it was possible to identify conditions where substantial hydrolysis was predicted to occur in nonpolar solvents. Figure 6 displays the degree of predicted hydrolysis as a function of the amount of added water and the log P of the media under conditions different than those used in Figure 5. The chief differences are that in Figure 6 the temperature is 51, not 42.5°C, the incubation time is extended from 6 to 11 h, and the amounts and ratio of substrate and catalyst are different. Under these conditions (Fig. 6) the degree of hydrolysis is decreased in the more polar solvents examined and is increased in nonpolar ones compared to Figure 5. As a result, nonpolar media support more hydrolysis than polar ones. It is apparent from this illustration that the hydrolytic rate is not absolutely determined by the polarity of the reaction media. Rather, it is dependent upon the interactive effects of several reaction parameters.

Response surface methodology allows estimation of the combinations of reaction conditions under which optimal hydrolysis is predicted. With Equation 2, the predicted hydrolysis was calculated for 2187 combinations of experimental factors. These calculations were conducted for the central one-half of the full range of each variable factor to avoid the regions of less certain predictive ability that lie near the edges of the factor ranges. The highest predicted hydrolyses were calculated to occur in reactions containing the least amount of PC and the highest amount of enzyme incubated for the longest time in the most polar solvent.

A more practically relevant set of conditions would involve hexane (commonly used in industry) as the solvent, the maximum amount of PC and the minimum amount of enzyme. For the 2187 combinations of reaction factors

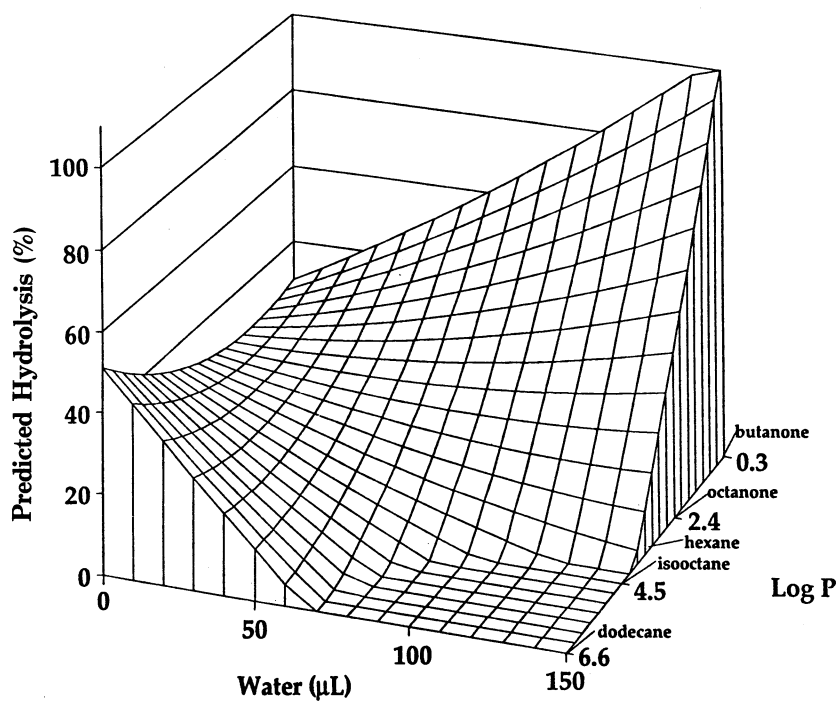

FIG. 6. The effects of added water and the polarity of the reaction media on the predicted degree of phosphatidylcholine hydrolysis by Lipozyme, calculated according to Equation 2, for pH, 7.0; incubation temperature, 51°C; incubation time, 6 h; substrate amount, 0.497; and Lipozyme, 0.047 g in an 8-mL reaction volume.

examined, Equation 2 indicated that maximum hydrolysis would occur in such a reaction at a pH of 5.25, with 37.5 μL of water added per 8 mL reaction and with the incubation conducted at the highest temperature in the range (51°C) for the maximum length of time (16 h).

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