NATIONAL BUREAU OF STANDARDS

COAXING ENZYMES TO ACT IN ORGANIC SOLVENTS

GAITHERSBURG, Md.—Enzymes and industrial processes have one basic incompatibility: most enzymes work in water while most processes in the chemical, petrochemical, and food industries (for example) simply don't. However, Alexander Klibanov (Massachusetts Institute of Technology) pointed out at a conference here in May on the "Chemical Aspects of Biotechnology" that as long as enzymes are surrounded by a thin shell of water, they *are* able to function in organic solvents. Such enzymes also become extremely thermostable and exhibit different substrate specificities. In fact, many enzymatic reactions that are not feasible in water take place readily in organic solvents.

All of the forces that maintain the catalytically active structure of an enzyme—including van der Waals interactions, hydrogen bonds, and salt bridges—require water either directly or indirectly. If the water is removed, the structure collapses. Klibanov explains that the relevant question is not *whether* water is required, but *how much* water is required. An enzyme doesn't "see" the entire 55.5 moles of water per liter that surround it in an aqueous solution; it only sees a shell of water around itself. Because water is *naturally* very tightly bound to an enzyme, a layer of essential water will remain with the enzyme and the rest of the solution can be replaced with an organic solvent.

Since it is imperative that the enzyme be surrounded with essential water, the most useful solvents are very hydrophobic—they will not pull water away from the enzyme. Less hydrophobic solvents will work, too, as long as they are resaturated with aqueous solutions so that their thirst for water is quenched. For most enzymes, hydrophilic solvents are out of the question; there are some, however, in which the water is bound so tightly that even hydrophilic solvents cannot usurp the water.

The pH of the solution is important, as well—a thorny problem because organic solvents *have* no pH. How does the enzyme know what the pH is, then? Apparently, an enzyme "remembers" the pH of the last aqueous solution it contacted.

Klibanov showed the importance of pH by comparing the activity of two subtilisin preparations, one taken straight off the shelf, the other freeze-dried from a pH 7.8 solution. The pH-adjusted enzyme was 300 times more active than the "bottled" enzyme.

Because enzymes are insoluble in organic solvents, they become heterogeneous catalysts. This raises the possibility of diffusion limitations—both inter- and intra-particle. Klibanov has surmounted these limitations by depositing the enzyme on the surface of small solid support particles—beads or sand—and then agitating them vigorously.

As long as enzymes are surrounded by a thin shell of water, they can function in organic solvents. They also become thermostable and exhibit different substrate specificities.

Enzymes often exhibit marked changes in substrate specificity when placed in organic solvents. Some substrates that are very good in aqueous solution become mediocre in organics, and some that are very poor in water become quite good out of it. Klibanov explains that this is due to changes in the free energy of catalysis. The total free energy is the difference between the free energy of binding to the substrate and that of binding to the medium. While protein engineers are working on altering the first term (by altering the active site to increase binding capacity), Klibanov has altered the second term (the medium). This essentially reverses the substrate specificity of enzymes. Whereas in water hydrophobic substrates will "escape" by entering the active site of an enzyme and hydrophilic substrates will remain in solution, in organic solvents there are no hydrophobic interactions, so now hydrophilic substrates have an incentive to enter the active site.

The enzymes also show greatly enhanced thermal stability. Apparently, all chemical and structural changes that lead to irreversible thermal inactivation have one thing in common—they require water. It follows that if enzymes are placed in an essentially water-free environment, they will become more thermostable. Klibanov compared the thermostability of porcine pancreatic lipase at 100°C in three water concentrations: 100 percent, one percent, and less than 0.02 percent. In water the enzyme inactivates almost instantaneously. In the one-percent concentration, the enzyme's half life is 10 minutes; when the water is reduced to 0.02 percent, the half life increases to 12 hours. Klibanov says that it's nice that the enzyme can withstand heating, but it's exciting that it will also *work* at this temperature. The enzyme is 10 times more active at 100°C than it is at room temperature.

Perhaps the most intriguing aspect of enzymes in organic solvents is that they can catalyze reactions they cannot in water. For instance, in water lipase catalyzes but a single reaction: hydrolysis. In a solvent, it can catalyze at least six other reactions, including transesterification, esterification, amidolysis, and acyl exchange. A number of commercial applications may evolve from these new enzymatic activities. Klibanov and his associates have already developed processes for lipase-catalyzed preparative production of optically active compounds (with commercial potential for herbicides, where only one isomer is biologically active) and peroxidase-catalyzed lignin depolymerization.

Lignin is the second most abundant organic chemical on the earth and the largest source of renewable aromatic chemicals. But, it is a complicated structure and difficult to degrade, chemically or biologically. Ligninase has been isolated—it occurs naturally in fungi, but in minute quantities. Klibanov reasoned that, since ligninase is a peroxidase, another peroxidase, such as horseradish peroxidase, should also degrade lignin. It doesn't in water, but it does in 95 percent dioxane, which also solubilizes the lignin. The kind of degradation is comparable to that observed with the natural ligninase, and about a third of the lignin is degraded.

—Jennifer Van Brunt