Solvent suitability for lipase-mediated acyl-transfer and esterification reactions in microaqueous milieu is related to substrate and product polarities

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Lipase-mediated esterification and acyl-transfer reactions were evaluated in a series of solvents, ranging in log P (partitioning coefficient of solvent between 1-octanol and water) values from - 0.33 to 8.8, and for combinations of substrates of differing polarities. Contrary to the prevailing view, some of the model reaction systems evaluated were most active in solvents having log P values well below the range of 2 to 4 generally believed to be most supportive of biocatalysis in microaqueous organic media. As the polarity of the most polar substrate in the reactive mixtures was progressively increased, there was an attendant decrease in the magnitude and range of solvent log P values yielding maximum activity. When glycerol (log P of —3.0) was used as an acyl-transfer acceptor, log P values of solvents most supportive of activity were - 0.33 to 0.80. For acyl-exchange reactions between triacetin (log P of - 0.075) and olive oil, solvents having log P of 0.60 to 1.9 were most supportive of activity. For esterification reactions involving benzyl alcohol (log P of 1.1) or dodecanoic acid (log P of 4.8) as the most polar substrate, solvents of log P values >2.5 and 3.5, respectively, were most supportive of reaction. Although there are likely some specific solvent effects, we conclude that the choice of solvent to serve as bulk phase for biocatalytic processes should be made in cognizance of the relative polarity of substrates (and products) of the designed reaction. A secondary feature that should also be considered is the macroscopic phase behavior of reactive mixtures, although this parameter appears to be dependent on the particular source of enzyme.

Keywords: Microaqueous enzymology; lipase; esterification; acyl-transfer; organic solvents; solvent suitability; substrate polarity

Introduction

A rapidly expanding area of research in the past decade has been enzyme action in microaqueous organic media.[1-3] Reactive mixtures can be configured as enzyme solubilized within reverse micelles or simply as particulate enzyme suspended in organic solvents. In addition to the proper choice, or source, of enzyme, great emphasis has been placed on the choice of solvent to serve as continuous phase for enzyme reactions in microaqueous systems. A rather dogmatic view that has emerged in this field is that solvents most suitable for serving as the bulk phase are those with a log P (where P is partitioning coefficient of solvent between 1-octanol and water) value of >2, and preferably >4.[3-7] This view was partially based on the general trend of solvents having log P values < 2 being able to distort[5] or strip[8] enzyme-associated water. Thus, in simplest terms, the ideal solvent should dissolve substrates and not desorb or distort enzyme-associated water that is essential for activity.

Other studies have focused on mechanistically and quantitatively accounting for varying abilities of solvents to support enzyme action in microaqueous media. Some studies have reaffirmed that an increasing ability of the chosen solvent to dehydrate the enzyme is associated with a decreasing ability to support enzyme action in microaqueous media.[7,9,10] This water-sorbing property of solvents was accounted for by deriving a modified log P value that reflected the tendency of the particular solvent to sorb water; the modified log P value decreased progressively as the solvent was more capable of sorbing water.[9] Another approach attempted to quantify the water-sorbing effect of solvents

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based on kinetic principles.[10] Alternatively, a log P value modified by a second dimension of the solvent character, either electron pair acceptance index or polarizability, was suggested to be an improved predictor of solvent suitability.[7] In the latter case, a marginal improvement was observed over the single parameter of log P, and moreover, the same general rule that the greater the log P, the greater the suitability of the solvent to support enzyme action, was implied. A three-dimensional parameter of solvent character was also suggested to predict which solvents would be suitable for supporting enzyme action, but there were insufficient experimental data to verify the model.[11]

Another research focus regarding solvent suitability for biocatalysis is the evaluation of fundamental aspects of solvent effects on enzyme structure-function and their ability to support enzyme action. Alcohol dehydrogenase becomes more rigid as the dielectric constant of the medium (solvent) decreases.[12] The same was found for α-chymotrypsin, and it was suggested that a change in active site conformation or flexibility may be responsible for the altered stereoselectivities of enzymes in microaqueous media.[13] Water-miscible solvents appear capable of inducing some degree of enzyme denaturation, but it is speculated that their impact on enzyme action may be mostly manifested by an effect of stabilizing (or destabilizing) the ground state of a substrate capable of undergoing enzymic transformation.[14]

Only recently has the view been challenged that solvents with a greater log P are more capable of supporting enzyme action in microaqueous media.[15] Over a narrow range of log P values (- 0.33 to 0.96), there was little clear distinction between the ability to support lipase and protease activity and log P of the selected solvents. In addition, water-immiscibility for the series of solvents selected was not clearly relevant to the extent of enzyme activity.

Much of the work summarized above was generated by studies on a single enzyme reaction in a variety of solvents. During the course of our studies on enzyme action in microaqueous media, we took an alternative approach where a single enzyme (lipase) was used to mediate reactions with combinations of substrates of differing polarity. We found that the relationship between log P of the solvent and the ability of lipase to mediate specific reactions was not as simple as previously implied in the literature.

Experimental

Materials

Lipases were obtained from microbial sources, including *Pseudomonas* sp. (type PS-30, lot No. LPSA009517, Amano Enzyme Co., Troy, VA) and *(Rhizo)Mucor miehei* (Lipozyme™ IM-20, lots No. LM70753 and LM70504, Novo-Nordisk Bioindustrials, Inc., Danbury, CT). All solvents used were high-performance liquid chromatography (HPLC) grade and obtained from Aldrich Chemical Co. (Milwaukee, WI). Anhydrous butteroil was acquired from Level Valley Dairy (West Bend, WI). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Reactive mixtures

Glycerolysis reactive mixtures contained 10 ml 25% (v/v) butteroil in the selected organic solvent, 1.0 g glycerol and 40 µ1 water. After preincubation of the mixture to the reaction temperature of 35°C, reactivity was initiated by the addition of 200 mg lipase PS-30 powder. Reactive mixtures were incubated with rotary agitation (300 rev min ~ f o r 1 h and reactivity was quenched by adding 1 volume of chloroform and passing the reactive mixture through a 0.45-µπ1 nylon membrane to remove particulate enzyme.

Esterification reactive mixtures contained 250 mM each benzyl alcohol and hexanoic acid and 20 µ1 water in 10 ml of the selected solvents. After preincubation of the mixture to the reaction temperature of 35°C, reactivity was initiated by the addition of 150 mg lipase PS-30 powder. Reactive mixtures were incubated with rotary agitation (300 rev min^{-1}) for 15 min and reactivity was quenched as described above for the glycerolysis reactive mixtures.

Acyl-exchange reactive mixtures contained 0.70 g triacetin and 1.30 g olive oil in the selected solvent to bring the total volume to 10 ml. After preincubation of the mixture to the reaction temperature of 60°C, reactivity was initiated by the addition of 200 mg Lipozyme™ IM-20 or PS-30 lipase powder. Reactive mixtures were incubated with magnetic stirring for 3.5 h and reactivity was quenched by adding 0.5 volume chloroform and passing the mixture through 0.45-µm nylon filter to remove enzyme.

Mixtures designed for monoester wax synthesis contained 100 mM each dodecanoic acid and dodecanol in 10 ml of the selected solvent. After preincubation of the mixture to the reaction temperature of 50°C, reactivity was initiated by the addition of 20 mg lipase IM-20 powder. Reactive mixtures were incubated with magnetic stirring for 30 min and reactivity was quenched by passing the reactive mixture through a 0.45-µm nylon membrane to remove particulate enzyme.

Periodically, quenched reaction mixtures were incubated for up to an additional 24 h to verify that reactivity was quenched by the filtration procedure, as was previously indicated.[16]

Reaction progress analysis

Products of the reactive mixtures were determined by HPLC, principally using a Waters Associates (Milford, MA) system of model 510 pumps, model 712 autosampler and a computer-interfaced baseline 810 software program for data analysis. Glycerolysis reactive mixtures were analyzed for acylglycerol species (triacylglycerol, TAG; diacylglycerol, DAG; monoacylglycerol, MAG) and fatty acid (FA) components on a normal phase silica column (250 mm x 4.6 mm, 5 µm; Econosil, Alltech Associates, Deerfield, IL) at 30°C with gradient elution and light scattering detection (model ELSD II; Varex, Rockville, MD) as described previously.[17]

Products of the esterification reaction between benzyl alcohol and hexanoic acid were analyzed on the same silica column just described at 30°C with isocratic elution with hexane:chloroform-:formic acid (60:40:0.2, v/v/v) and UV detection at 254 nm. Quantification was done relative to an external standard curve prepared with benzylhexanoate.

Acetylated acylglycerol components were analyzed using a reverse-phase column (Hibar™ Lichrosorb RP-18,250 mm x 4.0 mm, 5 µm; Alltech Associates, Deerfield, IL) at about 22°C. Separation was achieved with a linear gradient of 50% acetone in acetonitrile to 100% acetone over 10 min, followed by a 8 min hold at 100% acetone, and finally reequilibration at 50% acetone in acetonitrile for a final 7 min. Solvent flow rate was 1.2 ml min ~ except during the hold period with 100% acetone where it was 1.0 ml min^{-1}. Diacetylatedmonoacylglycerols (DAcMAG) monoacetylateddiacylglycerols (MAcDAG) and TAG were resolved and detected by light-scattering (triacetin was transparent). Quantitation of DAcMAG was done relative to an external standard curve prepared with a commercial DAcMAG product (Myvacet 9-08, prepared from corn oil; Eastman Chemical Co., Kingsport, TN).

Wax monoester products were analyzed using a reverse-phase column (Microsorb-MV C18, 3 μm, 4.6 mm x 100 mm; Rainin Instrument Co., Emeryville, CA) with isocratic elution with acetonitrile at 1 ml min^{-1}. Fatty acid, fatty alcohol, and ester components were resolved and detection was by refractive index (model R401 differential refractometer; Waters Associates, New Milford, CT). Quantification was done relative to an external standard curve prepared with dodecyldodecanoate.

Calculation of log P values

Log P values for solvents were obtained from literature sources.[6,15,18,19] If literature values were not available, log P was calculated on the basis of fragmental constants using the information compiled by Rekker and Mannhold.[20] Because of ambiguity in how calculation of proximity effects should be applied to these substrates and products, no correction for these effects was made. Such calculations are expected to have relatively minor impact on log P values.

Two examples of log P calculations for products yielded in this study follow: DAG derived from butteroil, assuming the average fatty acyl group to be tetradecanoic acid, can be represented as the composite of two -CH3 groups, 26 -CH2- groups, two -COO- groups, and one each of -CH- and -OH groups, or [(2 x 0.724) + (26 x 0.519) + (2 x -1.200) + (0.315) + (-1.448)] and a final log P of 11.4. Benzylhexanoate can be expressed as a composite of one phenyl group, one -COO- group, five -CH2- groups, and one $-CH_3$ group, or [(1.902) + (-1.200) + (5 x 0.519) + (0.724)] and a final log P of 4.02.

Results

Esterification reactions involving nonpolar substrates

Dodecyldodecanoate (wax monoester) synthesis. The initial rates of ester synthesis between dodecanol and dodecanoic acid were evaluated with selected solvents of log P values - 0.33 to 8.8 as the continuous phase. Solvents of log P values 3.0 to 8.8 were most supportive of dodecyldodecanoate synthesis (*Figure 1).* The lesser ability of solvents with log P values - 0.33 to 2.5 to support wax monoester synthesis was not attributable principally to any tendency to desorb water from the enzyme, as the addition of water had limited effect on the ability of these solvents to support the reaction. For example, maximum rates of monoester synthesis were limited to < 0.025 μmol min^{-1} mg^{-1} enzyme when 3-5% water (up to 40% water evaluated) was added to acetonitrile, and no rate enhancement was observed when up to 15% water was added to tetrahydrofuran (data not shown). Furthermore, the observed differences in solvent compatibility for the reaction were not correlated with apparent phase behavior of the reactive mixtures. Upon visual examination, all reactive mixtures contained a single liquid phase. Substrate levels in this case were each poised at 100 mM and would be expected to have little impact on phase behavior of reactive mixtures.

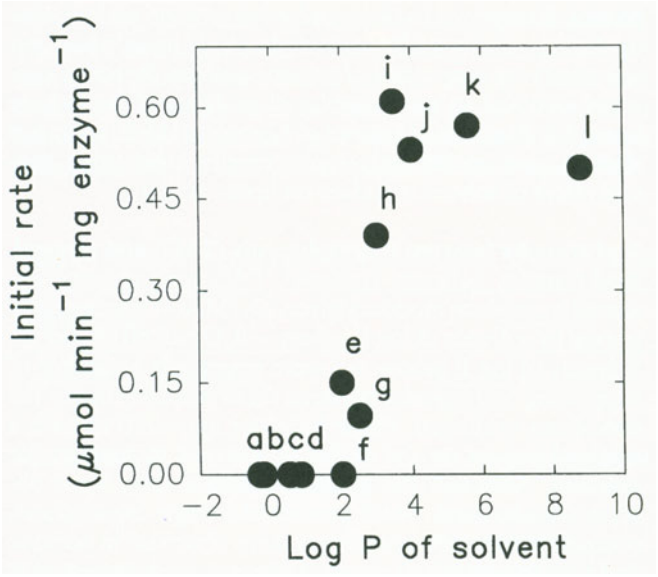

Figure 1 Initial rates of dodecyldodecanoate synthesis as a function of solvent log P value. Solvents used were (a) acetonitrile, (b) acetone, (c) tetrahydrofuran, (d) *t*-butanol, (e) benzene, (f) chloroform, (g) toluene, (h) pentane, (i) hexane, (j) heptane, (k) decane, and (l) hexadecane. All mixtures appeared to be a single-liquid phase. Coefficient of variation in analysis was 12% and the results are from two experiments

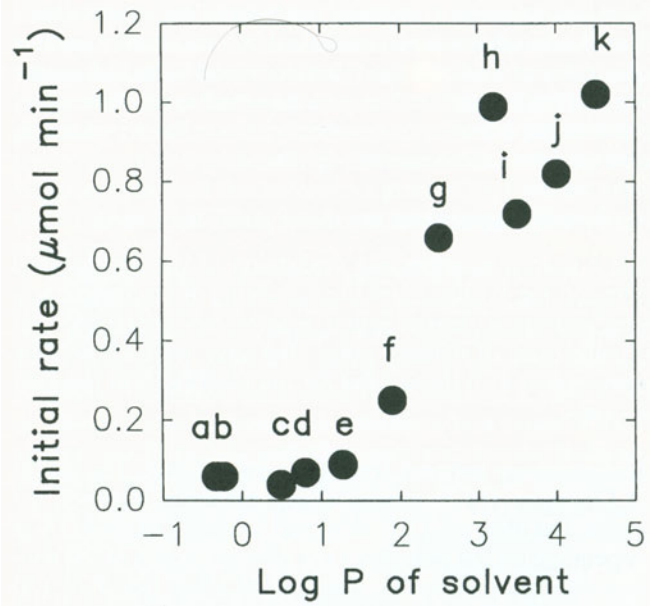

Figure 2 Initial rates of benzylhexanoate synthesis as a function of solvent log P value. Solvents used were (a) acetonitrile, (b) acetone, (c) tetrahydrofuran, (d) *t*-butanol, (e) *t*-butyl methyl ether, (f) diisopropyl ether, (g) toluene, (h) cyclohexane, (i) hexane, (j) heptane, and (k) octane. All mixtures appeared to be a single-liquid phase. Coefficient of variation in analysis was 7% and the results are from two experiments

Benzylhexanoate synthesis. Initial rates of ester synthesis with benzyl alcohol and hexanoic acid were evaluated with selected solvents, ranging from log P values - 0.33 to 4.5, used as the continuous phase. Greatest rates of benzylhexanoate synthesis were observed with solvents of log P values 2.5 to 4.5 serving as the continuous phase *(Figure* 2). By visual examination, all mixtures contained a single liquid phase, indicating apparent miscibility of solvents, substrates, and products for the reaction conditions used. The use of substrates at only 250 mM each would be expected to

have limited impact on phase behavior of the reactive mixture. Although it was not evaluated, the addition of greater quantities of water to the reaction mixtures including solvents of log P values < 2 may be expected to result in some, but limited, increase in reaction rate, similar to what was observed for wax monoester synthesis.

Acyl-transfer reactions involving polar substrates

For a more encompassing evaluation of the relationship between log P values and the suitability of an organic solvent to support enzyme action, reactive mixtures composed of substrates of distinct polar character were chosen for comparison with those used for the esterification reaction systems.

Acyl-exchange between triacylglycerols (TAG). The extent of Lipozyme IM-20-mediated acyl-exchange between triacetin and olive oil TAG designed to yield DAcMAG was dependent on the polarity of the solvent chosen as the continuous phase *{Figure* 3). Diisopropyl ether (log P of 1.9) and *t*-butyl methyl ether (log P of 1.3) were most supportive of acyl-exchange, whereas *t*-butanol (log P of 0.80) was less supportive and all other solvents evaluated were relatively nonsupportive of the reaction. These results clearly show an optimum range of log P values for acyl-exchange between the selected substrates. If olive oil TAG is modeled as triolein, the concentrations of substrates in the reactive mixture are 147 mM TAG and 321 mM triacetin. The molarities of substrates used in this case are similar to those used for benzylhexanoate synthesis. However, reactive mixtures with olive oil and triacetin as substrates had different phase

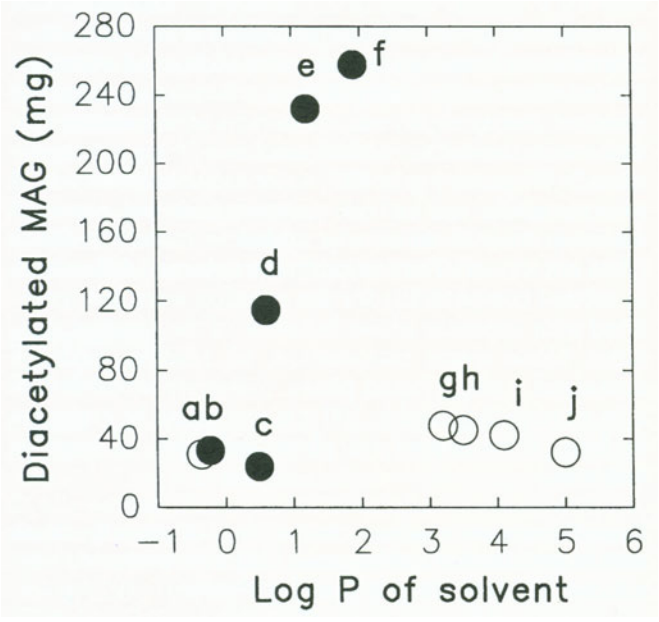

Figure 3 Yield of diacetylated monoacylglycerols after 3.5 h reaction as a function of log P value. Solvents used were (a) acetonitrile, (b) acetone, (c) tetrahydrofuran, (d) *t*-butanol, (e) t-butyl methyl ether, (f) diisopropyl ether, (g) cyclohexane, (h) hexane, (i) heptane, and (j) isooctane. (·) Single-liquid phase mixtures; (O) multiple-liquid phase mixtures. Coefficient of variation in analysis was 10% and the results are from two experiments

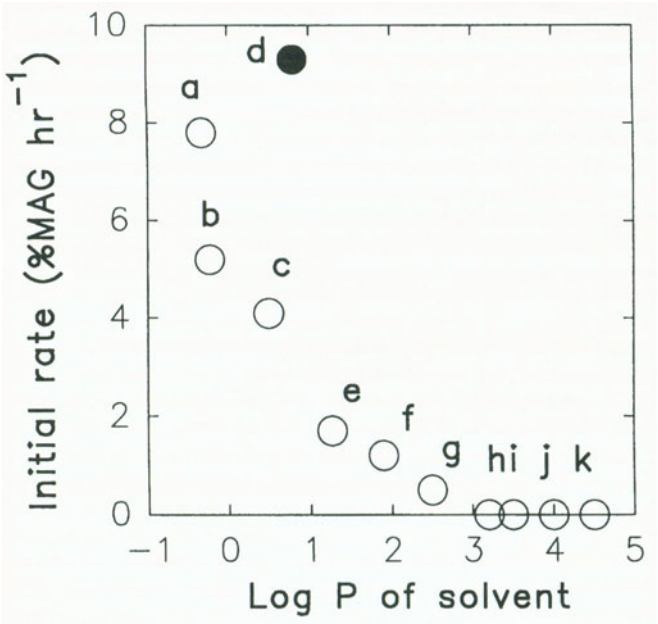

Figure 4 Initial rates of monoacylglycerol production as a function of solvent log P value. Solvents used were (a) acetonitrile, (b) acetone, (c) tetrahydrofuran, (d) *t*-butanol, (e) *t*-butyl methyl ether, (f) diisopropyl ether, (g) toluene, (h) cyclohexane, (i) hexane, (j) heptane, and (k) octane. (·) Single-liquid phase mixtures; (O) multiple-liquid phase mixtures. Coefficient of variation in analysis was 11 % and the results are from two experiments

behaviors, depending on which solvent was included as the bulk phase. It was apparent that there was an association between the promotion of an apparent single liquid phase and enzyme activity, and minimal enzyme activity was observed in reactive mixtures visibly containing at least two phases. Exceptions to this trend were acetone and tetrahydrofuran, which visually promoted a single liquid phase but were not very supportive of enzyme activity. Reactive mixtures identical to those displayed in *Figure 3,* but where lipase PS-30 was used instead of Lipozyme IM-20 (data not shown), gave qualitatively similar results, indicating a general effect of solvent selection on lipase action.

Acyl-transfer between triacylglycerols (TAG) and glycerol. Acyl-transfer between butteroil TAG and glycerol designed to produce monoacylglycerols (MAG) was best supported by solvents of log P values - 0.33 to 0.80 *(Figure 4).* Solvents of log P values > 3 were essentially incapable of supporting acyl-transfer between these reactants under the conditions evaluated. Phase behavior appeared to have a minimal impact on the ability of solvents to support the reaction. Although the greatest reaction rates were observed in *t*-butanol, which promoted a single liquid phase, comparable enzyme reactivities were observed in acetone and acetonitrile, which clearly promoted the existence of at least two liquid phases. Tetrahydrofuran and acetone were less supportive of the reaction than might have been expected based on the pattern that emerged with acetonitrile and *t*-butanol (log P values - 0.33 to 0.80). This may be attributable to a specific effect of acetone and tetrahydrofuran on enzyme structure and function, as a similar

observation was made for enzyme-mediated acyl exchange between triacetin and olive oil TAG *(Figure 3).* The concentration of substrates in this reaction system were 293 mM TAG and 988 mM glycerol (total of 879 mM acyl groups and 1281 mM glycerol, for an acyl group:glycerol 0.69, which is near-optimum for production of MAG[21,22]).

Discussion

The two examples of esterification reactions evaluated here conform to the prevailing view that organic solvents with log P values > 2 to 4 are best able to support enzyme action in microaqueous media.[3-7,23] However, in the present study, it is also apparent that substrates and products in reactive mixtures designed for esterification reactions were distinctly nonpolar, with calculated log P values ranging from 1.1 to 11.2 *(Table 1).* A closer examination of *Figures 1* and 2 seems to indicate that wax monoester synthesis was best supported by solvents of greater log P value than those fully supportive of benzylhexanoate synthesis. Likewise, substrates for, and products of, wax monoester synthesis have greater log P values than do those for benzylhexanoate synthesis *(Table 1).*

We believe it would be inappropriate to conclude that esterification, as a genre of reaction, should be expected to behave in a manner where solvents of log P values > 2 to 4 are most supportive of ester synthesis. Rather, we suggest that our observations, as well as those previously reported on the effect of solvent log P value and the ability to support reactions in microaqueous milieu,[5-7,23,26] are inseparable from the choice of substrates used. Brink and Tramper[23] concluded that solvents of greater nonpolarity and molecular weight are most supportive of biocatalysis. The substrates they subjected to epoxidation were propene and 1-butene, which have calculated log P values of 1.6 and 2.1, respectively, indicative of relatively nonpolar character. The rules developed later[4-6] for selecting solvents of log P values > 2 to 4 were based on the data of Brink and Tramper[23] and acyl-transfer reactions from tributyrin to heptanol. The calculated log P values for these latter two substrates are 3.0 and 2.4, respectively, both also of distinctly nonpolar character. Valivety *et al.*[1] studied ester synthesis between dodecanol and dodecanoic acid (log P values 5.0 and 4.8, respectively), as did the present study, and generally reaffirmed the rules for solvent selection proposed by Laane *et al.*[4-6] Studies on lipase-mediated intermolecular acyl-transfer of 1,3-dilaurin (calculated log P of 9.3) also indicated that solvents of greater log P values, over the range of -0.24 to 3.5, were more supportive of the reaction.[26]

However, there are clear indications in the literature that solvents with log P values < 2 are capable of supporting, sometimes at maximum levels, biocatalysis. Rates of lipase-mediated aminolysis using methyl butyrate and butylamine (respective calculated log P values of 1.3 and 0.94) as substrates were similar for solvents with a range of log P values -0.23 to 3.5, including acetone, tetrahydrofuran, pyridine, and hexane.[27] The greatest rates of peroxidase oxidation of *p*-anisidine in the presence of H_2O_2 took place in hydrated dioxane (log P of -1.1) and diethyl ether (log P of 0.85) compared to solvents of log P values ranging from 2.0 to 8.8 (benzene to hexadecane).[1] Over a narrow range of log P values (-0.33 to 0.96), there is little relation between a solvent's ability to support lipase and protease action on substrates (vinyl butyrate, benzyl alcohol, and *sec*-phenylethyl alcohol) with calculated log P values of 1.1 to 1.5.[15]

Although somewhat obscured by the authors' interpretations, our review of earlier work reveals that substrates of intermediate polarity may be most reactive with selected catalysts in solvents of intermediate log P values. For example, esterification reactions of butyric acid (log P of 0.81) with ethanol (log P of - 0.24), and isopentanol (log P of 1.3) with acetic acid (log P of - 0.23) are more rapid in hexane (log P of 3.5) relative to methylene chloride (log P of 1.5), octane (log P of 4.5) or decane (log P of 5.6).[28] Similarly, esterification of *N*-acetyl-phenylalanine with ethanol takes place at maximum rates when solvents of an intermediate log P value (1.6 to 2.0 over a range of 0.2 to 2.6 evaluated) are used.[18]

Laane *et al.*[5] had the wisdom to suggest that proper juxtapositioning of substrate and product polarities with that of the continuous phase is important to optimizing

Table 1 Reactive mixture substrates and products, and their calculated log P values

Reaction type	Substrates	Log P	Products	Log P
Acyl-transfer (glycerolysis)	Butteroil triacylglycerol	18.6[a]	Monoacylglycerol	4.2[a]
	Glycerol	-3.0	Diacylglycerol	11.4[a]
			Fatty acid	6.0[a]
Acyl-exchange	Olive oil triacylglycerol	23.6[b]	Monoacetylated diacylglycerol	15.7[b]
	Triacetin	-0.075	Diacetylated monoacylglycerol	7.8[b]
Esterification	Benzyl alcohol	1.1	Benzylhexanoate	4.0
	Hexanoic acid	1.9		
Esterification (wax ester synthesis)	Dodecanol	5.0	Dodecyldodecanoate	11.2
	Dodecanoic acid	4.8		

Calculation of log P for substrates and products was based on fragmental constants compiled by Rekker and Mannhold,[20] without any attempt to correct for proximity effects.

[a]Average molecular weight of fatty acyl group of butteroil acylglycerol was calculated to be 219[24] (and approximated as tetradecanoic acid), based on compositional data of Christie[25]

Representative fatty acyl group taken as 9-octadecenoic acid

biocatalysis. However, this criterion was clearly offered as a consideration secondary to selecting a solvent of log P value > 2 to 4. Our findings indicate that relative polarities of solvent and substrates may be of much greater importance than previously believed, upon considering the suitability of solvents to serve as reaction media. Of our reactive mixtures, that with the most polar substrate, glycerol, required solvents with lower log P values for maximum reactivity *(Figure 4* and *Table 1).* As the most polar reactant in the mixture tended to become progressively more nonpolar, the optimum solvent log P value(s) for supporting the reaction also progressively shifted to greater values *(Figures 1-3, Table 1).*

Product polarities, for the reaction systems we evaluated, may also have some impact on the abilities of solvents to support biocatalysis. Biocatalysis with polar substrates and/or products may be impeded in relatively nonpolar organic media by the lack of substrate solubility[29] or thermodynamic barriers to dissociation of a polar product from the enzyme active site into a relatively nonpolar medium.[30] Observations in our studies are consistent with the view that a lack of product dissociation into the continuous phase could have impeded reactivity in some solvents. For both esterification reaction systems, the product of reaction became more nonpolar than the corresponding substrates *(Table 1*), and this may have increasingly restricted enzyme-product dissociation as progressively more polar media were used as continuous phase *(Figures 1* and 2).

Another physical parameter that had some relevance to reaction efficiency was the phase behavior of the reactive mixtures. Phase behavior appeared to have a secondary effect on solvent suitability for supporting the model reaction systems evaluated. In most cases, single-liquid phase systems promoted the reaction more efficiently than multiliquid phase systems, as one would expect. However, this property may be enzyme source-specific, as here *(Figure 4)* and elsewhere[16,21,22] it was found that *Pseudomonas* lipase appears to be similarly efficient in single- or dual-liquid phase reaction systems. Also, there were some cases where solvents promoting a single liquid phase were not very supportive of biocatalysis (acetone and tetrahydrofuran, *Figure* 3). Some solvents may have a tendency to specifically perturb enzyme structure, and reaction thermodynamics, to the point where substrate transformation is not supported.[10,12-14] Alternatively, although mixtures with acetone and tetrahydrofuran designed for acyl-exchange reactions between triacetin and olive oil *(Figure 3)* visually appeared as a single phase, they may actually exist as a microemulsion. Microemulsions are well known to be formed under certain conditions where polar and nonpolar organic liquids are present with a surfactant.[31] The presence of a microemulsion in these cases could lead to a segregation of reactants, based on relative affinities for the two phases, and impeded reactivity because of an effective decrease in proximity of reactants. The source of surfactant for the reactive mixtures summarized in *Figure 3* could be deacylated triacetin or olive oil TAG intermediates and/or the fatty acids derived therefrom. The presence of particulate matter, in the form of the suspended enzyme, may further induce the formation of these noncontinuous liquid domains.

Acknowledgements

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