Control of Enzyme Enantioselectivity with Pressure Changes in Supercritical Fluoroform

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In the last decade the activity,[1] stability,[2] and specificity[3] of enzymes suspended in anhydrous organic solvents[4] have been studied in great detail. The ability of enzymes to catalyze reactions in unnatural media has also been extended to biocatalytic reactions in supercritical fluids.[5] Of particular note for those interested in nonaqueous biocatalysis is that the selectivity of enzymes can be affected by solvent type.[3] In this paper we describe how the pressure of a supercritical fluid (SCF) can be used to tune the enantioselectivity of the catalyst.

Until the late 1980s, protein engineering was one of the only approaches to controlling enzyme activity.[6] However, recent work has shown that in conventional organic media the solvent partition coefficient and dielectric constant can be used to alter the specificity of an enzyme.[3] Biophysical models have been proposed to explain the dependence of, in particular, enantioselectivity on the solvent physical properties.[7] Subtilisin, a serine protease, is the most commonly used enzyme for detailed studies of the effect of solvent on enzyme properties.[8] Indeed, given information regarding the mechanism,[1] rate,[9] and specificity[9] of subtilisin in many different solvents, "solvent engineering" has evolved into a realistic alternative approach to controlling subtilisin activity and specificity.

Unfortunately, predictable solvent engineering is inconsistent with the many physical property changes which accompany a change in solvent structure.[10] We have suggested that SCFs can alleviate such problems, since gradual changes in the pressure of a SCF result in manipulation of solvent properties.[11]

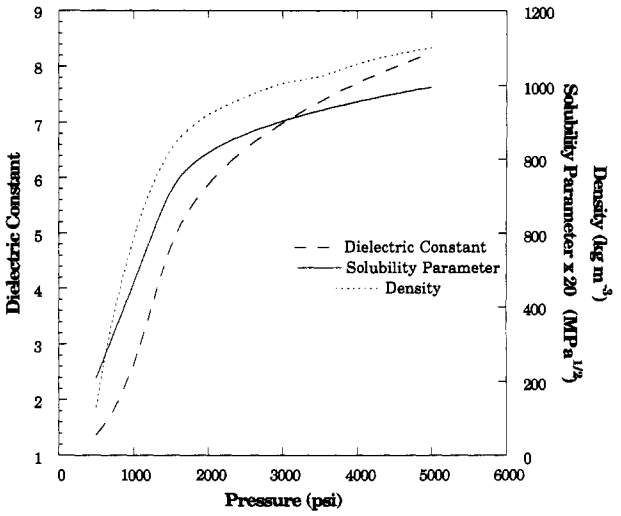

Figure 1. Effect of pressure on the physical properties of fluoroform.[11]

Irrespective of the effect of pressure on physical properties, SCFs are attractive nonaqueous media for biocatalysis[5] and chemical processing in general.[12] Our initial studies in SCFs have focused on the transesterification reaction between methyl methacrylate and 2-ethylhexanol catalyzed by lipase (*Candida cylindracea*).[10] While carbon dioxide is a potent inhibitor of the reaction, fluoroform is an ideal solvent. Further, the dielectric constant of fluoroform can be changed with pressure alteration. Previously we have used the pressure-tuned physical properties of fluoroform to elicit predictable effects on enzyme activity in the transesterification mentioned above.[11] We now determine whether similar pressure-induced changes in the physical properties of fluoroform can elicit predictable changes in the enantiospecificity, of enzymes.

We have examined the enantiospecificity of proteases from *Bacillus licheniformis* (subtilisin Carlsberg) and *Aspergillus* in fluoroform. The effect of pressure on selected physical properties of fluoroform is described by Figure 1. Clearly, pressure has a significant effect on the properties of the solvent. As pressure increases, the solvent becomes more hydrophilic in nature.[13] Changes of this order of magnitude in physical properties of conventional organic solvents have been shown to elicit alterations in specificity. For propane under similar conditions, there is little effect of pressure on solvent physical properties.[14]

Figures 2 demonstrates that as pressure is increased from 950 to 5100 psi, the activity of both enzymes (given as the specificity constant, k_{cat}/K_m) decreases. Interestingly, the activity of both enzymes is not affected by pressure when the solvent is propane.[15] As shown previously[11] for lipase-catalyzed reactions, the effect of pressure alone on the actual reaction rate is negligible; what exerts an effect on the rate of reaction is the altered physical properties of the fluid.

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(13) For fluoroform at 950 psi, the solubility parameter and dielectric constant are 21.19 MPa$^{0.5}$ and 2.36, respectively, whereas at 5100 psi they are 49.94 MPa$^{0.5}$ and 7.08, respectively.
(14) At 950 psi, the solubility parameter and dielectric constant of propane are 10.96 MPa$^{0.5}$ and 1.62, respectively, while at 5100 psi they are 12.91 MPa$^{0.5}$ and 1.71, respectively. the pressure dependence of enzyme enantioselectivity to be studied.
(15) For subtilisin in propane at 950 and 5100 psi, k_{cat}/K_m for the L ester was 1.47×10^{-2} min^{-1} mM^{-1}, and for the D ester 6.8×10^{-4} (950 psi) and 6.6×10^{-4} min^{-1}mM^{-1} (5100 psi). For *Aspergillus* protease in propane, the L-ester k_{cat}/K_m values were 5.95×10^{-1} (950 psi) and 5.76×10^{-1} min^{-1} mM^{-1} (5100 psi), and for the D-ester, 5.6×10^{-4} (950 psi) and 5.0×10^{-4} (5100 psi) min^{-1} mM^{-1}.

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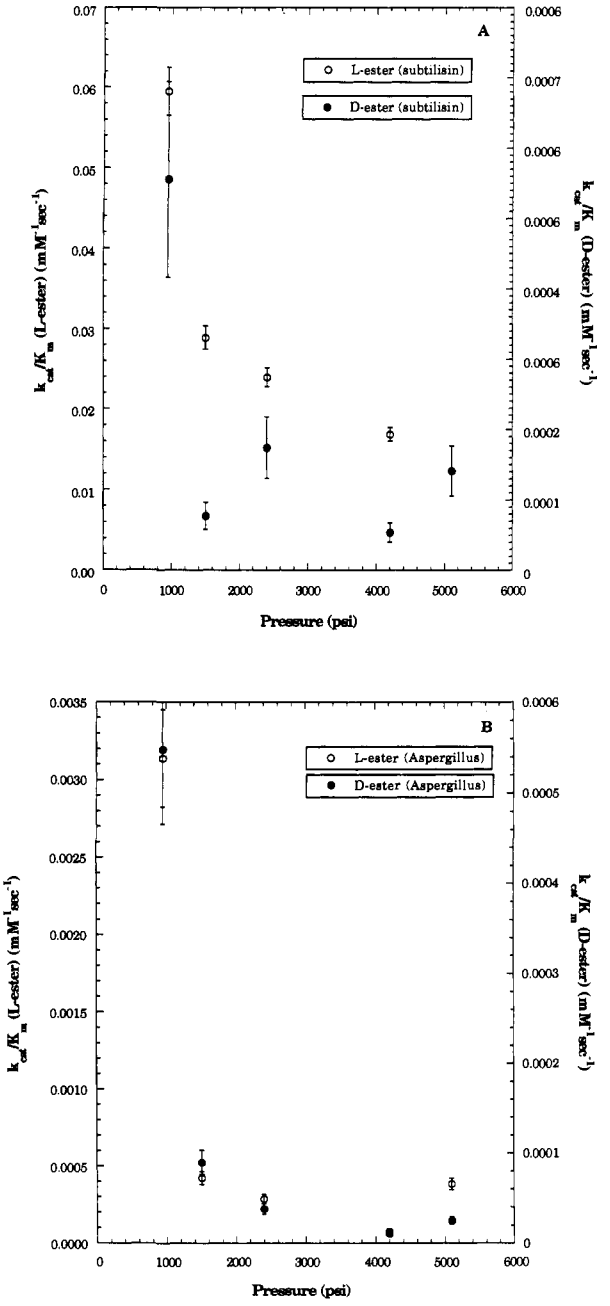

Figure 2. Effect of pressure on rate of reaction for *Aspergillus* protease- or subtilisin Carlsberg (1–20 mg/mL)-catalyzed transesterification of *N*-acetyl-(L or D)-phenylalanine ethyl ester (25 mM) with methanol (1 M) at 50 °C (± 3%) in fluoroform. A: Subtilisin B: *Aspergillus*.

Taking the ratios of the relevant pairs of fitted lines in Figure 2, we can generate a plot of enantioselectivity versus pressure for both enzymes in fluoroform and subcritical propane. Figure 3 shows the enantioselectivities of both subtilisin and *Aspergillus* protease over a pressure range of 950–5100 psi. While the actual selectivities of the two enzymes are distinct, both enzymes become more stereoselective as pressure increases. That is, as fluoroform becomes more hydrophilic, the enantioselectivity increases. There is no change in enantioselectivity for the enzyme-catalyzed reactions in propane.

Johnston and colleagues have investigated the effect of pressure on the selectivity. They report that for the reaction between methyl methacrylate and cyclopentadiene in pure carbon dioxide, an increase in pressure results in a change in selectivity from 2.8 to 2.88.[16] In a stereoselective enzyme-catalyzed process, the

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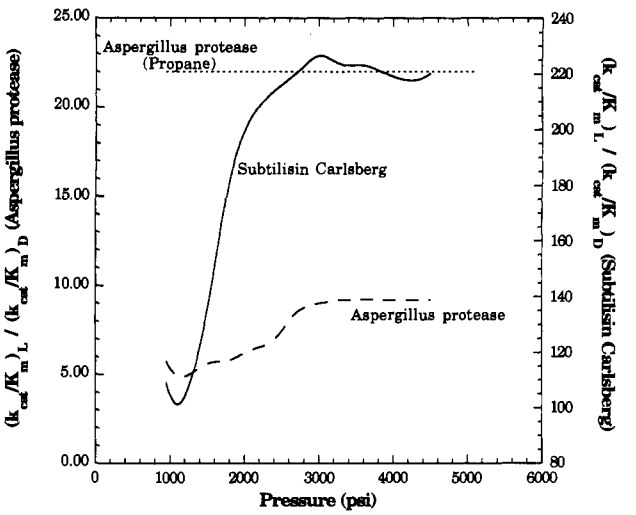

Figure 3. Effect of pressure on enantioselectivity in fluoroform and propane. The lines represent the calculated ratios of fitted lines to the data from Figure 2.

solvent can exert its effect directly on the catalyst, and, not surprisingly, the effect of pressure on specificity for the reactions studied herein is more significant.

Since the enzyme-catalyzed reaction is not diffusionally limited, the effect of pressure cannot be the result of altered rates of mass transfer of the solutes as a function of pressure.[17] Rather, the data are consistent with a hypothesis which has been described previously.[18] Briefly, when a hydrophobic substrate binds to the surface of subtilisin and similar enzymes, the driving force for the successful interaction is supplied by the entropy gain resulting from the release of ordered water molecules from the hydrophobic site on the enzyme. Not surprisingly, in organic solvents the binding of hydrophobic molecules to subtilisin is severely impaired, as determined from measurements of the actual binding constant.[1] The binding of substrates will, however, be more favored as the organic solvent becomes more polar. Since binding of each enantiomer will result in different amounts of water being expelled from the substrate binding pockets, one would expect that as the polarity of the solvent changes, so will the enzyme-enantioselectivity. The information provided by Figures 1 and 3 demonstrate that as the dispersent becomes more hydrophilic with increasing pressure, the enantioselectivity of the enzyme increases. The data are also consistent with the widely accepted notion that as solvent hydrophobicity decreases, the activity of enzymes will also decrease as a result of loss of water from the enzyme preparation. The data in Figure 2 show clearly that as dielectric constant increases, so the enzyme activity for the D and L esters decreases.

In conclusion, enzyme enantioselectivity can be controlled by changing the reaction conditions in SCFs, using pressure as the sole independent variable. Thus, without changing the solvent, yet using a solvent engineering approach, both enzyme activity and stereoselectivity can be predictably tailored in a supercritical environment. We are currently investigating the potential of pressure control to manipulate the regiospecificities and substrate specificities of proteins dispersed in compressible gases.

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