Feature

Research discovers new roles for lipases

The following article on research to develop lipases for enzyme technology was prepared by JAOCS newswriter Anna Gillis.

Scientists are discovering that nature's job description for lipases encompasses more than they originally thought.

Nature designed lipases mainly to cleave fatty acids from their glyceride backbones, but researchers in academia and industry have found that manipulation of the conditions under which lipases work results in a host of potentially profitable reactions. Lipases can be used in interesterification, transesterification, ester synthesis, peptide synthesis, biosurfactant production, and resolution of racemic mixtures to produce optically active compounds.

Alexander Klibanov of the Massachusetts Institute of Technology (MIT) and Chi-Huey Wong of Texas A&M University are among the researchers looking at lipase-catalyzed reactions with economic potential. In the early 1980s, Klibanov found that lipase activities were retained when the enzymes were tossed into nonaqueous solutions. "Conventional wisdom holds that enzymes work only in aqueous solutions. This is not so. That's exciting to know because water is not the ideal reaction medium for most organic processes," Klibanov wrote in *Chemtech* in June 1986.

The knowledge that lipases successfully catalyze reactions under anhydrous conditions widened the range of possible chemical reactions well beyond hydrolysis, Klibanov said. He explained that water, a normal environment for lipases, inhibits many reactions. When water is eliminated—except for an essential thin layer of water needed by the enzyme to maintain its integrity—the scope of biocatalysis can be expanded to include reactions such as the syntheses of esters from carboxylic acids and alcohols or of peptides from amino acids.

Klibanov cited several advantages of using enzymes in organics instead of water: (a) because enzymes are insoluble in organics, they don't need to be immobilized, and their recovery and reuse are easier; (b) product recovery from aqueous solutions can be more costly; (c) water does not favor the thermodynamics of certain reactions and can lead to unwanted side reactions, and (d) using organics allows some reactions to be carried out in one step instead of two.

Klibanov has found that lipases used in anhydrous solutions have increased stability dramatically at temperatures as high as 100 C and their substrate, regio- and stereospecificities are altered. The ability to use lipases at higher temperatures widens the processing options for the enzymes.

"This knowledge has enormous potential in the long term for a number of industries, including fats and oils, specialty chemicals and pharmaceuticals," Klibanov said. "Companies already are scaling up to use lipases to produce specialty chemicals."

Much of Klibanov's work on lipase-catalyzed resolution of racemic alcohols and acids, modification of sugars and peptide synthesis has been funded by W.R. Grace & Co.

Like Klibanov, Wong sees ever-increasing interest from companies, particularly pharmaceutical firms, lured by the possibilities presented by new developments in enzyme technology. "Companies are looking at the production of chemicals such as chiral alcohols that must be biologically pure to be used in pharmaceuticals or agricultural products. Lipases can be useful in the asymmetric transformation of symmetrical compounds and in the resolution of racemic compounds to find the isomer that is biologically active," Wong said. Wong and his colleagues have used lipases to develop a number of chiral alcohols, including chiral glycerol derivatives, furans, pheromones, organometallics and other compounds used in the manufacture of pharmaceuticals, perfumes, agrichemicals and liquid crystals.

Besides resolving chiral alcohols, Wong and Klibanov have used lipases in organic solutions to selectively modify sugars and to synthesize peptides. Wong, in work funded by Dow Chemical Co., used lipases to make peptide precursors to penicillin G and penicillin analogs. He took three commercially available lipases and showed that the lipases would use amino acids with D- or L-configurations as substrates.

Because these lipases work on the D-configuration of amino acids, Wong and others think lipases might aid in the synthesis of medically important peptides. "Although body proteins have L-configurations, a number of drugs, including antibiotics and neuroactive peptides, have unusual amino acids with D-configurations. Some of these can be made by fermentation, but not all can," Wong said. "In cases where fermentation can't be used to produce particular drug analogs, lipases are an option."

According to Wong, lipases have certain advantages in peptide synthesis that proteases, more natural candidates for protein synthesis, don't have. Lipases work well in peptide synthesis because they have esterase activity; unlike proteases, they have no amidase activity, so peptide bonds are not broken right after being made. Proteases generally are inactive or less active toward D-isomers.

Despite the advantages of using lipases, Wong noted that companies are not using them to produce peptides. "In cases where peptide antibiotics can be made through fermentation or with lipase catalysts, fermentation is the favored way to go due to cost," Wong said.

Both researchers said the acylation of sugars by lipases under anhydrous conditions has commercial applications in the drug and food sectors. "High-value chemicals and nucleosides can be made by selectively protecting sugars through acylation," Wong said. "A number of sugars could be useful in the production of nucleosides that might be antiviral."

Klibanov has used lipases to acylate sugar alcohols with fatty

acids from vegetable oils to make biosurfactants. The biosurfactants produced reduce surface and interfacial tension and stabilize emulsions. The latter trait could be useful in foods that separate, such as salad dressings, Klibanov said. "One advantage of using biosurfactants produced with lipases (in addition to their high potency) is that the foods can still be considered natural," he said.

Most of the patents covering lipase use in hydrolysis and interesterification of fats and oils are held by companies outside the U.S.

The newly formed, Boston-based Enzytech Inc. plans to use lipase technology based on work done in the Klibanov lab to produce chemicals for food ingredients, according to Akiva Gross, Enzytech's vice president of research. Enzytech is trying to modify commercially available lipases either to enhance or to alter their catalytic activities. The company hopes to use lipases to produce specialty fatty acids such as omega-3 fatty acids and surface-active materials for the food industry, Gross said.

Even though lipase-driven syntheses in nonaqueous solvents have practical applications, there is one disadvantage in their use. "Reactions in organic solvents can be 10,000 times slower than in aqueous solutions. The lipase may be stable in organics, but if the reaction is too slow, it may not be useful. For this reason, we have to provide a justification for lipase use," Wong said. He added that the use of lipases in organics might be warranted in reactions that involve water-sensitive substrates or products or in the production of compounds that can't be produced chemically or by fermentation.

One firm that has justified the use of lipases is Genzyme Corp. in Boston, Massachusetts. The company uses commercially available lipases to produce high-value optically active compounds for the pharmaceuticals industry. According to Alan Walts, the company's director of synthetic and biological chemistry, Genzyme has taken an academic process and optimized it for large-scale production. "The scientific literature abounds with examples demonstrating the use of lipases to resolve racemic mixtures, but only a limited number of companies are using lipases in a production sense," Walts said.

For the past two years, Genzyme has used an exclusively licensed process developed by George Whitesides at Harvard University to produce optically active glycidyl butyrate. The compound is a starting material in the production of beta blockers used to reduce hypertension.

Walts explained that the company uses a commercially available lipase to sort out one valuable isomer from an optically inactive racemic mixture. "The lipase hydrolyzes the D-isomer. The L-isomer that is left behind is then isolated, purified and sold," he said. As part of its research and development program, Genzyme has increased the lipase selectivity to produce L-glycidyl butyrate of 97% enantiomeric purity.

Although the company is considering protein engineering, its main focus is directed toward using lipases and other enzymes to produce high-value fine chemicals. Currently, Genzyme is capable of supplying ton quantities of glycidyl butyrate if necessary, Walts said. While that amount sounds small relative to commodity chemical production, Walts pointed out that optically active fine chemicals such as glycidyl butyrate cost from hundreds to thousands of dollars per kilogram.

"Our contribution has been in the optimization and scale-up of a particular lipase application. Genzyme is looking at other compounds in the pharmaceutical area to which that technology could be applied. There are a number of chiral alcohols and esters of interest to us," Walts said.

The fats and oils industries have been slower than some other industries in getting involved with lipase technology, according to Phil Sonnet, a U.S. Department of Agriculture (USDA) scientist based at the Eastern Regional Research Center in Philadelphia. "Lipase technology has had a limited adoption," he said.

Most of the patents covering lipase use in hydrolysis and interesterification of fats and oils are held by companies outside the U.S. However, none of the fats and oils companies have reported using lipases in commercial production. Nippon Oils & Fats, Fuji and Unilever have conducted pilot-scale work with lipases in hydrolysis and interesterification. Meanwhile, Henkel researchers have found that enzymatic hydrolysis of soybean oil can cost twice as much as conventional processing.

"Lipase interest is not a new topic for the industry. The (fats and oils) industry has known about its potential for 20 years but has been slow about using it," Sonnet said. "The industry might be more apt to accept enzyme technology once there's been commercial development elsewhere."

Outside the fats and oils area, Sonnet has investigated the creation of secondary alcohols for use in synthetic insect pheromones. In trying to develop a lipase-catalyzed method for liquefying tallow, Sonnet and other researchers at USDA's Eastern Regional Research Center found that some of the lipases had strong stereochemical biases in enzymatic reactions on triglycerides.

Sonnet thought those biases could be used to produce a number of chemicals, including synthetic pheromones. After screening many commercial lipases for stereobias, Sonnet experimented with several to find those that would resolve secondary alcohols for use in the preparation of synthetic sex pheromones. Primarily, Sonnet has

worked on synthetic sex attractants of several rootworm species that are corn pests.

Insect pheromones have structures with particular stereochemical orientations. It's the particular stereochemistry of a pheromone that attracts specific insects, Sonnet said. "Lipases could help produce the right 'bait' to trap insects in the field." The USDA scientist said several companies also are very much involved in looking at this method as a way to control insect pests.

There are advantages to using enzyme technology in the production of agrichemical products, according to Mark Empie, manager of technical applications for detergents, specialty enzymes and fine chemicals for International Bio-Synthetics Inc. (IBIS). IBIS, a joint venture between the Royal Dutch Shell Group and Gist-Brocades NV, will use enzyme technology to make biofine chemicals. Empie said lipases would be used to resolve racemic mixtures produced by conventional chemistry.

Lipase and other enzyme technology could lead to the production of more specific herbicides, Empie said. One class of compounds of interest to IBIS and other companies are the phenoxy propionate herbicides. "The herbicides might destroy some types of broadleaf plants, for example, but would not harm the grassy plants such as corn. They would be safe and would degrade rapidly in the soil," Empie said. To cut down on dosage requirements, companies also want to develop herbicides that are more potent than those manufactured conventionally. IBIS may use lipases to produce intermediates for these kinds of agrichemicals.

In addition to using lipases and other enzymes to manufacture intermediates for pharmaceuticals and agrichemicals, IBIS also plans to continue developing industrial enzymes for foods and detergents. According to Michael Crossin, the company's marketing manager for detergents, the company is developing a detergent lipase for use in current American detergents to augment its line of proteases and amylases.

The first detergent lipase for commercial use, Novo Industri A/S' Lipolase, made its market debut in March 1988. According to Steen Riisgaard, vice president of Novo's Detergent Enzyme Division in Denmark, several tons have been sold, but "the potential is difficult to predict at the present time." Novo said recombinant DNA technology was used to clone a detergent lipase from a fungal donor; the lipase then was expressed in a high-yielding *Aspergillus* host.

Novo reported that Lipolase is stable in water temperatures up to 60 C and in a pH range of 7-11. The enzyme "keeps its activity in combination with all other detergent ingredients, such as phosphates and surfactants," the company said, noting that it works in combination with proteases that act on protein stains.

The first detergent lipase for commercial use made its market debut in March 1988.

Amano International Enzyme Co. also plans to develop a surfactant-stable lipase. According to Terry Gallagher, sales manager for Amano, the company is "within a year" of introducing a detergent lipase. "We have found some lipases that work under wash conditions and at pH 9-10, but the cost of production is 10 times too high. We need to do some genetic manipulation to find an organism that will produce more of that lipase (once the lipase genes are introduced into the host organism) in fermentation," Gallagher said.

Besides working on lipases for detergent applications, the company remains active in enzyme development for the flavor industry and the fats and oils industries. Gallagher said Amano is considering development of fungal lipases for flavors. "Most fungally derived lipases have poor specificity toward shorter chain esters, the lengths important in flavorings. Development of fungal lipases with good specificity would cut the cost of flavorings and would make it easier to get kosher certification on foods," he said. (For kosher certification, animal lipases cannot be used in the production of dairy foods.) Now, many of the flavor lipases are made from pre-gastric or pancreatic lipases derived from animal organs. "By using fermentation to produce fungal lipases, the problems of cost and organ shortage can be avoided," Gallagher said.

Amano also produces lipases specific for α and α' esters in transesterification and for unsaturated fatty acid esters. Other lipases manufactured by Amano can be used in vegetable oil hydrolysis and monoglyceride synthesis.

Novo, meanwhile, has developed Lipozyme, an enzyme designed for interesterification of fats and for the synthesis of esters at 60-70 C. The immobilized, 1,3-specific *Mucor miehei* lipase has potential applications in the production of specialty fats and emulsifiers, according to Peter Eigtved, Novo's section head for the lipase satellite. However, he added, "Many factors determine whether it is advantageous or not to use the enzyme in a given industrial application. In the cases where alternative technologies exist for making a specialty fat or ester, cost and productivity of the enzyme are clearly important."

Novo also has introduced an experimental nonspecific, thermostable lipase for ester synthesis and interesterification.

As groups in academia and industry study the wide array of chemical reactions that can be catalyzed by lipases and the environments in which they must be carried out, Genencor Inc. in South San Francisco, California, is looking at "building" lipases to suit specific situations.

Genencor's main focus is to go beyond genetic engineering to protein or enzyme engineering, according to A.J. Poulose, a Genencor

scientist. "Genetic engineering occurs when you take the lipase gene from one organism and have it expressed in another; protein engineering occurs when you use knowledge about a lipase's structure and its active site to modify its gene and so create a new lipase," Poulose explained.

Genencor's first protein engineering breakthrough occurred with the protease subtilisin, which has a catalytic mechanism similar to some esterases and lipases. The company found that by substituting specific amino acids near the subtilisin active site, transesterification was favored over hydrolysis. This led to the engineering of a *Pseudomonas* lipase with altered specificity for the alcohol portion of an ester. Afterwards, the lipase was modified to change the specificity of the enzyme towards the acid portion of the ester. This resulted in a range of lipase enzymes with a wide variety of substrate specificities. "These enzymes will be useful in the transesterification reactions as well as in synthesis of specialty chemicals," Poulose said.

Patent applications have been submitted for the use of the lipases in carrying out transesterification reactions and on the modified enzymes themselves, according to Jonathan MacQuitty, Genencor's vice president of commercial development. The company has several other lipase patents pending. One is for a novel use of lipases in agrichemicals. "Our lipase for the agricultural sector will allow agrichemicals to be used more effectively by plants," MacQuitty said. "The lipase makes crop chemicals more effective and reduces the amount of spray per acre, with cost and environmental benefits."

Genencor also has been granted patents for lipolytic enzymes from *Aspergillus oryzae*. The enzymes are used to speed cheddar cheese aging.

There are two paths toward engineered enzyme production, Poulose said. The first, and more favored way, is to crystallize a lipase, determine its crystal structure using x-ray crystallography, and then modify the gene based on its 3-D structure. The other way is to identify functionally significant regions in the enzyme such as the active site and then change the amino acids in that region to alter the enzyme's properties.

After the enzymes are altered, they must be screened to determine if one of the new enzymes displays favorable activity. In either case, after the lipase activity has been altered, an appropriate organism has to be found that will produce the new enzyme in large quantities. "Genencor uses both approaches as a way to get to the goal the fastest way possible. You lose time waiting for the crystal structure," Poulose said. According to Poulose, Genencor used the second approach with its lipase work while waiting for the crystal structure to be determined.

"Genencor's success in protein engineering is significant because it is one of the first achievements in this area," according to Michael Haas of USDA's Eastern Regional Research Center. Haas pointed out that "the science of protein engineering still is in its infancy. There are very few general rules that allow us to rationally predict in which ways the amino acid sequence of a protein should be altered in order to achieve a desired modification." Haas predicted it will be five to ten years before the theoretical groundwork is laid so that protein engineering can be most effective.

In the meantime, Genencor plans to continue to develop enzymes in the fastest way it can for various uses, including fats and oils modification, production of specialty chemicals, flavor development in cheeses and some applications in the agrichemical area. In Poulose's opinion, it probably will be some time before lipases are used in large-scale hydrolysis. Even though the feasibility for large-scale work is there, Poulose said he believes greater lipase use will continue in the specialty chemicals area. "Enzyme use in the production of specialty products provides bigger margins, so this is the area that will grow while we develop more refined process technology to use enzymes for opportunities requiring large-scale hydrolysis."

Researchers seeking to alter lipases and to create new working environments for lipases are expanding the job description for lipases, particularly in the area of enzyme technology. As the knowledge about lipases increases, Klibanov said, "Applications that have been ruled out because of enzymes' shortcomings should be reevaluated."

Feature

USDA center studies lipase applications

Researchers at the U.S. Department of Agriculture's (USDA) Eastern Regional Research Center (ERRC) are studying the molecular biology, chemistry and direct applications of lipases that may be useful to the fats and oils industry.

In the molecular biology area, Michael Haas has constructed the genomic library of *Rhizopus delemar*, a fungus whose lipases display a 1,3 specificity. He believes that these enzymes eventually may be useful in modification of fats and oils. According to Haas, creating and examining the genomic library is one way to determine which genes control lipase production.

In this work, Haas and his colleagues inserted fragments of the chromosome into the bacterium *E. coli*. They screened 12,000 of the *E. coli* to determine whether any were expressing the lipase genes on the inserted *Rhizopus* fragments. However, the researchers failed to observe direct expression of the lipase genes.

One reason that lipase-producing clones were not detected could be that *E. coli* could not "read" the nucleic acid sequences that control lipase synthesis in *Rhizopus*, Haas said. "Another possible cause for the failure to express the fungal gene in the bacterium is that within genes of higher organisms (such as fungi), there are pieces of DNA that don't code for proteins. A higher organism can skip these portions and move on, but *E. coli* can't always process genetic material in the same way."

Haas is seeking a way around this problem by producing cDNA (copy DNA). The cDNA would have the non-coding portions that can't be read by *E. coli* removed. This could result in the expression of the lipase genes, Haas said. Once the genes are identified, Haas plans to identify the amino acid sequence of the *Rhizopus* lipase.

The development of better commercial lipases is possible once the lipases of organisms such as *Rhizopus* are characterized fully, Haas said, noting that the amino acid sequences for most enzymes sold commercially still are unknown. The knowledge gained from characterization work could be used in protein engineering, he added.

Information garnered from work with pseudolipids makes them a potentially useful tool in protein engineering.

In other work at the ERRC, Phil Sonnet has developed compounds that can be used to determine lipase structure and activity. According to Sonnet, these compounds act like lipids in hydrolysis and esterification reactions but chemically are diether esters of glycerine.

The chemical structure of these pseudolipids makes them useful probes for looking at the structure and activity of lipases, he said, explaining that the migration of groups does not occur in the diether esters as it does in triglycerides.

"A lipase sees and responds to the pseudolipid as it would to a triglyceride," Sonnet said. "Because migration doesn't occur (as it does in triglycerides), we can better judge the stereobias of lipases after examining which residues have been removed from the diethyl esters. We know the structure of the substrate (the pseudolipid). By having the lipase act on it, we gain information about the performance and possibly the active site of the protein."

Sonnet said the information garnered from work with pseudolipids makes them a potentially useful tool in protein engineering. He also is creating methyl branched fatty acids of high configurational purity as an aid to protein engineering.

On the applied side, Frank Taylor of USDA has developed an enzymatic process for use in the hydrolysis of tallow and vegetable oil. The process makes use of a catalytic membrane embedded with lipase from *Thermomyces lanuginosus* to carry out reactions between two immiscible fluids.

Taylor said researchers at ERRC found that the lipase was suitable because it remains thermostable at 50 C or higher. The *Thermomyces* lipase was a likely candidate because a lipase was needed that could work at temperatures at which contamination would be less likely to occur. "In any immobilized reactor system, it's necessary to have a high enough temperature and a low enough pH to prevent contaminants from growing in the oil or tallow," he said.

According to Taylor, the system is similar to a Japanese system that uses a microporous hydrophobic membrane. The main difference is that in the Japanese system, the oil and water are kept on opposite sides of the membrane thoughout the process. Taylor explained that his process begins with a positive pressure on the oil side. The oil goes through the membrane where hydrolysis occurs; the partially hydrolyzed tallow then goes to the aqueous side. Once on the aqueous side, the mixture is swept out by a recycle or sweep stream to remove the oil from the downstream side. Complete hydrolysis could be reached by running a series of reactors, he said.

Taylor has applied for a patent on the process and plans to begin work on pilot-plant scale in the next two years.