Control of Subtilisin Substrate Specificity by Solvent Engineering in Organic Solvents and Supercritical Fluoroform

Apurva K. Chaudhary,[†] Sanjay V. Kamat,[†] Eric J. Beckman,[†] David Nurok,[‡] Robert M. Kleyle,[§] Paul Hajdu,[‡] and Alan J. Russell*,[†]

Contribution from the Department of Chemical Engineering & Center for Biotechnology and Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and Departments of Chemistry and Mathematical Sciences, School of Science, Indiana University−Purdue University at Indianapolis, 402 North Blackford Street, Indianapolis, Indiana 46202.

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Abstract: We have investigated the effect of physical properties of 30 nonaqueous solvents on the specificity of Subtilisin Carlsberg for nucleophiles in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester by methanol, 1-propanol, and 1-butanol at fixed thermodynamic water activity. In organic solvents, enzyme activity and nucleophile specificity are solvent-dependent, while in supercritical fluoroform, the activity and specificity are pressure-dependent. Losses in catalytic efficiency and substrate specificity are observed when subtilisin is exposed to hydrophilic organic solvents such as dioxane, tetrahydrofuran, and acetonitrile as compared to hydrophobic solvents (hexane and heptane). Log *P* is an important descriptor for correlating both the rate and the specificity of deacylation with solvent properties. A linear model of log initial rate against both log *P* and nonpolar unsaturated area provides the best two-variable fit to the data for solvents of high log P. A nonlinear model of specificity against log *P* provides the best fit for the complete data set. Correcting the activity for partitioning of nucleophilic substrates shows a similar trend for the intrinsic activity dependence of nucleophiles as a function of log *P*. In propane, under subcritical conditions, there is no significant effect of pressure on either the activity or the nucleophilic specificity of subtilisin. In fluoroform, however, where the physical properties of the solvent are pressure-dependent, the specificity of the enzyme is solvent density-dependent.

Introduction

The activity and specificity of an enzyme can be altered by changing either the enzyme structure or the reaction medium.[1−4] Modification of proteins is time consuming and is not applicable to all enzymes. Since the free energy of desolvation of the substrate molecule is solvent-dependent, and enzymes utilize the free energy of substrate binding to drive catalysis, the substrate specificity of an enzyme depends on the solvents to which the enzyme is exposed.

There are numerous examples in the literature wherein solvent physical properties such as dielectric constant, dipole moment, and hydrophobicity are related to various effects on enzyme activity, specificity, and enantioselectivity.[3,5−8] These studies were performed with only relatively small sets of solvents and related the enzyme activity, specificity, and enantioselectivity to one physical property at a time. In a different context, we have shown that by using a large set of solvents, it is possible to construct good regression models for predicting the relationship between a dependent variable and two or more properties of the solvent.[9,10] This study was on the prediction of retention in liquid chromatography and has the solvation of the species of interest as a parallel with enzyme studies in nonaqueous media. Predictable alterations in the activity and specificity of enzymes have also been observed in other nonaqueous media such as supercritical fluids.[11,12]

Supercritical fluids are materials above their critical points (critical pressure and temperature) which cannot be liquefied. Because of the sharp dependence of physical properties (such as density, dielectric constant, and hydrophobicity) on temperature and pressure, supercritical fluids have attracted attention as media in which to conduct biocatalytic reactions.[12−16] Previously, we have demonstrated that the activity of *Candida cylindracea* lipase in various supercritical fluid environments (ethylene, ethane, sulfur hexafluoride, fluoroform, and carbon

* Address correspondence to Alan J. Russell, 300 Technology Drive, Center for Biotechnology and Bioengineering, University of Pittsburgh, Pittsburgh, PA 15219. Phone: 412-383-9740. FAX: 412-383-9710. E-mail: ajrche+@pitt.edu.

[†] University of Pittsburgh.

[‡] Department of Chemistry, Indiana University−Purdue University at Indianapolis.

[§] Department of Mathematical Sciences, Indiana University−Purdue University at Indianapolis.

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dioxide) changes smoothly with pressure.[11] In a separate study, we also observed a smooth alteration in the enantioselectivity of subtilisin with pressure in supercritical fluoroform.[1] Subtilisin, the most studied enzyme in organic solvents, offers an excellent model system in which to investigate whether there are general correlations between the physical properties of solvents and the specificity of enzymes, and when combined with the utility of supercritical fluids, one has a powerful system.

In nonaqueous media, in addition to solvent physical properties, enzyme structure and the amount of water associated with the enzyme are very important parameters in determining the activity of enzymes placed therein. Fourier transform infrared spectroscopy,[17a] solid state nuclear magnetic resonance, X-ray crystallography,[17b] and electron spin resonance (ESR) studies performed to date have shown that the local and global structure of enzyme particles suspended in organic solvents do not change significantly when compared with the structure of the same enzyme solubilized in aqueous solution (for review, see ref 52). Also, using Hammett analysis[18] and deuterium isotope exchange analysis it has been shown that the transition state structure of the serine protease subtilisin is the same in aqueous and organic media. Recent FT-IR work by Klibanov[19] has questioned the similarity of enzyme structure in aqueous and organic environments.

A variety of explanations of observed solvent effects on enzyme specificity have been presented in the literature. Most prominently, Klibanov and co-workers observed several different correlations between the specificity of enzymes and the physical properties of organic solvents in which they were suspended.[3−5,20] In none of Klibanov's studies was water activity controlled when changing solvent. In the subtilisin-catalyzed transesterification of *N*-acetyl-(L/D)-alanine chloroethyl ester by propanol, the enantioselectivity correlated well with the solvent hydrophobicity; this effect was interpreted in terms of solvent effects on the partitioning of water associated with the enzyme-substrate binding site.[8] For the same enzyme catalyzing transesterification of *N*-acetyl-L-phenylalanine ethyl ester and *N*-acetyl-L-serine ethyl ester by 1-propanol, a thermodynamic correlation between the substrate specificity and solvents to water partition coefficients was reported.[4] In another study with subtilisin-catalyzed transesterification of chiral alcohols (*sec*-phenethyl alcohol and vinyl butyrate), correlations between enantioselectivity and dielectric constant and dipole moment were reported.[5] No correlation was observed between the enantioselectivity and solvent hydrophobicity.[5] A similar correlation between enantioselectivity and solvent was observed for the closely related enzyme subtilisin BPN′, although the range of enantioselectivity was much lower than that for subtilisin Carlsberg.[5] For the same reaction catalyzed by porcine pancreatic lipase, a marked change in enantioselectivity was observed with changing organic solvent environment. In contrast to subtilisin, an opposite trend in enantioselectivity was observed, and with no correlation to either dipole moment or dielectric constant.[5] For the same reaction with chiral amines as nucleophiles (aminolysis), subtilisin exhibited no correlation between enantioselectivity and the dipole moment or dielectric constant of the solvent. The alteration of enantioselectivity upon changing reaction media is not limited to subtilisin. For example, in the transesterification of *N*-acetyl-(L/D)-phenylalanine 2-chloroethyl ester catalyzed by *Aspergillus oryzae*, Klibanov and co-workers observed a change in enantioselectivity which was dependent on the hydrophobicity of the solvent.[3] In all the solvents tested, the enantioselectivity of the protease correlated well with the log *P* of the solvent. Clearly, the alteration of substrate specificity by organic solvents is not trivial to predict. Several explanations have been proposed to explain the altered substrate specificity of enzymes in organic solvents. For a serine protease such as subtilisin, the loci of binding of ester and the nucleophile are known to be different. According to Klibanov and co-workers, the former locus is utilized in water and responsible for the preferred L enantioselectivity in aqueous medium.[5] However, the nucleophile-binding site has no distinct locus and thus it may vary with the structure of nucleophile, enzyme, and reaction system.

Another explanation for observed specificity of enzymes in organic solvents is proposed by Halling and co-workers. Halling suggests that solvation of substrates is a key determinant of specificity.[21] Klibanov has also suggested that there are two distinct ways in which a substrate can bind to subtilisin and still be correctly positioned for catalysis. However, for chymotrypsin, Faber[22,23] has suggested that the binding pockets for both D and L enantiomers are the same and the enantioselectivity observed in nonaqeous media is simply related to the difference in binding affinity of D enantiomer in nonaqueous media compared to water.

Although, increasing the water content of organic solvents normally accelerates enzymatic reactions, the enzyme is only affected by its bound water rather than by water dissolved in the solvent.[21,24,25] The amount of bound water necessary for optimal activity depends on the enzyme, and for many enzyme systems the amount of water needed has been well characterized. Nonaqueous media can strip water which is associated with the enzyme molecule and this tendency depends on the type of the solvent. More hydrophilic solvents have a greater tendency to strip the essential water from the enzyme molecule. Therefore, it is necessary to avoid the influence of water in a study of the effect of solvent. It has been proposed that water activity (a_w) provides a convenient means of monitoring enzyme hydration in organic solvents. There are two ways of maintaining constant water activity. Water can be added directly to the reaction system, or alternatively salt hydrates may be added as an indirect water source. According to Halling,[24a,26] salt hydrates act as buffering agents to maintain constant water activity in an organic solvent during enzymatic catalysis. When a salt hydrate is placed in nonaqueous media, the salt establishes an equilibrium between hydrated forms, thereby maintaining water activity. We therefore performed experiments on a single batch of enzyme at constant water activity and specificities were determined for each nucleophile in simultaneous experiments.

In both aqueous and organic solutions,[27] subtilisin follows an acyl−enzyme mechanism as described in Figure 1. The rate

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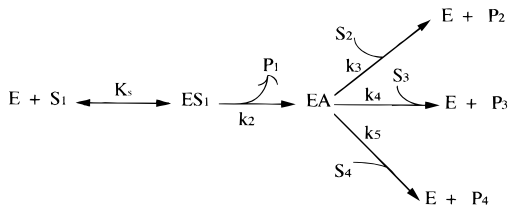

Figure 1. Acyl−enzyme mechanism for subtilisin-catalyzed transesterification. K_S is the dissociation constant for the binding of the ester substrate (S_1) to the enzyme, k_2 is the rate of acylation, and k_3, k_4, and k_5 are the rates of deacylation with different nucleophiles.

expression for formation of products for such a mechanism can be easily derived assuming that acylation and deacylation under initial rate conditions are irreversible.[27] The initial rate expressions for the formation of different esters are given by the following equations:

$$v_{P_2} = \left\{\frac{k_2k_3}{k_2 + k_3[S_2] + k_4[S_3] + k_5[S_4]}[E]_0[S_1][S_2]\right\}\Bigg/ \left\{K_S\left(\frac{k_3[S_2] + k_4[S_3] + k_5[S_4]}{k_2 + k_3[S_2] + k_4[S_3] + k_5[S_4]}\right) + [S_1]\right\}$$

$$v_{P_3} = \left\{\frac{k_2k_4}{k_2 + k_3[S_2] + k_4[S_3] + k_5[S_4]}[E]_0[S_1][S_3]\right\}\Bigg/ \left\{K_S\left(\frac{k_3[S_2] + k_4[S_3] + k_5[S_4]}{k_2 + k_3[S_2] + k_4[S_3] + k_5[S_4]}\right) + [S_1]\right\}$$

$$v_{P_4} = \left\{\frac{k_2k_5}{k_2 + k_3[S_2] + k_4[S_3] + k_5[S_4]}[E]_0[S_1][S_4]\right\}\Bigg/ \left\{K_S\left(\frac{k_3[S_2] + k_4[S_3] + k_5[S_4]}{k_2 + k_3[S_2] + k_4[S_3] + k_5[S_4]}\right) + [S_1]\right\}$$

The specificity of deacylation can be obtained by dividing the initial rates as follows:

$$\frac{v_{P_2}}{v_{P_3}} = \frac{k_3[S_2]}{k_4[S_3]}$$

$$\frac{v_{P_2}}{v_{P_4}} = \frac{k_3[S_2]}{k_5[S_4]}$$

$$\frac{v_{P_3}}{v_{P_4}} = \frac{k_4[S_3]}{k_5[S_4]}$$

where $[S_2]$, $[S_3]$, and $[S_4]$ are concentrations of substrate nucleophiles. Thus, these ratios are independent of the effect of K_m, and therefore any effect of K_m between solvents. In this paper, we focus on the solvent dependence of specificity of the enzymes toward nucleophiles in a set of 30 nonaqueous solvents, whereas a following paper will discuss specificity toward the ester.

Materials and Methods

Materials. Subtilisin Carlsberg, *N*-acetyl-L-phenylalanine ethyl ester, *N*-acetyl-L-phenylalanine methyl ester, methanol (MeOH), 1-propanol (PrOH), 1-butanol (BuOH), and sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Acetyl-L-phenylalanine propyl ester and butyl ester were synthesized by the method described in the literature.[25,26] Fluoroform was obtained from Air Products Inc. as a gracious gift.

Subtilisin used for experiments was lyophilized from aqueous solution at the pH optimal for the enzymatic activity in water (it has been shown that pH of 7.8 is optimal for subtilisin in organic solvent). Typically, 1 mg/mL of subtilisin was dissolved in pH 7.8, 0.01 M ionic strength phosphate buffer and lyophilized.

Activity Studies in Organic Solvents. Reactions in organic solvents were performed in 4 mL Wheaton vials. Typically, 0.4 g of sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$), 10 mM *N*-acetyl-L-phenylalanine ethyl ester, and 200 mM each methanol, 1-butanol, and 1-propanol were added to 2 mL of organic solvent. To this solution was added 2 mg of lyophilized subtilisin. Thereafter, the vial containing the reaction mixture was placed inside the incubator/shaker (300 rpm and 40 °C). At regular time intervals, 0.5 μL samples were taken from the reaction mixture with a Hamilton syringe. The samples were manually injected into a Hewlett Packard 5890 Series II gas chromatograph (equipped with an HP-1 cross-linked capillary column (30 m × 0.53 mm × 1.0 μm) for analysis. An initial temperature of 130 °C was maintained for 2 min, and then the temperature was raised at 25 °C/min to 180 °C. Under these conditions the retention times for *N*-acetyl-L-phenylalanine ethyl ester and *N*-acetyl-L-phenylalanine methyl ester were 4.25 and 5 min, respectively. Formation of the products (*N*-acetyl-L-phenylalanine methyl, propyl, and butyl esters) was monitored, and initial rates were determined. Since the K_M for subtilisin at this water activity far exceeds the substrate concentration, initial rates were used to calculate V_{max}/K_M. Also, since we have measured the rate for three substrates simultaneously, the active site content for each experiment will be identical. The use of all nucleophiles in every experiment also ensures that calculated specificities are only solvent-dependent, rather than enzyme active site concentration dependent.

Determination of Partition Coefficients of Alcohols in Organic Solvents. Partition coefficients of methanol, 1-propanol, and 1-butanol between water and organic solvents were determined in 4 mL Wheaton vials. Only in water-miscible solvents such as acetonitrile and tetrahydrofuran did the water phase contain 1 M NaCl to impart immiscibility with organic solvents. It is not known if addition of NaCl may affect the values of partition coefficients obtained. Typically, 200 mM of each alcohol were added to the organic solvents followed by the addition of 2 mL of water. Then the mixture was vigorously shaken and left overnight to separate into two phases. The concentrations of methanol, 1-propanol, and 1-butanol in both the phases were analyzed by gas chromatograph to determine the partition coefficients.

Activity Determination in Fluoroform. A 40 cm^3 high-pressure reactor was used in a batch mode to study the activity and nucleophilic specificity of subtilisin in supercritical fluoroform. Reactor design, reaction assembly and operating principles of the high-pressure reactor have been described previously.[30] Typically, *N*-acetyl-L-phenylalanine ethyl ester (10 mM), methanol, 1-propanol, 1-butanol (200 mM each), 10 mg of lyophilized subtilisin, and 2 g of sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) were placed inside the reactor. Precooled fluoroform was then introduced into the reactor through a syringe pump. The reactor was operated in a batch mode to maintain constant water content, and the reaction mixture was agitated with a magnetic stirrer. Samples from the reactor were taken via a four-way rheodyne valve and then analyzed gas chromatographically as described above.

Data Analysis. The regression coefficients and correlation coefficients for the exponential models in log *P* were calculated using STATISTICA for Windows, version 4.3, Statsoft Inc. Regression coefficients and R^2's for the two-variable linear models were computed using SAS for Windows, version 6.10, SAS Institute Inc.

Physical Properties of the Solvents. Our databank of physical properties[31−34] for the solvents includes log *P*, dielectric constant (ϵ),

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Table 1. Effect of Organic Solvent Environment on Subtilisin Activity at 40 °C and 300 rpm[a]

no.	solvent	log *P*	NPUA	initial rate (methanol) (mM/h)	ln(initial rate) methanol	initial rate (propanol) (mM/h)	ln(initial rate) (propanol)	initial rate (butanol) (mM/h)	ln(initial rate) (butanol)
1	hexane	3.9	0	1.43	0.357	0.31	−1.171	0.14	−1.966
2	heptane	4.397	0	1.38	0.322	0.29	−1.237	0.13	−2.040
3	toluene	2.641	43.7	0.0633	−2.759	0.0094	−4.667	0.0037	−5.599
4	carbon tetrachloride	2.875	0	0.117	−2.145	0.0178	−4.028	0.0083	−4.79
5	butyl ether	2.986	0	0.212	−1.551	0.0356	−3.335	0.0159	−4.141
6	benzene	2.142	45.9	0.0155	−4.166	0.0025	−5.991	0.0009	−7.013
7	cyclohexane	3.354	0	0.985	−0.015	0.184	−1.692	0.0814	−2.508
8	butyl acetate	1.769	0	0.069	−2.673	0.0343	−3.372	0.0248	−3.697
9	chloroform	1.952	0	0.117	−2.145	0.0178	−4.028	0.0083	−4.79
10	nonane	5.455	0	3.20	1.163	1.284	0.25	0.903	−0.102
11	octane	4.926	0	1.92	0.652	0.493	−0.707	0.241	−1.423
12	methylene chloride	1.249	0	0.0050	−5.298	0.0014	−6.571	0.0009	−7.013
13	1,4-dioxane	−0.497	0	0.0619	−2.782	0.0396	−3.228	0.023	−3.772
14	ethyl acetate	0.711	0	0.212	−1.551	0.076	−2.577	0.0513	−2.970
15	propyl acetate	1.240	0	0.115	−2.162	0.0515	−2.966	0.0266	−3.627
16	ethylbenzene	3.170	45.5	0.140	−1.966	0.0165	−4.104	0.0057	−5.1673
17	bromobenzene	3.005	45.1	0.0619	−2.782	0.0088	−4.733	0.0043	−5.449
18	chlorobenzene	2.855	42.1	0.06	−2.813	0.00858	−4.758	0.0033	−5.714
19	1-chlorobutane	2.523	0	0.0855	−2.459	0.0145	−4.233	0.0071	−4.948
20	2-chlorotoluene	3.354	38.9	0.106	−2.244	0.0158	−4.147	0.0065	−5.036
21	tetrachloroethylene	3.48	15.1	0.309	−1.174	0.0521	−2.954	0.0224	−3.799
22	1,1,1-trichloroethane	0.05187	0	0.0519	−2.958	0.0079	−4.840	0.0036	−5.6269
23	acetonitrile	−0.394	16.7	0.675	−0.393	0.294	−1.224	0.219	−1.518
24	tetrahydrofuran	0.456	0	0.230	−1.469	0.141	−1.959	0.867	−0.143
25	*N*,*N*-dimethylformamide	−1.038	0	0.0282	−3.568	0.0218	−3.826	0.0171	−4.069
26	nitromethane	−0.350	0	0.182	−1.703	0.0818	−2.503	0.0596	−2.82
27	acetone	−0.240	0	2.867	1.053	1.367	0.3126	1.00	0
28	*tert*-butylamine	0.923	0	0.724	−0.3229	0.635	−0.454	0.496	−0.701
29	pyridine	0.645	41.7	0.25	−1.386	0.30	−1.204	0.16	−1.833
30	triethylamine	1.395	0	0.96	−0.0408	0.41	−0.891	0.21	−1.561

[a] Reaction conditions are explained in the text. Also, log *P*, NPUA (nonpolar unsaturated area) and ln(initial rate) with various nucleophiles have been included in the table. To convert initial rate from mM/h to μM/min mg of enzyme, multiply by 8.33.

dipole moment, Kirkwood function $[(\epsilon - 1)/(2\epsilon + 1)]$, Hildebrand solubility parameter, molar refraction, nonpolar unsaturated area, polarizability, density, molar volume, viscosity, and boiling point (Table 3).

Log *P* values were calculated using the CLOG P methods as described by Leo[35] with Biobyte Corporation's MacLog P 1.0. The dipole moments were computed using the AM1 Hamiltonian implemented in MOPAC 6.0.[36] The values of polarizibility were computed from an algorithm based on a modification of Slater's rules.[37]

The surface area of each solvent was obtained by computation using PCMODEL.[38] A variety of different types of molecular surfaces have been defined for quantitative structure−activity relationships (QSAR),[39] and the solvent-accessible surface area was used in the current report. This surface area has been used in a variety of reports including the determination of protein folding and the prediction of solubility of drug molecules,[40] as well as in liquid chromatography,[9,10] where the surface area of the solvent was used as a descriptor in multiple linear regression to predict retention. The solvent-accessible area was first defined by Lee and Richards as the locus of the center of a solvent "sphere" which is rolled over the van der Waals surface of the solute.[41,42] For the current report, solvent accessible surface areas were calculated using the method and original parameters of Lee and Richards,[41] with the implementation of a grid spacing of 0.1 Å. Cartesian coordinates of solvent molecules needed for these calculations were determined by the MMX force field.

The solvent-accessible surface area may be partitioned into polar and nonpolar components, and the latter may be further divided into the accessible surface area contributed by saturated atoms and that contributed by unsaturated atoms such as the carbons in an alkene or aromatic compound. Carbon, hydrogen, and also halogen atoms are treated as nonpolar. Monovalent atoms attached to the unsaturated atoms of benzene are classified as saturated in the algorithm used.[41] As an example, toluene has a component of saturated (methyl and ring hydrogens) and unsaturated (carbon) atoms contributing to the total nonpolar surface area.

Results and Discussion

Subtilisin Activity and Nucleophilic Specificity in Conventional Organic Solvents. The transesterification reaction between nucleophiles and *N*-acetyl-L-phenylalanine ethyl ester was studied in 30 organic solvents, which were carefully selected to span a range of physical properties (including hydrophobicity, dielectric constant, and dipole moment). In order to ensure complete solubilization of *N*-acetyl-L-phenylalanine ethyl ester even in the most hydrophobic organic solvents a 20-fold excess of alcohols was used.

Preliminary experiments were performed to find the appropriate salt hydrate ($Na_4P_2O_7 \cdot 10H_2O$) concentration necessary to achieve optimal activity of subtilisin in organic solvents. In hydrophilic solvents (acetone and acetonitrile) at 40 °C, 0.15 g/mL salt hydrate was found to be sufficient to achieve optimal activity of subtilisin (this is consistent with the published data for subtilisin).[24b] The water activity attained at 40 °C using $Na_4P_2O_7 \cdot 10H_2O$ is 0.59. All the experiments in organic solvents described below were performed with 0.2 g/mL salt hydrate concentration.

Table 1 gives the activities of subtilisin in the transesterification between *N*-acetyl-L-phenylalanine ethyl ester and primary alcohols (methanol, 1-propanol, and 1-butanol) in 30 commonly used organic solvents. Reaction conditions in each of the experiments were identical, the only difference being the solvent

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in which the enzyme was dispersed. One can see from Table 1 that, under identical conditions of water activity, subtilisin activity changes with the solvent. The reaction is not diffusionally controlled, and the alteration in activity with solvent can be attributed to the changing solvent environment. The question that many groups are attempting to answer is how a particular solvent exerts its effect on enzyme activity. In all solvents, subtilisin favors methanol over 1-propanol and 1-butanol, although the extent of this preference is distinctly solvent-dependent. Among the solvents used, subtilisin displayed highest activity in nonane and lowest activity in methylene chloride.

In large number of solvents methanol is preferred and the preference is methanol > propanol > butanol. The hydrophobicity of the solvent also influences the partitioning of alcohol between enzyme and solvent. On the basis of hydrophobicity ($\log P_{\text{methanol}} = -0.79$, $\log P_{\text{propanol}} = 0.27$, and $\log P_{\text{butanol}} = 0.80$) methanol should partition most significantly into the enzyme environment, and therefore should show the highest reactivity. This is also consistent with the results shown in Table 1.

Dependence of Activity on Solvent Physical Properties. 1. Log *P* Model. Laane and colleagues proposed that log *P* (*P* is the partition coefficient of solvent between octanol and water) is a good indicator of solvent hydrophobicity. According to Laane, solvents with log $P < 2$ are not favorable for enzymatic system because they strongly distort the water associated with enzymes, which is essential for activity.[43]

In the present case, although the amount of water associated with subtilisin in all organic solvents was kept constant ($a_w = 0.59$), the activity of subtilisin in various organic solvents did not follow the "log *P*" model proposed by Laane. Interestingly, subtilisin showed high activity in acetone, nitromethane, and acetonitrile which possess negative log *P* values. Toluene, benzene, and chlorobenzene with relatively high log *P* values promoted a rather poor enzyme activity. Similar results contradicting the "log *P* rule" have appeared in the literature, indicating that log *P* cannot be used as a universal predictor of enzyme activity in the organic solvents.[25,44−47]

An attempt was made to use multiple linear regression, performed in the stepwise mode, to study the relationship between initial rate and the descriptors (solvent properties) listed in Table 3. No satisfactory model was found for the complete set of 30 solvents. The solvents were then divided into the subsets of 15, with values of log *P* being above 2.0 in one subset (the high log *P* subset) and less than 2.0 (the low log *P* subset) in the other. The value of 2.0 is based on Laane's suggestion, discussed above, that solvents with lower log *P* values distort the water associated with the enzyme.

The following model predicts the natural log of the initial rate for each of the three alcohols in the high log *P* solvents:

$$\ln \nu = -a + b \log P - c\text{NPUA}$$

where, for methanol, $a = 5.17$, $b = 1.24$, $c = 0.03$, $R^2 = 0.9201$; for propanol, $a = 7.61$, $b = 1.47$, $c = 0.03$, $R^2 = 0.9464$; and for butanol, $a = 8.67$, $b = 1.56$, $c = 0.03$, $R^2 = 0.9578$.

NPUA is the solvent-accessible nonpolar unsaturated area. Seven of the solvents in the high log *P* set do not have any unsaturation, seven have a benzene ring in the structure, and the fifteenth solvent is tetrachloroethylene.

All terms in the model for methanol are statistically significant with 97.5% confidence; all terms in the other two models are significant with 99.9% confidence. These models are not overfitted using the criterion of five data points (i.e., the solvents) for each term in the model. The high value of R^2 demonstrate that most of the variability in log initial rate about the mean is described by these models. Thus for butanol the model predicts 95.78% of the variability of log initial rate about the mean.

These models show that log initial rate increases with increasing log *P* of the solvent and decreases with an increase in its nonpolar unsaturated area. The model indicates that solvents such as toluene, benzene, and chlorobenzene will promote a lower initial log rate than solvents of similar log *P* that lack unsaturation. This explains the apparent anomalous behavior of these solvents referred to earlier in this report.

While these three regression models have excellent fits for data generated with solvents of log *P* greater than 2.0, the fits are exceedingly poor for the solvents of low log *P*.

2. Dielectric Constant or Dipole Moment. Dielectric constant has been used (as an indicator of solvent polarity) for predicting enzyme behavior when a partially charged transition state is involved.[48,49] Clark observed a linear relationship between catalytic efficiency and the reciprocal of the dielectric constant and concluded that solvent effect is primarily electrostatic in origin. For our data, no correlation exists between subtilisin activity at constant water activity with solvent dielectric constant or dipole moment.

The activity of enzymes in organic environments is a complex function that is governed by the physical properties of the system and specific chemical nature of the system. Well-known physical properties of the solvents may not be ideally suited for correlating enzyme behavior in organic solvents. In addition, we have not measured the amount of active enzyme within the system, which can vary from solvent to solvent.

Dependence of Specificity on Solvent Physical Properties. The nucleophilic specificity of subtilisin Carlsberg (expressed as a ratio of rates of deacylation, Table 2) was significantly affected by the nature of the organic solvents. In order to understand why subtilisin nucleophilic specificity depends on the reaction medium, we attempted to correlate substrate specifcity with physical properties of the solvents.

There are only weak correlations between specificity and any single descriptor for the complete set of 30 solvents. As an example the correlation coefficient (for a linear model) between the methanol/butanol specificity (given by the ratio of the respective initial rates) and each of the descriptors in Table 3 varies between 0.136 and 0.511. The latter value is for the correlation with log *P*. Plots illustrating these relationships are available as Supporting Information.

There is a little improvement in using higher order models in log *P* (Figure 2). The following normal distribution model, however, has a moderately good value of R^2 with all parameters statistically significant:

$$S = a \exp(-((\log P - b)/c)^2) \qquad R^2 = 0.7822$$

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Table 2. Effect of Organic Solvent on Nucleophilic Specificity of Subtilisin-Catalyzed Transesterification of *N*-Acetyl-L-phenylalanine Ethyl Ester[a]

		nucleophilic specificity		
no.	solvent	MeOH/PrOH	PrOH/BuOH	MeOH/BuOH
1	hexane	4.61	2.21	10.21
2	heptane	4.76	2.23	10.62
3	toluene	6.71	2.54	17.02
4	carbon tetrachloride	6.57	2.15	14.10
5	butyl ether	5.96	2.24	13.33
6	benzene	6.27	2.76	16.92
7	cyclohexane	5.36	2.25	12.10
8	butyl acetate	2.25	1.40	2.02
9	chloroform	6.57	2.16	14.10
10	nonane	2.49	1.42	2.57
11	octane	3.89	2.05	7.94
12	methylene chloride	3.61	1.57	5.66
13	1,4-dioxane	1.56	1.72	2.68
14	ethyl acetate	2.80	1.48	4.13
15	propyl acetate	2.23	1.94	4.32
16	ethylbenzene	8.44	2.91	24.60
17	bromobenzene	7.00	2.06	14.38
18	chlorobenzene	7.00	2.60	18.20
19	1-chlorobutane	5.89	2.05	12.08
20	2-chlorotoluene	6.66	2.44	16.27
21	tetrachloroethylene	5.92	2.33	13.76
22	1,1,1-trichloroethane	6.55	2.20	14.41
23	acetonitrile	2.30	1.34	3.09
24	tetrahydrofuran	1.63	1.63	2.65
25	*N*,*N*-dimethylformamide	1.30	1.28	1.65
26	nitromethane	2.22	1.38	3.08
27	acetone	2.10	1.37	2.87
28	*tert*-butylamine	1.14	1.28	1.47
29	pyridine	0.83	1.88	1.56
30	triethylamine	2.34	1.95	2.34

[a] Reaction conditions are explained in the text. Specificity is defined as the ratio of initial rates of deacylation measured experimentally.

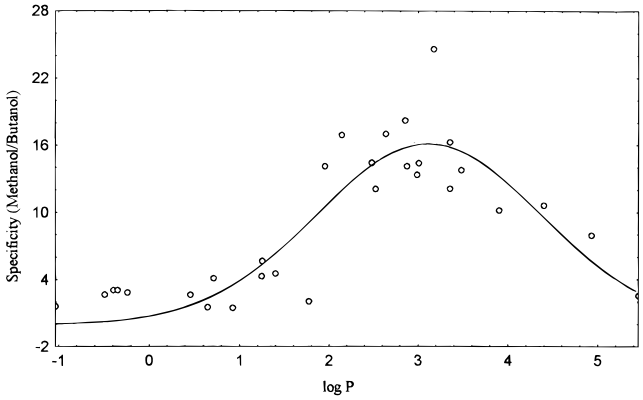

Figure 2. Subtilisin substrate specificity (methanol/butanol) against solvent log *P* for transesterification of *N*-acetyl-L-phenylalanine ethyl ester with primary alcohols.

$$a = 16.11, \quad b = 3.15, \quad c = 1.78$$

It is often possible to find a distinct trend within a small group of solvents. Figure 3 shows that there is a parabolic relationship between the methanol/butanol specificity and log *P* for the alkanes. This regular relationship is not demonstrated by a more diverse set of solvents. Relationships between log *P* and other solvent descriptors can be found by restricting the class of solvent considered. Such relationships, however are unlikely to yield insight into the mechanism of enzyme action or to predict a solvent that would give an optimum yield for an enzyme catalyzed reaction performed in a nonaqueous solution.

The possibility of obtaining a fortuitous relationship between specificity and a solvent dependent variable when using only a small data set is illustrated by the following short study in which subsets of five solvents were randomly selected from the complete set of 30 solvents. There are 145 206 possible combinations of these five solvents. The solvent dipole moment, dielectric constant, Kirkwood function, and log *P* were each used separately as the independent variable for predicting the methanol/butanol specificity. Log *P* was found to be the best predictor of specificity, even though the correlation was weak. The regression equations for about 28% of the subsets of five solvents have R^2 of 0.04 or less. Nevertheless the regression equations for about 5% of these subsets have R^2 between 0.80 and 1.0. Clearly, a study involving only five solvents could give a misleading correlation. A two-term equation based on five data points represents substantial overfitting. This short study illustrates that it is imperative to use regression equations which do not overefit the data and where all terms in the model are statistically significant.

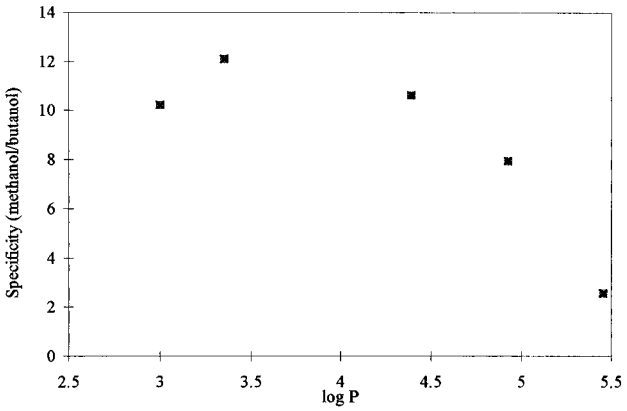

Figure 3. Nucleophilic specificity of subtilisin Carlsberg against log *P* for alkanes as solvents.

Determination of Partition Coefficients for Substrates. We have performed our experiments at relatively high values of water activity. It has been previously hypothesized[50] that, at high values of a_w, water can be bound to the enzyme as essentially free water. This can be replaced by other solvents, with large voids in protein crystals. Moving from low to high water activity values, solvents can partition into weaker binding sites followed by the formation of secondary hydration layers.[51] These water molecules are, therefore, likely to resemble bulk water because of the poor interaction with the enzyme residues on the surface. Since a constant amount of water is associated with subtilisin in all our experiments, consideration of partition coefficients of alcohols between water and organic phase may be appropriate. Alcohols must partition out of the solvent into microaqueous phase surrounding the enzyme before reaction can occur. In order to test the effect of partitioning of alcohols on reaction rate, we determined the partition coefficient of methanol, 1-propanol, and 1-butanol between water and the organic phase (Table 4).

Due to the miscibility of alcohols with some of these solvents and/or analytical difficulties for separation using gas chromatography, it was not possible to determine partition coefficient values in all cases. Rather than mix calculated and experimental values, we concentrate the following discussion on those solvents for which partition coefficients were obtained (19 solvents in total).

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Table 3. Physical Properties of Organic Solvents

solvent	log P^a	dielectic constant (ϵ)	dipole moment^b ($\times 10^{30}$) (Cm)	Kirkwood function^c	Hildebrand solubility parameter (J/cm^3)$^{1/2}$	molar refraction (cm^3/mol)	polarizability (10^{-25} cm^3)	nonpolar unsaturated area (Å^2)	density (g/mL)	molar volume (cm^3/mol)	viscosity (cp)	boiling point (°C)	E_t^n (refer to legend)^d
hexane	3.900	1.89	0.00	0.1862	14.9	29.87	139.8	0	0.66	130.5	0.271	68.7	0.009
heptane	4.397	1.92	0.00	0.190	15.1	34.54	161.8	0	0.68	146.5	0.341	98.4	0.012
toluene	2.641	2.38	0.867	0.2396	18.2	31.1	129.9	43.7	0.87	106.5	0.471	110.6	0.099
carbon tetrachloride	2.875	2.23	0.00	0.225	17.6	26.46	101.1	0	1.59	96.5	0.965	76.7	0.052
butyl ether	2.986	3.06	4.34	0.289	16.0	41.24	186.8	0	0.76	170.4	0.75		0.071
benzene	2.142	2.28	0.00	0.230	18.8	26.34	108.8	45.9	0.87	89.4	0.647	80.1	0.111
cyclohexane	3.354	2.02	0.00	0.2024	16.8	27.71	131.8	0	0.78	108.1	0.980	80.7	0.006
butyl acetate	1.769	5.01	5.67	0.364	17.0	31.51	148.7	0	0.88	131.7	0.563		0.241
chloroform	1.952	4.81	3.80	0.3587	19.0	21.53	83.3	0	1.48	80.5	0.50	61.3	0.259
nonane	5.455	1.97	0.00	0.196	15.7	43.81	205.7	0	0.72	178.6	0.711	150.8	0.009
octane	4.926	1.95	0.00	0.194	15.6	39.19	183.8	0	0.70	162.6	0.433	125.0	0.009
methylene chloride	1.249	8.90	5.00	0.420	19.8	16.36	65.5	0	1.33	64.0	0.393	40.7	0.309
1,4-dioxane	−0.492	2.21	0.00	0.223	20.5	21.67	94.1	0	1.03	85.2		101.1	0.164
ethyl acetate	0.711	6.02	5.8	0.385	18.6	22.23	94.5	0	0.90	97.7	0.375	77.1	0.228
propyl acetate	1.240	6.00	5.9	0.385	18.0	26.91	116.4	0	0.89	115.0	0.44	101.8	0.210
ethylbenzene	3.170	2.41	0.8	0.2423	18.0	35.78	151.9	45.5	0.87	122.5	0.69	136.2	
bromobenzene	3.005	5.40	4.84	0.373	20.3	33.96	135.2	45.1	1.50	105.0	0.985	156.2	0.182
chlorobenzene	2.855	5.71	4.34	0.3792	19.4	31.19	125.6	42.1	1.11	101.8	0.756	132.2	0.188
1-chlorobutane	2.523	7.39	5.7	0.4049	16.7	25.45	113.6	0	0.89	104.5		77.8	
2-chlorotoluene	3.354	5.50	3.8	0.375		35.82	147.4	38.9	1.08	116.9		159.3	
tetrachloroethylene	3.480	2.50	0.00	0.250	19.0	30.35	114.8	15.1	1.62	102.2	0.839	120.8	
1,1,1-trichloroethane	0.05187	7.50	5.8	0.4063		24.38	105.12	0	1.33	92.8	0.858	74.1	0.170
acetonitrile	−0.394	36.20	9.64	0.4795	24.3	11.09	47.3	16.7	0.78	52.09	0.35	81.8	0.460
tetrahydrofuran	0.456	7.32	6.41	0.404	18.6	19.88	90.9	0	0.88	81.1		66.0	0.207
N,*N*-dimethylformamide	−1.038	36.70	11.2	0.4798	24.8	20.01	80.2	0	0.94	77.4		153.0	0.404
nitromethane	−0.350	35.94	13.9	0.479			43.6	0	1.12		0.576	101.2	0.481
acetone	−0.240	20.70	9.74	0.465	20.2	16.17	68.6	0	0.791	73.5	0.28	56.5	0.355
tert-butylamine	0.923		4.87			24.3	107.2	0	0.69	105.2		44.5	
pyridine	0.645	12.30	6.57	0.441			97.0	41.7	0.98		0.974	115.4	0.302
triethylamine	1.395	2.42	3.10	0.243			150.8	0	0.73			88.9	0.052

^{a} Partition coefficient of solvent is the ratio of concentration of solvent in octanol to that in water at equilibrium. ^{b} 1 debye = 3.336×10^{-30} Cm. ^{c} Kirkwood function = $(\epsilon - 1)/(2\epsilon + 1)$. ^{d} Normalized E_t^n values derived from transition energy of the long-wavelength UV/vis absorption band of a negative solvatochromic pyridinium *N*-phenoxide betaine dye (measured at 25 °C and 1 bar). $E_t^n = [E_{t(solvent)} - E_{t(tetramethylsilane)}]/[E_{t(water)} - E_{t(tetramethylsilane)}]$. For a more detailed discussion see ref 33.

Table 4. Partition Coefficients Expressed as the Ratio of Concentration of Species in Organic to Water Phase

no.	solvent	partition coefficient of methanol	partition coefficient of propanol	partition coefficient of butanol
1	hexane	0.055	0.138	0.396
2	heptane	0.0324	0.098	0.242
3	toluene	0.0408	0.2688	1.115
4	carbon tetrachloride	0.082	0.215	28.00
5	butyl ether	0.0585	0.36	1.50
6	benzene	0.0552	0.331	1.299
7	cyclohexane	0.0129	0.073	137.8
8	butyl acetate	0.0967	1.114	4.124
9	chloroform	0.124	0.903	3.54
10	nonane		0.0954	0.306
11	octane		0.089	0.286
12	methylene chloride	0.158	0.857	2.71
13	1,4-dioxane			
14	ethyl acetate	0.261	1.62	5.06
15	propyl acetate	0.17	1.628	5.6
16	ethylbenzene	0.0699	0.288	0.948
17	bromobenzene	0.0296	0.314	1.324
18	chlorobenzene	0.0923	0.346	1.143
19	1-chlorobutane	0.0394	0.269	0.885
20	2-chlorotoluene	0.0191	0.2385	0.9170
21	tetrachloroethylene	0.019	0.184	0.674
22	1,1,1-trichloroethane	0.0202	0.395	41.16
23	acetonitrile	0.784	1.525	2.111
24	tetrahydrofuran	0.57	3.032	7.29
25	dimethylformamide			
26	nitromethane	0.205	0.927	2.33
27	acetone			
28	*tert*-butylamine			
29	pyridine			
30	triethylamine			

Specificity, which is the ratio of deacylation, is given as

$$\frac{v_{P2}}{v_{P4}} = \frac{k_3[S_2]}{k_5[S_4]}$$

where $[S_2]$ and $[S_4]$ are concentrations of methanol and butanol, respectively. The total concentration of the alcohols in the system was maintained at 200 mM; however, if alcohols were to partition in the organic and microaqueous phases, then the microaqueous concentration of alcohols will depend on their partition coefficient between solvent and the water phase and could be different. The concentrations of alcohols in the bulk organic and microaqueous phase can be determined as follows, with the assumption that the properties of microaqueous phase will mirror those of bulk water. This assumption is a simplistic one, but necessary for the analysis.

$$P_{S_2} = \frac{[S_2]_{solvent}}{[S_2]_{water}}$$

$$[S_2]_{solvent} + [S_2]_{water} = 200$$

Thus,

$$\frac{v_{P2}}{v_{P4}} = \frac{k_3(1 + P_{S_4})}{k_5(1 + P_{S_2})}$$

Therefore, a correction for partitioning of substrates into microaqueous environment will be given by

$$\frac{k_3}{k_5} = \frac{v_{P2}(1 + P_{S_2})}{v_{P4}(1 + P_{S_4})}$$

The ratio of rate constants which is the nucleophilic specificity is then the product of two terms, the observed specificity (determined experimentally) and the partitioning factor. The effect of log *P* on nucleophilic specificity (after correcting for partitioning) is given in Figure 4.

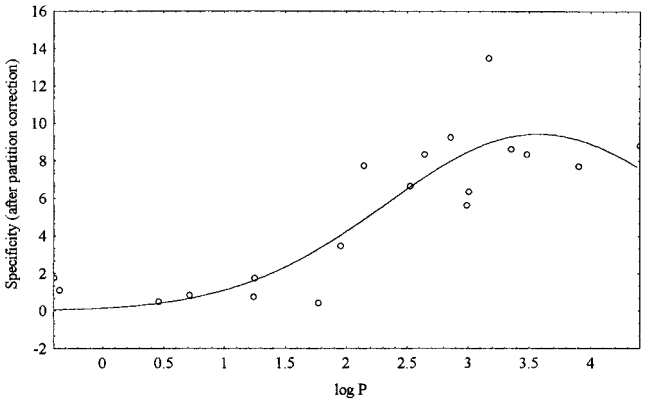

Figure 4. Subtilisin substrate specificity (methanol/butanol) against solvent log *P* (after correcting for partitioning) for transesterification of *N*-acetyl-L-phenylalanine ethyl ester with primary alcohols.

The model, discussed earlier in this report, relating specificity to log *P* holds after correcting for substrate partitioning and the value of R^2 is essentially the same for the model with uncorrected specificity.

$$S_{cor} = a \exp(-((\log P - b)/c)^2) \qquad R^2 = 0.7691$$

$$a = 9.45, \quad b = 3.57, \quad c = 1.76$$

Since, all experiments were performed at constant water activity, the behavior of specificity with solvent log *P* cannot be explained by the difference in solvent's ability to strip water from enzyme. Partitioning of solvent itself within the enzyme microenvironment must play a role in determining the enzyme−substrate interaction. Solvents with higher log *P* value are more able to change the nature of interactions at the enzyme−substrate

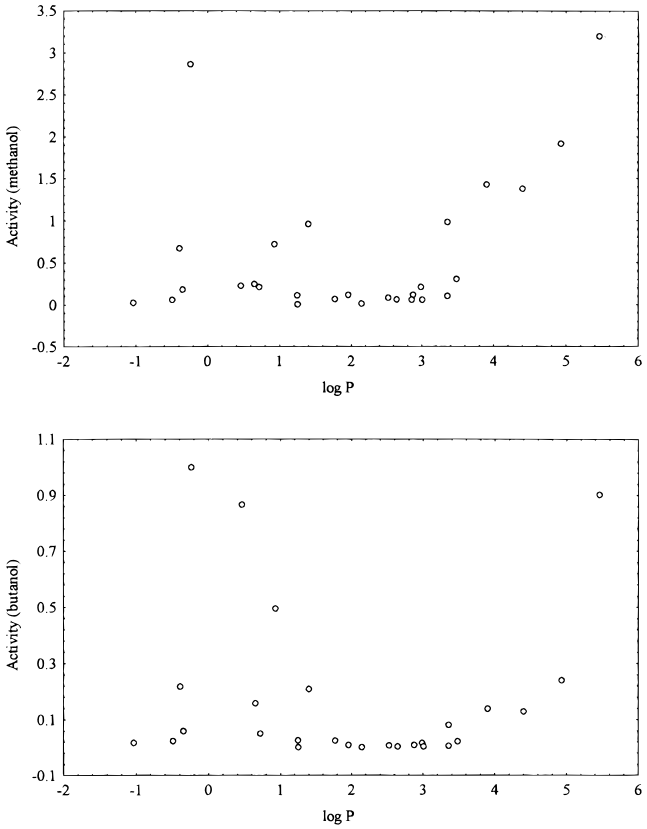

Figure 5. (a) Subtilisin activity for transesterification of *N*-acetyl-L-phenylalanine ethyl ester with methanol against log *P* for different solvents. (b) Subtilisin activity for transesterification between *N*-acetyl-L-phenylalanine ethyl ester with butanol against log *P* for different solvents.

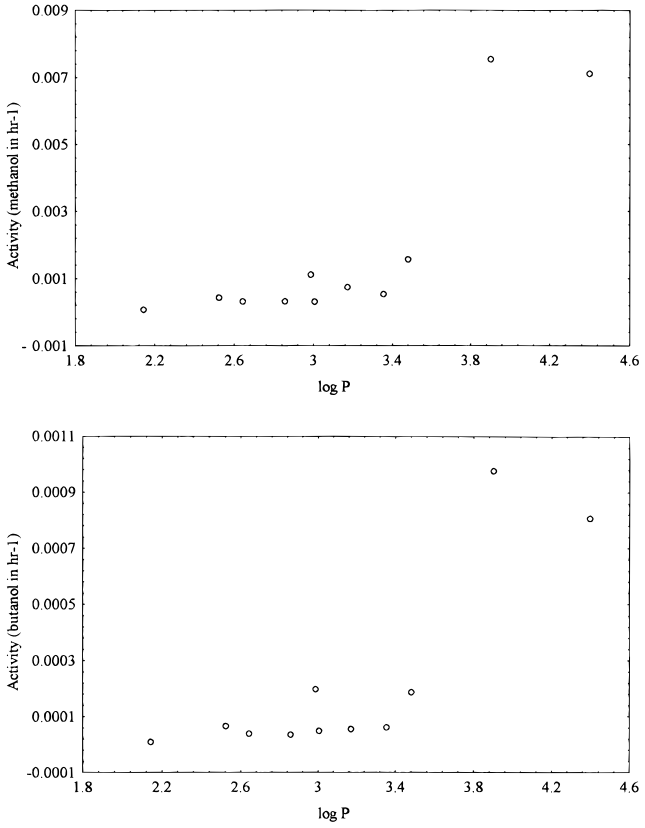

Figure 6. (a) Plot of subtilisin activity for transesterification of *N*-acetyl-L-phenylalanine ethyl ester with methanol after correcting for the partitioning. (b) Plot of subtilisin activity for transesterification of *N*-acetyl-L-phenylalanine ethyl ester with butanol after correcting for the partitioning.

interface than the ones with low log *P* values, and hence higher specificity values are observed.

Plots of deacylation activity with methanol and butanol are shown in Figure 5. It is interesting that, when activity data are corrected, the profiles of the activity curves for each nucleophile become similar (Figure 6). While it is tempting to interpret this as a proof that the partitioning exists in the way we describe, since few solvents selected for this study had log *P* values within the range 3.4−4.2, we must be careful not to overinterpret this data and future studies will be performed within the log *P* range where one observes an increase in the activity with log *P* value.

The data presented in the current report are important for a number of reasons, and present a case for how to move forward. If we are to understand the details of enzyme−substrate−environment relationships the data sets used must be as simple as possible. The system we describe here is very simple. We are attempting to model the attack of similar nucleophiles on a single enzyme−substrate complex with a fixed water activity. The respective regression models, discussed earlier in this report, for predicting log initial rate for each of the three alcohols show that it is possible to relate enzyme activity to simple properties of the nonaqueous solvent. These models are statistically very significant and have very good predictive ability (high R^2). These models apply only to solvents with log *P* values greater than 2.0. A remaining challenge is to find models for predicting specificity with the same high R^2 and statistical significance, and this may be a difficult goal to achieve. We have presented a statistically significant equation that describes the relationship between specificity and log *P*. This equation is of only moderate R^2. As in previously descibed systems, the utility of such statistically derived equations will probably be limited to the chosen enzyme−substrate pair. We are therefore left without a physically based explanation for the observed behavior in organic solvents.

For the reasons described, we decided to simplify the system further and assess the effect of a single solvent with variable physical properties on enzyme specificity. Supercritical fluids have a unique feature in that one can tailor their density-dependent physical properties by changing pressure.

For organic solvents, the specificity contributions derive from changes in physical properties (p_i) as well as interactions induced by changes in chemical structure (c_i)

$$\mathrm{d}S = \sum_{i=1}^{n}\left(\frac{\partial S}{\partial p_i}\right)\mathrm{d}p_i + \sum_{i=1}^{m}\left(\frac{\partial S}{\partial c_i}\right)\mathrm{d}c_i$$

where d*S* is specificity change in going from one solvent to another in which there are a total of *n* physical properties (p_i, *i=1 to n)* and *m* chemical nature induced interactions (c_i, *i=1 to m)*.

For supercritical fluids, the variation of chemical structure (and therefore variety of chemical structure induced interactions) is replaced by pressure (*P*):

$$\mathrm{d}S = \sum_{i=1}^{n}\left(\frac{\partial S}{\partial p_i}\right)\mathrm{d}p_i + \left(\frac{\partial S}{\partial P}\right)\mathrm{d}P$$

Ideally, however, one would be interested in the system where a single physical property p_i can be altered while all the others are kept constant such as

$$\mathrm{d}S = \left(\frac{\partial S}{\partial p_i}\right)\mathrm{d}p_i$$

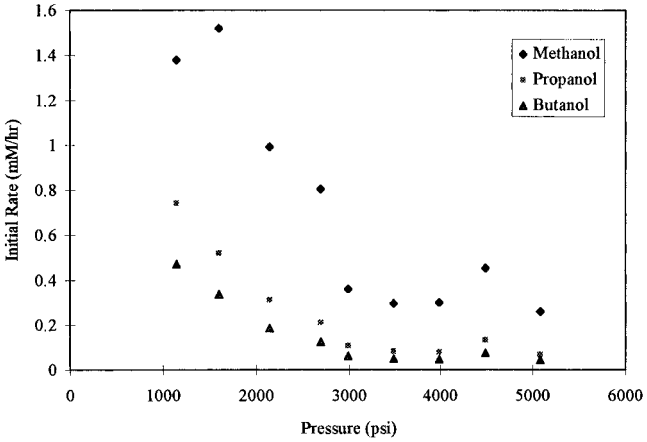

Figure 7. Effect of pressure on the initial rate of subtilisin catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester by primary alcohols (methanol, 1-propanol, and 1-butanol) in supercritical fluoroform. The experimental conditions are as explained in the text.

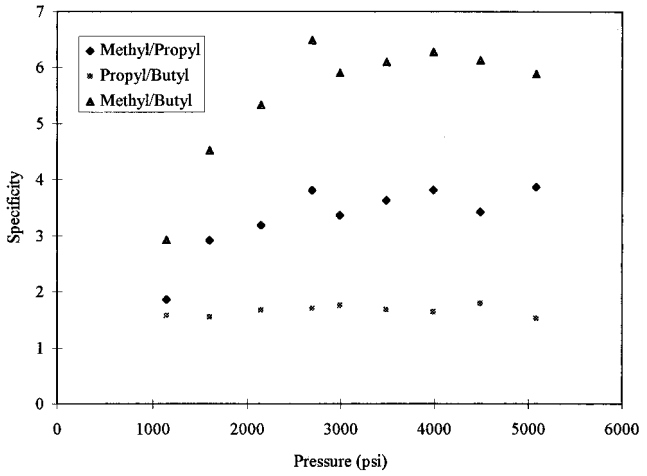

Figure 8. Effect of pressure on nucleophile specificity of subtilisin Carlsberg in transesterification of *N*-acetyl-L-phenylalanine ethyl ester by primary alcohols (methanol, 1-propanol, and 1-butanol) in supercritical fluoroform. The substrate specificity is expressed as the ratio of initial rates as explained in the text.

We have demonstrated previously that the pressures needed to accomplish these alterations in physical properties, and thus a tailoring of enzyme function, are low enough to have no direct effects on the enzyme. We report below the specificity data collected for fluoroform at varying pressures.

Subtilisin Activity and Nucleophilic Specificity in Fluoroform. In order to understand how much salt hydrate ($Na_4P_2O_7 \cdot 10H_2O$) was required to attain the optimal enzyme activity, we screened the subtilisin activity for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester by methanol at 5100 psi and 50 °C with varying $Na_4P_2O_7 \cdot 10H_2O$ concentrations. We observed, for the same substrate concentration, that enzyme activity varied with the changing concentration of salt hydrate and maximizing at a concentration of 0.025 g/mL. Therefore, all further experiments were performed with salt hydrate concentration of 0.05 g/mL.

The effect of pressure on the activity of subtilisin in supercritical fluoroform at 50 °C is shown in Figure 7. An order of magnitude change in subtilisin activity was observed over the small pressure range studied. The change in activity parallels smooth alterations in physical properties of fluoroform which occur with changing pressure.

Figure 8 shows the effect of pressure on the nucleophilic specificity of subtilisin Carlsberg at 50 °C in supercritical

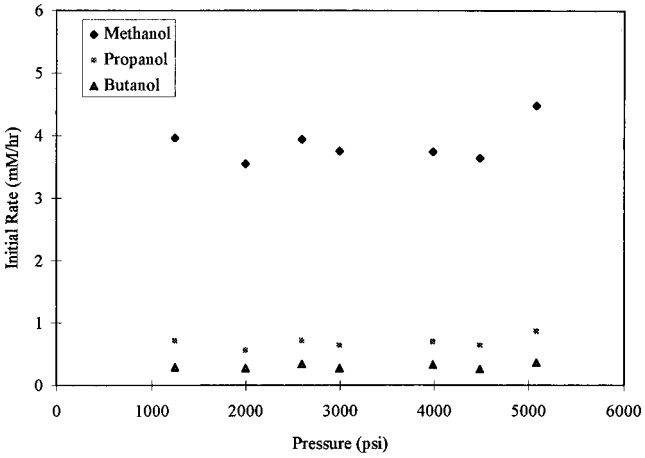

Figure 9. Effect of pressure on the initial rate of subtilisin-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester by primary alcohols (methanol, 1-propanol, and 1-butanol) in near-critical propane. The experimental conditions are as explained in the text.

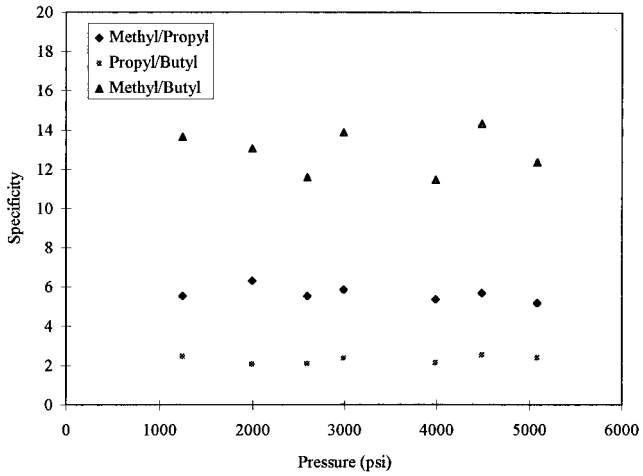

Figure 10. Effect of pressure on nucleophile specificity of subtilisin Carlsberg in transesterification of *N*-acetyl-L-phenylalanine ethyl ester by primary alcohols (methanol, 1-propanol, and 1-butanol) in near-critical propane. The substrate specificity is expressed as the ratio of initial rates as explained in the text.

fluoroform. Substrate specificity for methanol increases with increasing pressure. The increase in substrate specificity up to 3000 psi is mainly due to a lower drop in the catalytic efficiency of methanol compared to drop in catalytic efficiencies for 1-propanol and 1-butanol. Indeed, the maximum drop in catalytic efficiency of methanol is 80% while the drop in catalytic efficiency of the other two alcohols is 90%. This represents a one order of magnitude difference in the effect of pressure on each.

In conventional solvents such as liquid propane, increasing pressure from 100 to 5100 psi has no significant effect on the physical properties of the solvent or subtilisin activity and specificity (Figures 9 and 10). The intrinsic effect of pressure alone on enzyme activity is not significant. Further, since the reaction is not diffusionally limited, any changes in the activity and specificity of subtilisin in supercritical fluoroform are solely the results of solvent physical properties alterations rather than the pressure of the system.

The physical properties of fluoroform which are most relevant to biocatalysis, such as solubility parameter and dielectric constant, change by 1 order of magnitude between 850 and 5100 psi.[11] In particular, the dielectric constant changes from 1 to 8 and the solubility parameter increases from 12 to 50 $MPa^{1/2}$. Since, with increasing pressure, enzyme specificity smoothly

changes with pressure, it is quite tempting to believe that either dielectric constant or solubility parameter or any other density-dependent physical property could be used to predictably control subtilisin specificity. However, to say that this is the effect of a change in only one physical property is not advisable because these properties are interrelated. As can be seen from our data, the general correlations (if derived) from our results in fluoroform are not likely to hold if extended to organic solvents, where there is a discontinuity in the values of the different physical properties between solvents. The lack of such a system in which the changes in physical properties are not interrelated prevents us from making any definitive conclusions about the cause and effect relationship between enzyme behavior and the changing physical property in a supercritical fluid. Nevertheless, we are currently evaluating the use of mixtures of supercritical fluids to generate solvents in which one physical property at a time can be altered and tested.

Conclusions. Subtilisin activity and nucleophilic specificity for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester by primary alcohols in nonaqueous media (organic solvents and supercritical fluoroform) can be altered by changing the solvent environment. For the solvents with log *P* greater than 2.0, the activity of the enzyme is positively correlated with log *P* and is negatively correlated to the presence of unsaturation in the solvent molecule. The reaction was studied at constant water activity and is not diffusionally limited, indicating that the change in activity and substrate specificity of subtilisin is wholly the result of the changing solvent environment. In organic solvents, the partitioning of substrates into the microaqueous phase is related to solvent log *P*. Correcting for partitioning, one can say that, while the intrinsic activity of subtilisin is higher with methanol as compared to butanol, changing the solvent environment appears to affect this intrinsic activity in a similar manner (Figure 11).

In supercritical fluoroform, both activity and nucleophilic specificity of subtilisin change with the pressure. We have shown that these changes are related neither to pressure nor to changing water solubility in fluoroform with pressure. The change in activity and specificity of subtilisin in supercritical fluoroform parallels the change in physical properties of the solvent. Supercritical fluoroform is an ideal medium for basic studies in nonaqueous biocatalysis since the enzyme specificity can be easily tailored by changing pressure and/or temperature of the system, without changing the molecular structure of the solvents.

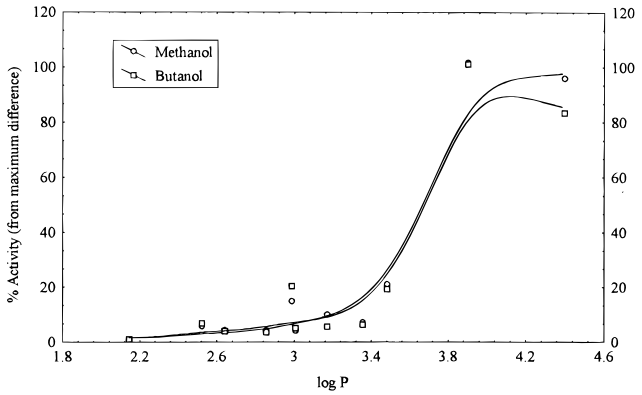

Figure 11. Plot of percentage activity (activity$_{(solvent)}$/(highest activity$_{(observed)}$ − lowest activity$_{(observed)}$) for different solvents after correcting for partitioning against log *P* for transesterification of *N*-acetyl-L-phenylalanine ethyl ester by methanol (left) and butanol (right).

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Supporting Information Available: Plots described in Dependence of Specificity on Solvent Physical Properties (11 pages). See any current masthead page for ordering and Internet access instructions.

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