Biocatalytic synthesis of acrylates in supercritical fluids: Tuning enzyme activity by changing pressure

(enzymes/lipase/transesterification)

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ABSTRACT Supercritical fluids are a unique class of nonaqueous media in which biocatalytic reactions can occur. The physical properties of supercritical fluids, which include gas-like diffusivities and liquid-like densities, can be predictably controlled with changing pressure. This paper describes how adjustment of pressure with the subsequent predictable changes of the dielectric constant and Hildebrand solubility parameter for fluoriform, ethane, sulfur hexafluoride, and propane, can be used to manipulate the activity of lipase in the transesterification of methylmethacrylate with 2-ethyl-1-hexanol. Of particular interest is that the dielectric constant of supercritical fluoriform can be tuned from approximately 1 to 8, merely by increasing pressure from 850 to 4000 psi (from 5.9 to 28 MPa). The possibility now exists to predictably alter both the selectivity and the activity of a biocatalyst merely by changing pressure.

When the temperature and pressure of a material exceed, or approach, the critical points for that material, the physical properties of the “solvent” become sharply dependent on the pressure encountered by the material. Since density is controllable via adjusting either pressure or temperature, it is possible to tune the solvent physical properties by changing reaction conditions, rather than the solvent itself. In addition to these benefits, supercritical fluids (SCFs) are recyclable solvents that aid significantly in sample preparation (1). SCFs are also ideal dispersants in which to investigate the effect of solvent physical properties on an enzyme-catalyzed reaction (2–7).

Enzymes are known to function in both aqueous and organic media (for instance, see ref. 8 or ref. 9). Studies of biocatalysts in nonaqueous environments have led to a deeper understanding of how enzymes function in unnatural surroundings as well as in water (10). A detailed understanding of environment/structure/function relationships for proteins suspended in organic media may enable predictive control of enzyme function merely by “solvent engineering.” Much research has been dedicated to furthering our understanding of how enzymatic activity is related to the physical properties of the solvent in which it is placed. The driving force for much of this research is the fact that when a lyophilized enzyme particle is suspended in an organic solvent, the activity, specificity, and stability of the catalyst depend on the solvent. If one could predict how a particular solvent would affect an enzyme, then, as described above, rational solvent engineering would be a simple way to tune biocatalyst function.

Although determining the effect of changing solvent on activity and specificity can suggest interesting correlations between solvent and enzyme properties, the question of why similar solvents can have such different abilities to support biocatalytic reactions always remains. For example, lipase-catalyzed transesterification of acrylates in hexane, heptane, octane, nonane, and decane gives significantly differing rates that no simple model can explain (11). If, however, one could predictably and significantly alter the physical properties of a solvent without changing the solvent molecular structure, it might be possible to elucidate more clearly the relevance of various physical properties in predicting the activity of an enzyme. In particular, the distinct differences in enzyme–solvent interactions, which are dependent on solvent structure, will not obscure the meaning of any enzyme environment/function relationships which exist.

As previously indicated, the physical properties of SCFs are sharply dependent on pressure. Enzymes, however, are generally unaffected by small (<10,000 psi; 1 psi = 6.89 KPa) pressure increases. Clearly, the possibility exists to perform biocatalysis in SCFs at various pressures. In such a system, the effect of pressure on the reaction rate should be the result of changes in the physical properties of the fluid rather than changes in the structure of the bulk solvent. Naturally, for this approach to be successful, the physical properties of the fluid must be tunable over a range which will affect the activity of an enzyme.

This paper reports the effect of pressure on some SCF physical properties and how these alterations change the activity of an enzyme. Our model reaction is the lipase-catalyzed transesterification of methylmethacrylate with 2-ethyl-1-hexanol, to produce 2-ethylhexylmethacrylate and methanol. We have previously reported the activity of the enzyme (EC 3.1.1.3; from Candida cylindracea) in 14 conventional solvents, near-critical propane, and supercritical ethane, carbon dioxide, fluoroform, sulfur hexafluoride, and ethylene (11).

The use of fluoroform as a solvent for nonaqueous biocatalytic reactions is particularly interesting because there is a marked effect of pressure on the solvent dielectric constant. Experimental data have shown that the dielectric constant of fluoroform is dependent on pressure, increasing from approximately 1 to 8 over a relatively small pressure change (850–4000 psi) (12). Recently, Clark, Dordick, and colleagues (13, 14) have reported that the majority of significant alterations in the flexibility of a protein occur when the solvent dielectric increases from 1 to 10. Given that changes in dielectric constant have also been related to the flexibility, hydration, intrinsic activity, specificity, and stability of enzymes in anhydrous media, fluoroform appears to be an ideal solvent in which to study such effects in detail.

EXPERIMENTAL PROCEDURES

Materials. All substrates were purchased from Aldrich. Gases were purchased from Airco. Lipase (Candida cylindracea) was purchased from Sigma.

Abbreviation: SCF, supercritical fluid.

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Correlation of Physical Property Data for SCFs. For each of the six fluids examined in our studies, a pressure-explicit equation of state was used to calculate density. In the cases of ethylene and carbon dioxide a multiparameter virial equation was employed (15, 16), whereas a modified Benedict-Webb-Rubin expression was used for ethane, propane, sulfur hexafluoride, and fluoroform (17, 18). Density (\(\rho\)) was calculated as a function of temperature and pressure by using a Newton-Raphson iterative procedure.

The Hildebrand solubility parameter (\(\delta\)), a first approximation for the solvating power of a particular material, was derived from the cohesive energy density as shown in ref. 19. Whereas for conventional liquids the cohesive energy density (\(\rho_{\text{ce}}\)) is expressed as a function of the heat of vaporization, because we employ SCFs we have employed the thermodynamic definition of the \(\rho_{\text{ce}}\) as shown in ref. 19. Therefore, we have determined the Hildebrand solubility parameter for each fluid versus temperature and pressure.

We have used the following expression to correlate literature data (12, 16, 20–22) for dielectric constant (\(\epsilon\)) with density and temperature (\(T\)):

\[
[(\epsilon - 1)/(\epsilon + 2)](1/\rho) = C_1 + C_2(\rho) + C_3(\rho)^2 + C_4\ln[1 + (T/T_c)],
\]

in which \(C_1\) to \(C_4\) are virial coefficients and \(T_c\) is the critical temperature. Densities for each fluid were calculated as described above, and thus dielectric constant was calculated for each fluid versus temperature and pressure. In cases where the fit of experimental data to this equation was not available in the literature, fitting was performed prior to calculation of the dielectric.

Measurement of Enzyme Activity. All initial rate determinations were performed as described previously in a specially designed high-pressure reactor (11). For all experiments the initial concentrations of methymethacrylate and 2-ethylhexanol were fixed at 20 mM, enabling complete miscibility in the pressure range utilized (580–4000 psi). The reaction was followed for approximately 3 hr, with a 5% conversion of substrate to product.

RESULTS AND DISCUSSION

The effects of pressure on density, Hildebrand solubility parameter, and dielectric constant of propane, ethane, fluoroform, and sulfur hexafluoride were predicted as described above. Fig. 1 is an example of the ability of our program to accurately predict physical properties for some of the solvents. Fig. 2 demonstrates the dependence of physical properties of these solvents on pressure in the range within which we wished to test enzyme activity. Propane is subcritical under our reaction conditions, and thus the effect of pressure on solvent physical properties is negligible. The use of fluoroform as a solvent enables control of the solvent dielectric over a large range (1.5–7) with a relatively small change in pressure. The Hildebrand solubility parameter for both ethane and sulfur hexafluoride increases with pressure, while there is essentially no change in dielectric constant. Sulfur hexafluoride is a particularly hydrophobic, nontoxic, high-density solvent in which detailed studies with enzymes have never, to our knowledge, been reported.

The kinetic parameters for the lipase-catalyzed transesterification of methymethacrylate have been reported previously (11). Given the high \(K_m\) (ester), at low substrate concentrations the initial rate is proportional to \(V_{\text{max}}/K_m\), and thus the dependence of initial rate on solvent physical properties follows the effect of solvent on free enzyme and free substrate. We chose an initial substrate concentration of 20 mM methymethacrylate so that all substrate would be soluble over the entire pressure range selected. The temperature for all reactions was 50°C, which exceeds the critical temperature for ethane, fluoroform, and sulfur hexafluoride.

Our initial experiments were performed in ethane. If the effect of solvent physical properties on enzyme activity is related to changes in density, then one should observe the same trend in enzyme activity whether density is altered by

<table>
<thead>
<tr>
<th>Temp., (^{\circ}\C)</th>
<th>Pressure, (\text{psi})</th>
<th>(\Delta) Initial rate, %</th>
<th>(\Delta) solubility parameter, %</th>
<th>(\Delta) (\rho), %</th>
<th>(\Delta) (\epsilon), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>1600</td>
<td>71.5</td>
<td>-4.4</td>
<td>-3.51</td>
<td>-1.3</td>
</tr>
<tr>
<td>50</td>
<td>1600</td>
<td>91.6</td>
<td>-8.8</td>
<td>-7.38</td>
<td>-2.6</td>
</tr>
<tr>
<td>55</td>
<td>1600</td>
<td>113</td>
<td>-13</td>
<td>-11.3</td>
<td>-4.2</td>
</tr>
<tr>
<td>60</td>
<td>1600</td>
<td>212</td>
<td>-18</td>
<td>-15.5</td>
<td>-5.5</td>
</tr>
</tbody>
</table>

For changing temperature, values reported are relative to those at 40°C. For changing pressure, values reported are relative to those at 1600 psi.

![Fig. 1. Comparison of theoretical prediction (line) and measured data (triangles) for effect of density on dielectric constant of fluoroform at 323 K. For fluoroform experimental data are available only at 325 (△) and 292 K (▼) (12).](image)

![Fig. 2. Effect of pressure on Hildebrand solubility parameter (A) and dielectric constant (B) for ethane (curve 4), propane (curve 3), fluoroform (curve 2), and sulfur hexafluoride (curve 1) at 30°C.](image)
changing temperature or pressure. Table 1 describes the effect of changing only either temperature or pressure on enzyme activity. It is clear that increasing density causes decreased rates for both temperature and pressure alterations. Naturally, there will be an intrinsic effect of increasing temperature on the rate of the reaction, and thus one cannot compare the actual rates achieved when using temperature and pressure to control density. For the same reason, further experiments requiring density control have been performed with pressure fluctuations which would not be expected to alter rate, a priori, in the range used. The results imply that the effect of density on enzyme activity is independent of the method of density control. The effect of pressure on enzyme-catalyzed reactions in conventional solvents (including water) is often reported in terms of a volume of activation. Activation volume can provide important information regarding the effect of pressure on a reaction (23), especially if the reaction is diffusionally limited. Care must be taken, however, when interpreting activation volumes that are determined from only two rate determinations at different pressures.

Solvent physical properties which have been reported to affect activity include partition coefficient, dielectric constant, and Hildebrand solubility parameter (24). For individual classes of solvent, the partition coefficient between octanol and water (log $P$) can be related to Hildebrand solubility parameter as shown in Fig. 3. Our data base was designed to determine only Hildebrand solubility parameter and dielectric constant, since log $P$ determination would be significantly more complex. Since, as shown in Fig. 3, the solubility parameter and log $P$ for a given solvent are related, the effect of solubility parameter should mirror that of log $P$. Interestingly, since log $P$ and solubility parameter are related, and solubility parameter varies to a first approximation with $\varepsilon = (e - 1)/(e + 2)$, there should also be a relationship between dielectric constant and log $P$. Although the experiment described in Table 1 indicates that controlling density can control the activity of the enzyme, the information generated does not enable one to distinguish how, for instance, solvent dielectric constant and Hildebrand solubility parameter each could be used to predict the activity of the enzyme. We stress that simply because a solvent property can be used to predict activity does not mean a priori that this property is the sole determining factor in controlling the activity of the catalyst. Fig. 4 describes the effect of pressure on the lipase-catalyzed transesterification reaction in supercritical ethane, fluorocarbon, and sulfur hexafluoride in and subcritical propane. It should be noted that the extreme hydrophobicity of sulfur hexafluoride probably results in partitioning of the reaction product onto the enzyme surface (without inhibition). This increases the experimental error of the system but could result only in underestimation of reaction rates. It may be tempting to correlate the rate of reaction in supercritical sulfur hexafluoride to its extremely high solubility parameter, but caution should be exercised. The solubility parameter is so high because of the unusually high density of sulfur hexafluoride, and it is not related to a property which is more likely to affect the rate of reaction.

In a conventional solvent such as hexane, or a subcritical compressible fluid such as propane, increasing pressure from 850 to 4000 psi has little or no effect on the physical properties of the solvent. Our results indicate that a similar pressure increase does not affect the catalytic activity of the enzyme in propane or hexane (data for hexane not shown here), as measured by changes in $V_{\text{max}}/K_{\text{m}}$ (which is proportional to initial rate at the substrate concentrations used). We have also reported previously that this enzyme-catalyzed reaction is not diffusionally limited in either SCFs or conventional solvents (30). Thus, any changes in the activity of the enzyme are solely the result of solvent physical property alterations rather than the pressure of the system. It is, however, worth summarizing once again the reasons why we can say with certainty that this reaction is not diffusionally limited. The rate of reaction in conventional solvents is maximal with the agitation chosen; the rate of reaction in sulfur hexafluoride is not altered with pressure, while the solvent mass transfer coefficient does change over this range; the effect of temperature fits the data expected for a kinetically controlled reaction; and the estimated rate of reaction for a mass transfer limited reaction with these particles is approximately five orders of magnitude faster than the rates observed. Fig. 5 combines the data generated for the effect of Hildebrand solubility parameter, dielectric constant, and density on enzymatic activity in supercritical ethane, fluorocarbon, and sulfur hexafluoride.

In the case of both ethane and sulfur hexafluoride, the change in pressure will not result in any significant alterations in the solubility of water. Indeed, both dispersants are very hydrophobic at all pressures, and water introduced into the system via an enzyme particle will undoubtedly partition onto the enzyme at all pressures. Fluorocarbon, however, exhibits marked changes in its ability to solubilize water as pressure is increased. As the dielectric and Hildebrand solubility parameter of fluorocarbon increase with pressure, so will the solubility of water in the system. Thus, at high pressures more water could be partitioned from the enzyme particle into the solvent. To determine the role of any changes in water solubility in fluorocarbon upon the activity of lipase, we have investigated the effect of pressure at various water.
solubility parameter. In the case of sulfur hexafluoride, over the pressure range of our experiments, the Hildebrand solubility parameter more than doubles. The activity of the enzyme, while difficult to measure exactly, remains within a constant range, indicating that for this fluid the increase of solubility parameter is not rate limiting. Both the solubility parameter and dielectric constant for fluorofom increase over our pressure range (4 times and 5 times, respectively). Given that the increase in solubility parameter matches that for sulfur hexafluoride (see Fig. 2), we can see that the decreased activity of the enzyme (90%) at high pressure can be predicted by increased solvent dielectric constant. The intrinsic activity of the enzyme in each fluid is, of course, different, and therefore one should be cautious when comparing the effect of pressure in different solvents. Ethane is also a hydrophobic solvent, and in our pressure range the activity decreases with increasing dielectric and solubility parameter. The solvent physical property which can be used to predict activity for all three solvents is dielectric constant. In determining the meaning of this data set, we should not be tempted to state that only solvent dielectric constant controls activity. Fig. 3 demonstrates that the meaning of Hildebrand solubility parameter varies with different solvent types when compared with other typical scales of solvent hydrophobicity. Thus, when attempting to predict activity based on solvent Hildebrand solubility parameter, one should not expect correlations between activity in different classes of solvents (like sulfur hexafluoride and ethane).

Our data indicate that for this enzyme–substrate pair, the effect of solvent on activity of the enzyme can be predicted from changes in solvent dielectric constant. While we are not stating that the dielectric constant is the sole determining factor of lipase activity in these SCFs, it is interesting to consider how dielectric constant could affect enzyme activity. Traditionally one examines the effect of solvent character on rate constants by using the Kirkwood expression (26, 27). As a first approximation, the Kirkwood expression predicts the following dependence of rate on dielectric constant for simple homogenous reactions:

$$\ln \text{rate} \propto \frac{\epsilon - 1}{\epsilon + 1}.$$  

While a complete discussion of this relationship is beyond the scope of this particular paper, the data do fit this equation well (Fig. 7).
CONCLUSION

We have shown that the physical properties of SCFs are tunable over a range which can significantly affect enzyme activity. In particular, the dielectric constant of fluoriform can be predictably altered with small changes in pressure. Indeed, at 600 psi the physical properties of fluoriform resemble those of propane at its vapor pressure, while above 4000 psi fluoriform is more like methylene chloride. We have shown that the effect of pressure on this lipase-catalyzed transesterification is neither a pressure effect (see discussion of results with propane and hexane) nor solely an effect of changes in water solubility (the same trends are observed when solvent is water saturated). In addition, the reaction we are studying is not diffusionally rate limited, and changes in solvent mass transfer coefficients with pressure will not affect activity. Although it is not possible to separate out the effect of pressure on $V_{\text{max}}$ and $K_m$, we have hypothesized that the activity of this enzyme will be best predicted by models incorporating changes in solvent dielectric constant. Other researchers have also shown that changes in dielectric constants of conventional solvents can be used to manipulate enzyme activity and specificity (28), and we propose that supercritical fluoriform is an ideal fluid for further investigation and utilization of these “solvent engineering” phenomena. Indeed, by using enzymes for which microscopic rate constants can be determined in nonaqueous media (29), it should be possible to determine the effect of solvent physical properties on each step of the transesterification mechanism.

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