

# Food Biotechnology Microorganisms

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# Lipases of the Genera *Rhizopus* and *Rhizomucor*: Versatile Catalysts in Nature and the Laboratory<sup>1,2</sup>

Mono-, di-, and triglycerides are esters of glycerol containing, respectively, one, two, or three fatty acids. Glycerides are the major components of depot, or storage, fats in plant and animal cells. Those that are solid at room temperature are termed *fats*, while those that are liquid at room temperature are known as *oils*. The natural or biotechnological synthesis and hydrolysis of glyceride ester bonds play a variety of roles in relation to food flavor, quality, appearance, and wholesomeness. For example, the desirable organoleptic properties of aged cheeses are, in part, due to the accumulation of short chain length fatty acids (three and four carbons) released by the enzymatic hydrolysis of endogenous triglycerides. On the other hand, the enzymatic release of longer chain fatty acids (6–12 carbons in length) from triglycerides results in off-flavors characterized as goaty, soapy, and bitter. The ability of mono- and diglycerides to emulsify oil–water mixtures has led to their extensive use in the food and nonfood sectors. In the biotechnological realm, the addition to foods, especially cheeses, of the enzymes that catalyze glyceride hydrolysis has been employed for the past several years to accelerate the development of desirable flavor profiles. More recently, these enzymes have been widely employed as catalysts for the production of a great variety of ester and ester-like compounds for applications in the food, confectionary, pharmaceutical, cosmetic, and other industries.

The enzymes catalyzing the hydrolysis of glyceride ester bonds are termed *acylglycerolases*, *acylhydrolases*, or *lipases*. Their substrate ranges are wide, and compounds other than acylglycerols are also hydrolyzed. These enzymes can be considered as transferases since the fatty acid released is transferred to water or to some other compound with a free hydroxyl group or related moiety. They have been assigned the identifier EC 3.1.1.3 by the Enzyme Commission (80). The biological role of lipases is the initiation of glyceride metabolism. Therefore, they are widespread throughout the living world, being produced by virtually every organism in its attempt to exploit the rich energy stores that lipids represent.

Lipases form a diverse and well-studied group of enzymes. Several monographs and reviews are available, providing a comprehensive overview of this useful group of enzymes (10, 29, 32, 60, 89, 90, 108). Their defining common feature is the prerequisite that their substrates be water insoluble. Their activities against such molecules generally exceed their activities toward water soluble esters by 1,000-fold. (Enzymes that hydrolyze the carboxylic ester bonds of water soluble molecules are termed *esterases*.)

Lipases can be classified into three broad groups on the basis of their abilities to hydrolyze secondary esters. A large number of enzymes, including those produced by *Rhizopus* and *Rhizomucor*, are unable to hydrolyze such esters. They cleave only the terminal positions of triglycerides, and are therefore said to be 1,3-specific. It is more useful and accurate to regard these enzymes as being capable of hydrolyzing primary, and not secondary, esters since their substrate ranges are not limited to triglycerides. Some lipases hydrolyze both primary and secondary esters, and are referred to as nonspecific. The third group of lipases consists of those few enzymes that are positionally nonspecific but exhibit fatty acid selectivity, cleaving only ester bonds wherein the fatty acid is of a particular type. The most thoroughly characterized of these enzymes are those produced by the fungus *Geotrichum candidum*. These lipases preferentially hydrolyze the esters of 9, 10 unsaturated fatty acids (13, 14, 84). Ungerminated oat seeds also contain a lipase with this specificity (137). Lipases may also exhibit chain length selectivity. For example, some (e.g., those involved in cheese flavor development) hydrolyze glyceride esters of short chain, but not medium and long chain, fatty acids. Others display the opposite specificity.

Interest in lipases is not solely the result of their role in food flavor development. These enzymes are versatile catalysts for the initiation and completion of many reactions, including the hydrolysis of fats and oils, and other carboxylic acid esters. Many of these enzymes retain their activity in nonpolar organic solvents. This has fostered their use in the hydrolysis of water-insoluble esters, such as in the resolution of racemic mixtures through stereospecific hydrolysis. These enzymes have also recently proven useful in the catalysis of the reverse reaction, the synthesis of useful organic esters.

The lipases of the genera *Rhizopus* and *Rhizomucor* are the most thoroughly characterized of all microbial lipases, having been under study for more than 30 years. Both intra- and extracellular enzymes have been described. The extracellular enzymes are the most thoroughly characterized and are produced in much higher amounts. They constitute a family of quite closely related proteins, and exhibit many similarities in structure, genetics, and biochemistry. Basic studies on them have provided detailed and generally applicable information on lipases and their genes, including crystallographic data and an associated model that explains, in molecular terms, the require-

ment of lipases for water insoluble substrates. Lipases from these organisms have also proven useful in ester synthesis and hydrolysis, and have been widely employed as biotechnological catalysts. This chapter will summarize current knowledge regarding the production, biochemistry, molecular biology, and applied enzymology of these prevalent and useful enzymes.

## Taxonomy

The genera *Rhizopus* and *Rhizomucor* are ubiquitous in nature, and occupy in many diverse environments. They are members of the order Mucorales, which is within the class Zygomycetes of the division Amastigomycota (11). They are mainly terrestrial saprophytes or parasites of plants, fungi, or mammals, or predators of microscopic animals. Zygomycetes have a haploid aseptate mycelium with cell walls composed of chitin and lacking cellulose. Reproduction can be either sexual or asexual. The term *Zygomycetes* itself refers to the thick-walled resting spore ("zygospore") that results from sexual reproduction. Asexual reproduction is by nonmotile spores (sporangiospores). The Zygomycetes are members of the division Amastigomycota (11), and are mainly terrestrial saprobes or parasites of plants, fungi, or mammals, or predators of microscopic animals. The majority of the Mucorales live on such substances as dung and decaying plant or animal matter. A description of their anatomy, modes of reproduction, and general life history is provided by Alexopoulos and Mims (11). Most humans are passingly familiar with *Rhizopus stolonifer* (*Rhizopus nigricans*), the common bread mold. Various members of the order are weakly parasitic to fruits, green plants, fungi, and animals. Some are capable of infecting humans. The first human cases of infection by Zygomycetes were described nearly 125 years ago (59). There have been additional reports since that time (4, 140). These organisms are opportunistic pathogens, typically infecting individuals who are immunocompromised or weakened by severe trauma. Infection most commonly occurs in the acidotic diabetic or with malnourished children, burn patients, or others with debilitating disease. The organisms are not generally considered to be strongly pathogenic. They are not listed as pathogens by the Recombinant DNA Advisory Committee of the National Institutes of Health (Federal Register). Therefore, genetic engineering experiments involving these organisms can be conducted under P1 conditions, the lowest level of containment.

*Rhizopus* and *Mucor* (a previous genera designation for *Rhizomucor*) are differentiated taxonomically on the basis of whether their sporangiophores are grouped (*Rhizopus*) or emerge singly (*Mucor*). The classification of these organisms has undergone refinements over the past 15 years. The recognition of the separate genus *Rhizomucor* was proposed by Schipper in 1978 to include thermophilic, stolon- and rhizoid-bearing organisms previously classed within the otherwise nonthermotolerant genus *Mucor* (142). The presently recognized species of *Rhizomucor* are *pusillus*, *miehei*, and *tauricus* (142).

The genera *Rhizopus* has also undergone recent reclassification, again as a result of the work of Schipper (143, 144). This work established the essential identity of several isolates originally thought to be distinct species, and therefore caused a great reduction in the number of recognized species. Based primarily on features of the mycelia (such as the height of the sporangiophores and the degree of branching of

**Table 15.1.** Previous Designations of *Rhizopus* Isolates  
Currently Classified as Species *Rhizopus oryzae*<sup>1</sup>

<i>Rhizopus arrhizus</i>	<i>Rhizopus pseudochinensis</i>
<i>Rhizopus oryzae</i>	<i>Rhizopus chungkuoensis</i>
<i>Rhizopus japonicus</i>	<i>Rhizopus shangheiensis</i>
<i>Rhizopus tonkinensis</i>	<i>Rhizopus peka</i>
<i>Rhizopus tritici</i>	<i>Rhizopus thermosus</i>
<i>Rhizopus nodosus</i>	<i>Rhizopus boreas</i>
<i>Rhizopus norvegicus</i>	<i>Rhizopus fusiformis</i>
<i>Rhizopus batatas</i>	<i>Rhizopus suinus</i>
<i>Rhizopus delemar</i>	<i>Rhizopus achlamydosporus</i>
<i>Rhizopus kansanensis</i>	<i>Rhizopus bahnensis</i>
<i>Rhizopus usamii</i>	<i>Rhizopus delemar</i> var. <i>minimus</i>
<i>Rhizopus formosaensis</i>	<i>Rhizopus javanicus</i>
<i>Rhizopus maydis</i>	<i>Rhizopus semarangensis</i>
<i>Rhizopus liquefaciens</i>	<i>Rhizopus sontii</i>
<i>Rhizopus hangchao</i>	<i>Rhizopus javanicus</i> var. <i>hawasakiensis</i>
	<i>Mucor arrhizus</i>

the mycelium), sexual compatibility, and the maximum temperatures at which the organisms grew, Schipper proposed the recognition of three species groups:

1. *Rhizopus microsporus* group—Sporangiophores mostly not exceeding .8 mm in height; rhizoids simple; sporangia up to 100  $\mu$ m in diameter; usually growth at 45°C.
2. *Rhizopus stolonifer* group—Sporangiophores often more than 1 mm in height; rhizoids with secondary branching; sporangia commonly up to 275  $\mu$ m in diameter; no growth at 36°C.
3. *Rhizopus oryzae*—As for no. 2, but sporangia not exceeding 240  $\mu$ m in diameter; growth at 36°C.

Within the complex *Rhizopus microsporus* were identified the species *Rhizopus homothallicus* and *Rhizopus microsporus*, the latter with three additional varieties: *chinensis*, *oligosporous*, and *rhizopodiformis*. These groups include organisms originally known by these names, as well as other isolates previously categorized as other *Rhizopus* species and, in a few cases, as *Mucor* species. Two species, *stolonifer* and *sexualis*, were identified in the *Rhizopus stolonifer* group, to which *Rhizopus nigricans* was also renamed. To the species *Rhizopus oryzae* were assigned numerous isolates previously thought to constitute 30 separate species, including *delemar* (Table 15.1).

Recent issues of the *Catalog of Filamentous Fungi* of the American Type Culture Collection, Rockville, MD, reflect these modifications (12). On the basis of DNA-DNA complementarity, Ellis proposed that several isolates previously thought to be unique species were actually varieties of *Rhizopus arrhizus* (50). The now outdated species names will be employed here, however, in deference to the substantial body of published work in which they are employed. It is clear from the work of Schipper and Ellis, however, that there is a high degree of relatedness within this group of organisms. It is useful, and in accord with existing data, to consider their lipases as a family of closely related enzymes.

## Propagation, Storage, Growth, and Lipase Production

A variety of isolates of *Rhizopus* and *Rhizomucor* are available from the standard culture collections, such as the American Type Culture Collection, the Commonwealth Mycological Institute (Kew, Surrey, England), and the Central-Bureau Voor Schimmel Cultures, (Baarn, The Netherlands). Their growth and propagation in the laboratory are straightforward. *Rhizopus* species grow readily on malt extract media (Medium 325 in reference 12) at room temperature. *Rhizomucor* can be grown on potato dextrose agar at 37°C (Medium 336 in reference 12). Simpler media will also support growth. The minimum nutritional requirements of *Rhizopus delemar* are a simple carbon source; the amino acids glutamate, aspartate, phenylalanine, and serine; biotin; and inorganic salts (68). However, lipase production is poor under these conditions. We routinely grow this organism in a liquid media consisting of inorganic salts, hydrolyzed casein, biotin, and a simple carbon source (65), and obtain acceptable growth and lipase production. This can be solidified with 1.5% agar as required. This and similar media should support the growth of all *Rhizopus* and *Rhizomucor* species.

Sporangiospores are produced by *Rhizopus delemar* after about 3 days of growth on malt extract agar media. Sporulation is indicated by the appearance of readily visible black spores above the white mycelium. The spores can be teased free into sterile salts solution such as M9 (117) with a sterile glass rod bent into the shape of a hockey stick. The spore-laden solution is then aseptically transferred to a sterile screw-capped tube. Stored at 4°C, the spores will retain their viability for at least 6 months. Fresh spore preparations are produced simply by spreading a small aliquot of spores on media and incubating at 30°C. Longer-term storage has been achieved on silica gel containing skim milk media (136).

*Mucor*, but not *Rhizopus*, converts from a mycelial to a single-celled growth pattern when grown in an atmosphere of CO<sub>2</sub> (16). This dimorphism could greatly facilitate genetic and molecular genetic studies, but has not yet been exploited in the study of the lipases of these organisms. In semisolid media, net lipase yields are low, apparently due to the degradation of the enzymes by proteases that are also synthesized (84). Both *Rhizopus* and *Rhizomucor* grow and produce lipase readily in submerged liquid culture. The inoculation of liquid media with spore preparations to initial densities of 10<sup>7</sup>/ml and growth with shaking under ambient atmosphere at, or slightly above, room temperature result in good enzyme yields. It is common to provide nitrogen in an organic form, such as enzymatically hydrolyzed casein or soybean meal (84). Small amounts of inorganic nitrogen may also be added to such media.

Since lipases are not obligatorily required for survival, it seems reasonable that their synthesis would be regulated according to the availability of fats and oils, their substrates, in the environment. Thus, it initially seems surprising that many laboratories have observed lipase production by *Rhizopus* and *Rhizomucor* during growth in media in which glucose is the major carbon source (35, 53, 58, 64, 100, 162). However, in most of these cases, lipase levels were not determined until 4 or 5 days after inoculation. It is likely that the simple carbohydrate had been completely metabolized by that time. In *Rhizopus delemar* (64) and in the *G. candidum* (108), it has been shown that extracellular lipase does not appear until after glucose levels have fallen due to metabolic consumption. Furthermore, the addition of glucose to a media that normally supports lipase production has been shown to reduce intra-

cellular lipase levels in *Rhizopus chinensis* (127) and extracellular activity in *Rhizopus delemar* (68). These data indicate that glucose represses lipase production.

Because of differences in the experimental techniques employed in various laboratories, it is difficult to assess the ability of lipids to increase (induce) lipase production. Variations in technique include such factors as culture age at the time of harvest, the type of nitrogen source employed (some of which, such as soybean meal, can contain endogenous lipids), and the measurement of extra- or intracellular lipase levels. Several reports indicate reductions in lipase levels, and often the inhibition of growth, in media containing lipids. Growth and extracellular lipase activity were reduced in *Rhizopus japonicus* (2) and *Rhizopus stolonifer* (34) by the addition of glycerides or fatty acid to a medium containing 1% glucose as the main carbon source. In the former case, lecithin increased extracellular lipase levels nearly eightfold. The data indicated that this effect was due to a facilitation of the release of lipase into the medium, not to a stimulation of lipase production. Akhtar et al. (5, 9) demonstrated that replacement of glucose by olive oil in the growth media caused a one-half- to fourfold increase in mycelial lipase activity and as much as a threefold decrease in the soluble activity in *Rhizopus arrhizus*, *Rhizopus japonicus*, and *Rhizopus oryzae*. These researchers also determined that the extracellular lipase was inactivated by oleic acid, which could have contributed to the observed decrease in extracellular activity. Similarly, Nakashima et al. (127) found extracellular lipase activity in *Rhizopus chinensis* to be decreased as much as 10-fold by growth in lipids. However, intracellular/cell-bound lipase levels were increased up to 16-fold. They postulated that substrate-related compounds retarded the release of lipase into the medium. However, one must be cautious in regard to generalizations in this matter. In other fungi, including *Mucor* (7), the presence of lipids rather than carbohydrates appears to cause an increase in lipase activity.

Espinosa et al. (53) achieved maximum extracellular lipase levels in *Rhizopus delemar* when carbohydrate polymers, especially dextrin, were used as the carbon source. These may be hydrolyzed slowly, providing a sustained low concentration of monosaccharide, while avoiding levels sufficient to repress lipase synthesis. Their data indicate that lipids provide higher lipase levels than simple carbohydrates, but that this is due to a stimulation of growth rather than of lipase production per se. The surfactant Tween, which contains fatty acyl groups, also increased extracellular lipase activity (53). The methods employed were insufficient to determine if this was the result of an increase in enzyme production or a stimulation of enzyme release.

In *Rhizopus*, the substrate specificity of the overall lipase activity produced does not appear to change in response to the type of triglyceride in which the organism is grown (6). In *Mucor*, such substrate-dependent changes in lipase specificity have been observed (8). The issue has not been examined in *Rhizomucor*.

In *Rhizopus delemar*, lipase production appears to be subject to catabolite repression by glucose (64). Lipase activity and lipase cross-reacting protein are undetectable until glucose in the medium is metabolized. Lipase is produced during growth on triglycerides. The use of triglycerides in growth media introduces several difficulties. Their low water solubilities can limit the availability of carbon and metabolic energy, retarding growth. Maintenance of acceptable emulsions may be difficult. Visual or optical monitoring of the culture for growth and bacterial contamination is impeded by the turbidity of the oil-media mixture. Finally, the lipid may be difficult to remove from the sample during downstream processing or purification. Glycerol, a water

soluble metabolite of glycerides, also supports growth of the organism and lipase production (64). Furthermore, in glycerol media, enzyme production and peak lipase activity are obtained sooner (about 40 h after inoculation) and maintained longer (until approximately 90 h postinoculation) than during growth on glucose or olive oil (64). We have repeatedly used this simple, single-phase media in the production of consistently high levels of *Rhizopus delemar* lipase. The enzyme can be readily purified from these preparations without interference from components of the growth media.

Simple media and growth conditions are also suitable for the growth of these organisms as sources of genetic material. A media composed of yeast extract, protease-digested casein, and sodium chloride, was successfully employed in isolating *Rhizopus delemar* DNA to establish a genomic library of the fungal sequences in a bacterium (67). Expression of the lipase gene in the fungus during growth for DNA isolation was not required in this situation. In establishing a cDNA library, however, expression is required if the library is to contain lipase sequences. The Casamino Acid-glycerol media described previously was used successfully to create a *Rhizopus delemar* cDNA library from which a lipase-encoding cDNA was isolated (63). Some basal expression of the lipase gene must occur in the absence of lipids, however, since a lipase cDNA was successfully synthesized and cloned using mRNA isolated from *Rhizopus niveus* grown on peptone-yeast extract-glucose media (100). Also, Boel et al. (27) isolated a lipase cDNA from a *Rhizomucor miehei* library prepared from cells grown on media optimized for protease, not lipase, production.

A variety of lipases are commercially available. Most enzyme suppliers offer the lipase of at least one member of the Mucorales. Principal suppliers include Sigma Chemical Co. (St. Louis, MO), Amano Enzyme USA (Troy, VA), Gist-Brocades (King of Prussia, PA), Solvay Enzymes (Elkhart, IN), Biocatalysts Ltd. (Mid Glamorgan, UK), and Novo Nordisk BioIndustrials (Danbury, CT). Most of the available preparations are enzyme powders. Their protein content is generally low, sometimes below 5%.

Novo offers an immobilized form of *Rhizomucor miehei* lipase, marketed under the trade name Lipozyme IM (88). This was one of the first, and remains one of only a few, commercially available immobilized lipases. Among the other suppliers are Sigma Chemical Co., Novo, and Biocatalysts Ltd. Immobilization facilitates the ready recovery of the catalyst from a reaction mixture. This allows reuse of the enzyme, which is of obvious interest from an economical and biotechnological standpoint (23). Since its introduction in the mid-1980s, Lipozyme has been used in numerous applications, and is one of the most frequently employed of all lipases.

## Biochemistry

### 15.4.1. Determination of Activity

Lipases are a type of ester hydrolase. Their common defining feature in terms of enzymatic activity is that their substrates are water insoluble. In fact, the activity of these enzymes is negligible when the substrate is present below its solubility limit. This is apparently due to the necessity of interaction between the lipases and a hydrophobic surface in order to expose the active site of the enzyme (see Sections 15.4.2

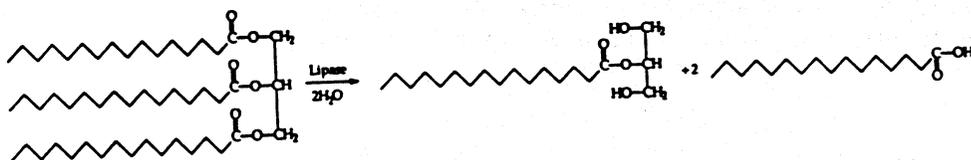
and 15.4.5). Numerous approaches have been taken in the measurement of hydrolytic activity. Those used most frequently are discussed here.

Agar plate methods have long been employed in detecting and measuring lipase activity. They are appropriate for screening organisms for lipolytic activity, but lack the precision and consistency for more quantitative work. The technique is simple, relatively inexpensive, and can be used to simultaneously examine large numbers of samples. It has proven useful in the detection of lipase-producing microorganisms, and also has seen some use in the study of the cell-free enzymes themselves. Two basic strategies are employed in agar plate methods. The first relies upon the formation and detection of zones of clearing as a result of lipase activity on a turbid substrate emulsion solidified by agar. The second exploits the pH drop that accompanies fatty acid liberation, incorporating a pH indicator dye into the substrate emulsion. Numerous variations of these approaches have been devised (152). An extremely sensitive variation on this theme exploits the fact that the fluorescence spectrum of rhodamine B is altered in the presence of free fatty acids (99). By incorporating this dye into media containing a lipase substrate, enzyme activity is readily detected and quantitated by examining the plates under fluorescent light for the presence of bright fluorescent pink zones. This method was sufficiently sensitive to allow the identification of a recombinant *Escherichia coli* strain expressing a cloned *Rhizopus delemar* lipase cDNA at low levels (63). This method has also been useful for the quick detection of lipase during purification, for example, in fractions collected from chromatography columns, as an activity stain for lipase following nondenaturing electrophoresis.

Lipase activity can also be detected by monitoring the release of a fluorescent moiety, such as 4-methylumbelliferone, from the corresponding nonfluorescent fatty acid ester (86, 121, 141). However, such methods lack specificity for true lipases and will also detect esterases. Therefore, these assays are appropriate only when contaminating esterases are absent. Probably for this reason, they are rarely used in lipase research.

End-point assays have been used by some laboratories to quantitate lipase activity (58, 125). The substrate is typically a glyceride that has been emulsified in buffered water. An emulsifier, typically gum arabic, is added to stabilize the mixture and thereby maintain a sufficient amount of the aqueous-oil interfacial area wherein catalysis takes place. Following the addition of enzyme and an incubation period, the degree of hydrolysis is determined by extracting and titrating the free fatty acids that have been released. This end-point type of assay suffers from the disadvantages that the lipase can be inactivated by the acidification of the reaction mixture due to ionization of the product fatty acids, the method cannot provide initial enzyme rates, and it is relatively insensitive to the addition of excessive amounts of enzyme.

Continuous titrating pH-stat methods do not suffer from these deficiencies, and are therefore the preferred means of determining lipolytic activity (65, 95). In such assays, the pH of the reaction mixture is maintained at a preset value by the addition of dilute base in response to the liberation of fatty acids by the lipase. Enzyme activity, expressed as moles of fatty acid released per unit time, is calculated from the rate of consumption of titrant. This method allows the determination of initial velocities, and is amenable to computer-assisted calculation and storage of the results (40). Its disadvantages are the cost of the equipment required (\$10,000–\$20,000 [U.S.]) and the fact that only one reaction can be run at a time.



**Figure 15.1.** The hydrolysis of a triglyceride by a 1,3-specific lipase. Diglycerides are formed as intermediates in this reaction.

In addition to quantitating net lipase activity, it is often of interest to determine such parameters as fatty acid or positional selectivity. A detailed review of these considerations is beyond the scope of this presentation, but has been adequately dealt with elsewhere (89, 90, 172). The calculation of kinetic data is possible for lipases. However, due to the biphasic nature of the reaction mixture, special steps must be taken to ensure the validity of the data (171).

#### 15.4.2. Catalytic Specificity

In virtually all cases examined, the *Rhizopus* and *Rhizomucor* lipases have demonstrated high selectivity for the hydrolysis of primary, and not secondary, glyceride ester bonds (79, 129, 137). Thus, in aqueous systems they catalyze primarily the hydrolysis of the fatty acid esters at the 1- and 3-positions of tri-, di-, and mono-glycerides, but not at the 2-position (Figure 15.1). The positional selectivity is sufficiently high that the enzymes have been used as diagnostic tools in determining the structures of glycerides.

However, this regioselectivity is not absolute. Under some conditions the enzymes hydrolyze, or appear to hydrolyze, secondary esters. In aqueous solution, this is often the result of the migration of acyl groups from the secondary ester position to a free primary hydroxyl group generated by lipolytic action, with subsequent enzymatic hydrolysis (89). Actual hydrolysis or synthesis of secondary esters also occurs (114), although at rates substantially lower than those demonstrated for primary esters. It is our experience that the degree of regioselectivity exhibited by the Mucorales lipases is among the highest of any lipases, and is modulated by the reaction under study, the solvent system, and the fungal source of the enzyme.

It has been reported that in aqueous solution the extracellular lipases from *Rhizomucor miehei* show little chain length preference in the hydrolysis of fatty acid esters. They hydrolyze esters with fatty acyl components between at least 4 and 22 carbons long (79). Other investigations have found variations of approximately three-fold in activity as a function of fatty acid chain length. For the *Rhizopus delemar* lipase, maximum activity is obtained with medium chain fatty acids on the order of eight carbons in length (93). The latter situation appears to also occur in organic solvents when lipases catalyze ester-exchange reactions (19). For ester synthesis in organic solvents, optimal activity has been observed with fatty acids above six carbons in length (116). The glyceride esters of saturated and unsaturated fatty acids are hydrolyzed at approximately equal rates in aqueous reactions (79), although the unsaturated esters appear to be about 10-fold poorer substrates in some reactions in organic solvents (19).

The enzymes have been reported not to display stereoselectivity in the hydrolysis of triglycerides in aqueous solution (79). This differs from their behavior in organic solvents, in which stereoselectivity is observed (see section 15.6.2.6). Under appropriate conditions their substrate ranges are quite broad. As noted previously, they are capable of hydrolyzing the glyceride ester bonds in lipoproteins. They also hydrolyze phospholipid ester bonds in aqueous and organic solvents, and are capable of hydrolyzing and synthesizing a variety of other ester and ester-like bonds (see Section 15.6.2.1).

### 15.4.3. Enzyme Multiplicity

The production of multiple lipases is the rule rather than the exception in the fungi. To our knowledge this phenomenon has been detected in every organism appropriately examined. The families of lipases arise via any of several mechanisms, including gene duplication followed by sequence divergence, proteolytic maturation of lipolytically active precursor forms, the binding of small molecules to the lipases, intracellular versus extracellular compartmentalization, and differential glycosylation. Except for the first of these mechanisms, all appear to contribute to the formation of multiple lipases in the Mucorales. To date, there has been no indication of multiple lipase genes in these organisms.

The binding of small molecules (i.e., phospholipids and peptides), has been shown to generate different forms of lipase in *Rhizopus delemar*. Iwai and Tsujisaka (83) isolated three extracellular lipases, termed A, B, and C, from cultures of this organism grown in defatted soybean meal-glucose media. The B form was separated from the others on the basis of its insolubility at pH 4.5. The A and C forms were separated from one another by cation exchange chromatography at pH 5. The pH versus activity and pH versus stability profiles of the enzymes were comparable. There were differences of 5 to 10°C in their optimal temperatures. The A form was stable to a 15 min incubation at 60°C, while the B and C forms were inactivated above 40 and 35°C, respectively. It was shown that the presence of phospholipid accounted for the difference between the B and C forms, with the noncovalent binding of phospholipid by the C form converting it to the B enzyme (82). This increased the hydrolytic activity of the enzyme toward lipoprotein by 10- to 15-fold (85) without affecting its activity against triglycerides. Phospholipid binding also caused a 3- to 5-fold increase in hydrolytic activity toward water soluble esters, and a 19-fold increase in the hydrolysis of Tween 80 (153). The enzyme displayed a more acidic isoelectric point and a decreased  $\alpha$ -helical content (81). The differences between forms B and C, and the A form enzyme were not determined.

Binding or dissociation of peptides has also been shown to occur with the lipases, generating families of enzymes. Working with *Rhizopus arrhizus*, Semeriva et al. (149) reported that freshly prepared crude lipase contained two forms, separable by ion exchange chromatography, designated I and II. Gel filtration indicated that Form I had a molecular weight of approximately 40,000 and Form II had a mass of approximately 30,000. Upon aging of Form I at 4°C, Form II appeared and the amount of Form I decreased. Subsequent work (150) demonstrated that this conversion involved the dissociation of a noncovalently bound glycopeptide of molecular mass approximately 8,500.

Another source of multiplicity in the lipases is posttranslational modification. In some fungi, such as *Geotrichum* (13, 87), differential glycosylation by the producing organisms accounts for the occurrence of distinct lipases. This phenomenon has not yet been shown to occur in the *Rhizomucor* and *Rhizopus*. Multiplicity has been shown to occur, however, in the course of manipulation of the enzymes in the laboratory. For example, Høge-Jensen et al. (79) isolated two extracellular lipases from *Rhizomucor miehei*. The enzymes exhibited a high degree of antigenic relatedness, and had similar pH-activity profiles toward tributyrin. On the basis of differences in their affinities for concanavalin A, the authors concluded that one form was generated by partial deglycosylation of the other. Exposure of the lipase to acidic pHs during purification was postulated to have triggered the partial deglycosylation.

Physical location is also a differentiating factor among the lipases of these fungi, with both intra- and extracellular activities being known. Little is known concerning the intracellular lipases, whose total activity is approximately one-tenth that of the extracellular enzymes (9). The relationship between these two classes of enzymes has not been investigated. Tahoun and co-workers identified and purified three intracellular lipases from *Rhizopus delemar* (162). Electrophoresis indicated that these enzymes had molecular weights of 76, 60, and 45 kDa, whereas the extracellular enzyme has a mass of approximately 30,000. The enzymes were all 1,3-specific in triglyceride synthesis and hydrolysis (162, 163).

#### 15.4.4. Biochemical Properties

Extracellular lipases from *Rhizopus japonicus* (3), *Rhizopus nodosus* (125), *Rhizomucor miehei* (27), *Rhizopus delemar* (65), and *Rhizopus niveus* (100) have been purified to homogeneity. The purification schemes involved combinations of traditional methods, such as gel filtration and ion-exchange chromatographies, with hydrophobic interaction chromatography (27), reverse-phase high-performance liquid chromatography (100), and affinity chromatography on resins containing covalently bound fatty acids (65, 125), heparin (3), or concanavalin A (27).

The purified lipases from *Rhizopus delemar*, *Rhizopus niveus*, and *Rhizomucor miehei* were all shown by electrophoresis to consist of single polypeptide chains, with estimated molecular masses between 30 and 34 kDa. The cloning and sequencing of the corresponding genes has validated these determinations (see Section 15.5). The enzyme from *Rhizopus japonicus* was estimated by gel filtration to have a mass of 42,000 (3). Curiously, the *Rhizomucor miehei* lipase was also estimated by gel filtration to be 42.5 kDa, although electrophoresis under denaturing conditions indicated a mass of 32 kDa (27). The estimation of larger masses by nondenaturing methods may be due to the adherence of a peptide to the lipase, as has been described for the *Rhizopus arrhizus* enzyme (see Section 15.4.3.). The isoelectric point (pI) of the *Rhizopus delemar* lipase has been experimentally determined to be 8.6 (65) and calculated from its predicted amino acid sequence to be 8.0 (63). The enzyme from *Rhizomucor miehei* is considerably more acidic, with a measured pI of 3.9 (79).

These enzymes are not extensively glycosylated. The *Rhizomucor miehei* lipase contains approximately 11% carbohydrate on a weight basis (27). The *Rhizopus delemar* lipase, isolated from the fungus, was determined to contain only one monosaccharide per lipase molecule (65). This enzyme was produced in a fully active form when a cDNA encoding it was introduced into *E. coli* (63). Since *E. coli* is incapable

of protein glycosylation, this observation indicates that glycosylation is not a prerequisite for enzymatic activity.

The enzymes are most active near neutral pH values. Slightly acidic pH optima have been reported for some members of this family (83, 125), while in other cases, neutral (79) or slightly basic pH optima are reported (65, 122). This may be a function of the use of different types of assays in different laboratories for the determining pH optima. The enzymes are stable over a range of pHs, exhibiting full activity following exposure to pHs between 4 and 9.

The enzymes are not particularly temperature resistant (3, 65, 122, 125). Optimal activities generally occur between 30 and 40°C. Denaturation occurs rapidly above about 45°C. It has been shown that the proenzyme form of the *Rhizopus delemar* lipase displays enhanced temperature stability, surviving a 15-min incubation at 70°C with little loss of activity (92).

The lipases are generally resistant to inhibition by sulfhydryl-directed inhibitors such as *p*-chloromercuribenzoic acid and *N*-ethylmaleimide, indicating that free sulfhydryl groups are not required for activity (65, 122). They can be inhibited by serine-directed modifiers, indicating the essential role of at least one serine (31).

A detailed study of metal ion requirements was reported for the *Rhizopus delemar* lipase (65). Polyvinylpyrrolidone was employed to emulsify the substrate in order to eliminate interference by the metal ions present in gum arabic. It was found that the enzyme requires divalent cations (barium, calcium, or manganese) for activity. Activation by calcium was maximal at and above 10 mM. Such a requirement has been previously noted for other lipases. The concentration required to see a stimulatory effect is relatively high. This has been interpreted to indicate that the metal ion affects the suitability of the substrate emulsion, perhaps by binding to and removing free fatty acids from its surface, rather than playing an integral role at the active site of the lipase. Slight to moderate inhibition of the enzymes from *Rhizopus nodosus* (125) and *Rhizomucor miehei* (122) by metal ions has been noted.

### 15.4.5. Tertiary Structure

The *Rhizomucor miehei* (RM) lipase was the first lipolytic enzyme for which a high-resolution structure was determined by x-ray crystallography (Figure 15.2A). This development (31) has been followed by high-resolution structures for the lipases from the human pancreas (174) and *G. candidum* (145). The RM lipase is an  $\alpha/\beta$  type protein, with a central eight-stranded  $\beta$  pleated sheet made up of predominantly antiparallel strands. These are connected to one another by helical segments, loops, and hairpins. These connecting structures are located on one side of the sheet, and a single N-terminal  $\alpha$ -helix provides support for the distal side of the sheet. The structure is stabilized by three disulfide bonds. A triad of amino acids, consisting of Ser144, His257, and Asp203, was identified whose geometric arrangement was reminiscent of the catalytic triad of the protease trypsin. It was postulated that this was also the catalytic unit in the lipase, a proposal subsequently supported by site-directed mutagenesis.

One of the loops connecting the  $\beta$ -sheets of the structure forms a 15 amino acid long "lid" (Residues 82–96) over the active site (31). Upon contact with the hydrophobic surface of a substrate micelle, it is postulated that the lid shifts position, exposing its hydrophobic face and additional hydrophobic residues on the surface of



**Figure 15.2.** Diagrammatic representation of the proposed three-dimensional structure of the *Rhizomucor miehei* lipase, determined by x-ray crystallography (31, 33, 44). The  $\beta$ -sheet structures are indicated by ribbons,  $\alpha$ -helical regions are depicted as cylinders. The active site residues (Ser144, His257, and Asp203) are at the top of the figures, with each nonhydrogen atom being depicted by a sphere. The "lid" loop is indicated in boldface print. (A) The uninhibited enzyme; (B) structure of the enzyme when complexed with active site-directed inhibitors. The exposure of the active site by movement of the lid upon inhibitor binding is clearly illustrated by comparing these two figures. Generously provided by Drs. Z. Derewenda and A. Sharp.

the protein to the substrate. Subsequent studies employing active site-binding irreversible inhibitors, especially diethyl *p*-nitrophenyl phosphate, have validated this proposal (Figure 15.2B) (33, 44). The structure of the RM lipase complexed with this agent shows that the six amino acids of the central portion of the lid moved as a rigid body, with the amino acids at the ends of the lid dramatically changing their conformational dihedral angles while acting as hinges for this motion. As a result, some atoms of the lid are moved by over 1.2 nm relative to their original position, the active site of the enzyme is uncovered and a hydrophobic area of about 80 nm<sup>2</sup> becomes exposed.

The structure of the enzyme in the presence of these inhibitors is thought to closely resemble the actual structure during binding to substrate micelles and catalysis. This model of lipase activity, wherein the active site is buried under the lid until the enzyme is in the proximity of substrate, whereupon the lid rolls away to expose the site, would seem to explain the biochemical observation that lipases are active only on water insoluble esters. Since many lipases have sequences that suggest the presence of a lid, it is believed that this model for the activation of the enzyme at the oil-water interface is likely to be generally applicable to all lipases. As mentioned previously, the three active site residues of the lipase are identical to those of some proteases. In the initial publication of the structure of the RM lipase, where the presence of the lid was first noted, it was speculated that by blocking the active site in the absence of substrate, the lid may serve to inhibit a potential proteolytic activity of the active site (31).

The amino acid residues forming the lid of the RM lipase are only partially conserved in the sequence of the proposed lid of the *Rhizopus delemar* (RD) enzyme. A striking difference is the replacement of Trp with Ala at Position 88 in the RD lipase (63). It was thought initially that Trp88 might play an important function in shielding the active site of the RM enzyme. However, the absence of this residue in the lids of both the RD and *Humicola lanuginosa* (HL) (26) lipases (Figure 15.3) indicates that there is no universal requirement for this residue in the interfacial activation process.

Data obtained from crystals of the RM lipase containing the covalently bound active site directed inhibitor, *n*-hexylchlorophosphonate ethyl ester, revealed additional features of the substrate-binding site (33). One residue (Ser82) appears to stabilize the oxy-anion through hydrogen bonding. The corresponding residue in the RD lipase is a threonine. Replacement by site-directed mutagenesis of the threonine residue with alanine resulted in an inactive enzyme (93). This indicates that Thr83 from the RD lipase also plays an important role, either structurally or in the processes of substrate binding and/or catalysis.

Analysis of the structure of the RM lipase-*n*-hexylchlorophosphonate ethyl ester complex also revealed that the *n*-hexyl moiety is located within a shallow hydrophobic groove in the enzyme (33). This groove is formed by residues residing in the body of the enzyme and on the exposed side of the "opened" lid (33). The groove is thought to harbor the scissile leaving fatty acid of the substrate molecule. The residues of this hydrophobic surface (Ile85, Trp88, Ile89, Leu92, Phe94, Val205, Leu208, Phe213, Val254, Leu255, Leu258, and Leu267) have functionally equivalent counterparts in the RD lipase. The major differences between the two enzymes at these sites are that in the RD lipase, Ala89 substitutes for Trp88 of the RM enzyme, and the replacement of Leu in Position 267 of the RM enzyme with Ser in RD. The structural basis of the 1,3-specificity to these enzymes, or of the strict mono- and diglyceride specificity

RD	mvsfisisqgvslcllvssmmlgssavpvsgksgssntavsasdnaalpp	-74
RM	mvlkqranylgflivfftaflv--eavpikrqsntvds-----lpp	-55
HL	-----	
PC	-----	
RD/RN	lissrcappsnkgsksdlqaepynmqkntewyeshggnltsigkrddnlv	-24
RM	lipsrtsapssspsttdpeapam-----srngplps----dvetk	-19
HL	-----	
PC	-----	
	-1/+1	
RD/RN	ggmtldlpsdappislsstnsaSDGGKVAATTAQIQEFTKYAGIAATA	27
RM	ygmalnatsypdsv----vqamSIDGGIRAATSQEINELTYTTLANS	27
HL	-----EVSQDLFNQFNLFAQYSAAA	20
PC	-----DVSTSELDQFEFWQYAAA	20
	* . . . . .	
RD/RN	YCRS---VVPGNKWDCV--QC-QKWVPDGKIIITFT-SLLSDTNGYVLS	70
RM	YCRT---VIPGATWDCI--HC-DA-TEDLKI IKTWS-TLIYDTNAMVARG	69
HL	YCGKNNDAPAGTNICTGNACPEVEKADATFLYSFEDSGVGDVTGFLALD	70
PC	YYEADYTAQVGDKLSCKGNCPEVEATGATVSYDFSDSTITDTAGYIAVD	70
	* . . . . .	
RD/RN	DKQKTIYLVFRGTNSFRSAITD-IVFNFSYKPV-KGAKVHAGFLSSYEQ	118
RM	DSEKTIYIVFRGSSSIRNWIAD-LTFVPSYPPV-SGTKVHKGFLLDSYGE	117
HL	NTNKL <sup>1</sup> VLSFRGSRSEETGSGNLFDLKEINDICSGCRGHDGFTSSWRS	120
PC	HTNSAVVLAFRGSYSVRNWDADA-TF-VHTNPGLCDGCLAEFGWSSWKL	118
	* . . . . .	
RD/RN	VVNDYFPVVQEQLTAHPTYKVI VTGHSLGGAQALLAGMDLYQREPRLSPK	168
RM	VQNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSS	167
HL	VADTLRQLVEDAVREHPDYRVVFTGHSLGGALATVAGADL--RGNGY-DI	167
PC	VRDDIIKELKEVVAQNPNYELVVVGHSLGAAVATLAATDL--RGKGYPSA	166
	* . . . . .	
RD/RN	NLSIFTVGGPRVGNPTFAYYVES-TGIPFORTVHKRDIVPHVPPQSGFGL	217
RM	NLFLYTOGQPRVGDPAFANYVVS-TGIPYRRTVNERDIVPHLPPAAFGL	216
HL	DVFSY--GAPRVGNRAFAEFLTVQTGGTLYRITHTNDIVPRLPPREFGYS	215
PC	KLYAY--ASPRVGNAAALAKYITAQ--GNNFRFTHTNDVPVKLPLLSMGYV	212
	* . . . . .	
RD/RN	HPGVESWIKSGT-----SN-VQICTSEIETKDCSNSIIVP-FTSILDHLSY	260
RM	HAGEEYWITDNS-----PETVQVCTSDLETSDCSNSIIVP-FTSVLDHLSY	260
HL	HSSPEYWILSGTLVPVTRNDIVKIEG---IDATGGNNQPNIPDIPAHLWY	262
PC	HVSPEYWITSNNATVSTSDIKVIDGDVDFDNGTGTGLPLLTDFEAHIWY	262
	* . . . . .	
RD/RN	FDINEG-----SCL	269
RM	FGINTG-----LCT	269
HL	FGLIGTC-----L	270
PC	FVQVDAGKGPLPFRV	279
	* . . . . .	

**Figure 15.3.** Comparison of the predicted amino acid sequence of the *Rhizopus delemar* (RD) lipase (63) with the predicted sequences of the *Rhizomucor miehei* (RM) lipase (27), the *Humicola lanuginosa* (HL) lipase (26), and the *Penicillium camembertii* (PC) mono- and diglyceride lipase (179). The predicted preproenzyme sequences from the RM and RD enzymes are written out in lowercase letters in the one-letter amino acid code. The amino acid sequence of the *Rhizopus niveus* (RN) lipase, based on analysis of the lipase and the corresponding cDNA, has been reported (100). The protein begins with an Asp residue corresponding to Asp28 of the RD lipase amino acid sequence and is identical to the amino acid sequence predicted from RD lipase cDNA. The sequence comparison was generated using the alignment program CLUSTAL from the IntelliGenetics PC/GENE program package. Residues identical in all four sequences are marked by asterisks (\*). Amino acid residues with a functional relationship are marked by the symbol ".". The amino acid sequences of the mature enzymes are given in uppercase letters. The residues in the prepropeptide sequences were given negative numbers. The N-terminal residues of the mature enzymes were given the number +1.

of the related lipase from *Penicillium camembertii* (179), are not presently known. It is anticipated that structural analysis of additional lipases, along with subsequent model building and site-directed mutagenesis, will lead to further insights into the molecular bases of substrate specificity.

## Molecular Genetics

### Lipase cDNA Cloning and Expression

The first studies on the molecular structure and genetics of lipases were concerned with enzymes of mammalian origin. Data on bacterial and fungal lipases began to appear after 1988. However, progress since then has been considerable. Nucleotide and amino acid sequences are presently available for several lipases, including a number of Mucorales enzymes. In conjunction with the development of detailed three-dimensional structural models by x-ray crystallography (Section 15.4.5), this information has served as a powerful tool in understanding lipase activity.

A cDNA was produced from RD mRNA that specified the production of lipolytic protein in the heterologous host *E. coli* (63). Analysis of the nucleotide sequence of this cDNA suggested that cDNA encodes a pre/propeptide consisting of a 26 amino acid signal sequence, a 97 amino acid propeptide, and a 269 residue mature enzyme. The predicted amino acid sequence of the mature lipase is identical to the sequenced portion of the authentic lipase purified directly from RD (65). The enzyme exhibits little similarity to lipases from mammalian and bacterial sources, but it is identical to a lipase from *Rhizopus niveus* IF04759 (100), and similar to lipases from RM (27), HL (26), and the mono- and diglyceride lipase from *P. camembertii* U-150 (178) (Figure 15.3). The virtually identical nucleotide sequence (difference of one nucleotide) and the identical amino acid sequence of the RD and the *Rhizopus niveus* (RN) lipases suggest either a close taxonomic relationship between the two organisms or, as noted previously, the difficulty in the classification of fungal isolates.

There are substantial homologies between the lipase genes, and the corresponding proteins, of the fungi listed previously. The highest degree of similarity is found between the RD and RM enzymes. Both are apparently produced initially as pre-proenzymes. The nucleotide sequences encoding the pre-, pro-, and mature portions of the enzymes exhibit a considerable degree of identity (47, 53, and 59%, respectively). At the amino acid level, 29% of the residues of the pre/propeptides and 68% of the residues of the mature enzymes from RD and RM are either identical or functionally similar (63). Only a single gap must be introduced in the sequences of the two mature lipases to bring them into optimal alignment, and this gap is only one amino acid in length (63).

The RD and RM lipases both contain a consensus pentapeptide (—Gly-X-Ser-Y-Gly—), which is conserved in all known lipases. The central serine residue is the active site serine. Crystallographic data from the RM lipase showed that this residue (Ser144) is part of a catalytic triad that also includes an aspartate (Asp203) and a histidine residue (His257) (31). The same residues occupy identical positions in the predicted amino acid sequence of the RD lipase (Figure 15.2). The relatively high degree of amino acid sequence similarity, including the conservation of six cysteine residues

that have been found by crystallography to form three disulfide bonds in the RM enzyme, suggests that the enzymes have similar three-dimensional structures (63).

The amounts of lipase produced by the fungi are, of course, balanced by the nutritional needs of these organisms, and thus they are often insufficient for industrial or even scientific demands. Genetic manipulations to improve yields in fungal systems were virtually impossible until a few years ago. Even today such manipulations are not easily accomplished with some fungi. Nevertheless, one of the first reported expression systems for a fungal lipase was that for the production of RM lipase in the fungus *Aspergillus oryzae* (78). In this work the cDNA encoding the RM prelipase was placed under the control of an  $\alpha$ -amylase promoter on a plasmid vector. After introduction of the recombinant plasmid, the *A. oryzae* host strain produced a lipase with the characteristics of the original RM enzyme. The recombinant lipase was found in the culture medium and, except for some heterogeneity of the N-terminal amino acid residue, appeared to be the same size as mature RM lipase. Thus, *A. oryzae* was able to process and export the recombinant RM lipase.

The cDNA encoding the RD lipase was originally identified by its ability to specify production of a lipolytic enzyme in *E. coli* (63). In these expression constructs, the RD cDNA, a 1.3-kb *EcoRI* fragment, was inserted into *EcoRI* sites within the multiple cloning regions of the vectors  $\lambda$ -gt11 and pUC8.2. This allowed the production of fusion proteins containing a few residues encoded by the  $\beta$ -galactosidase gene of the vectors, some residues specified by the region 5' to the presumed translation initiation codon of the fungal preproenzyme mRNA, and the residues of the preproenzyme itself. Immunological analysis indicated that *E. coli* was incapable of processing this fusion protein to the mature fungal lipase.

As initially isolated, the cloned RD lipase cDNA was capable of producing only low levels of lipase activity and of lipase cross-reacting material. The use of lipases as biocatalysts will require the availability of significant amounts of the enzymes. Therefore, expression systems were sought that would allow the production of larger quantities of enzyme. Successful high-level expression was obtained using a combination of *E. coli* BL21 (DE3) and the plasmid vector pET11-d (92). To date this is the only report of the high-level expression of a cloned fungal lipase gene. The cloning system used is characterized by tight regulation of the transcription of the cloned gene. Such regulation was necessary in this case because of prior indications that small amounts of active lipase severely jeopardize cell viability. Even the low levels of expression typical of the uninduced state of a standard gene cloning system are apparently quite toxic to excretion-deficient hosts (15). The RD lipase cDNAs, after alteration by site-directed mutagenesis to produce DNAs encoding either the mature or the proenzyme form of the enzyme, were, therefore, cloned into the pET11-d expression system. In this manner, high levels of the proteins encoded by these genes were successfully produced and cell viability was not compromised. Enzyme production constituted about 20% of total cell protein and was sufficiently high to cause the now well-known phenomenon of inclusion body formation. These intracellular bodies form when polypeptides synthesized at high levels aggregate into inactive and insoluble particles. In the cases of both the pro- and mature forms of the lipase, the inclusion bodies were isolated, providing a substantial enrichment for the proteins within them. These were solubilized with urea and detergent, renatured to yield active enzymes, and purified to homogeneity. Using this expression route, a 100-fold increase

in the yield of mature lipase, relative to production by RD, was achieved. Furthermore, this offered the first opportunity to isolate and study the prolipase.

Both the mature and prolipases were enzymatically active. Their specific activities were comparable to that of the authentic fungal enzyme, indicating that proper refolding had occurred in vitro. The mature form exhibited the characteristics of the fungally produced enzyme with respect to temperature and pH optima and thermal stability. The proenzyme had the same temperature optimum as the mature enzyme, about 30°C, but exhibited an acidic shift (approximately .5 pH unit) in its pH optimum, to 7.5. This may be correlated with the fact that the calculated isoelectric point of the prolipase (7.2) is nearly a full pH unit more acidic than that of the mature enzyme. Thus, both the mature and prolipase display highest activity when their net charges are near zero. Such behavior is not unexpected considering the apolar nature of the substrate. The thermal stability of the proenzyme was considerably greater than that of the mature enzyme; whereas the latter loses appreciable activity during a brief incubation at 45°C, the proenzyme survives exposure to 70°C with no activity loss. This suggests that the propeptide is able to delay the onset of thermally induced denaturation or assists the enzyme in regaining an active conformation after heat exposure. It is possible that the propeptide plays some analogous role in stabilizing the lipase or facilitating folding within the cell.

### 15.5.2. Genomic Lipase Sequences

Comparatively little is known concerning the structure, location, and control of lipase genes in the Mucorales. There has been no report of the isolation and characterization of a lipase gene directly from one of these organisms. In one case, a genomic library of RD DNA was established in *E. coli* and scanned for lipase production (67). However, no lipolytic activity was detected. This failure could indicate the presence of introns within the fungal lipase gene. Alternatively, the fungal sequences regulating transcription or translation may not function in *E. coli*. Friesen and Baker (57) have isolated DNAs homologous to lipase cDNA from this genomic library. The nucleotide sequences of these DNAs are not interrupted by introns, indicating that in at least some cases the lipase gene does not contain introns.

In their isolation of RM lipase cDNAs, Boel et al. obtained three clones (27). One of these contained 74 additional nucleotides, in a single block, which were not present in the other clones and for which there is no analog in other lipase cDNAs. The authors postulated that these corresponded to an unspliced intron.

The mono- and diglyceride lipase of *P. camembertii* is 29% homologous to the RM lipase (179). The gene for the *P. camembertii* enzyme has been shown to contain two small introns, each about 55 bp in length. These lie near the region encoding the N-terminus of the mature lipase. Therefore, it appears possible, but by no means universal, that fungal lipase genes contain introns.

## 15.6. Biotechnological Applications

Several features of enzymes render them attractive for the catalysis of organic reactions. Among these is the fact that they are active under ambient conditions. This eliminates the energy expenditure required to conduct reactions at elevated temper-

atures and pressures, reduces the destruction of labile reactants and products, and avoids the formation of by-products. In addition, enzymes often display desirable substrate-, regio-, or stereo-specificities, and the reactions that they catalyze are of substantial and diverse utility in the food, pharmaceutical, and industrial chemical sectors. In addition, for lipases, the facts that they do not require cofactors for activity, and generally are active as monomeric polypeptides with moderate molecular weights, add to their potential utility. For these reasons, enzymes, including lipases, have been widely studied as applied catalysts (94, 139, 176). However, they are not yet broadly employed in industry. This can be attributed to relatively high costs, instabilities under some conditions, the limited availability of some of the enzymes, and deficiencies in basic knowledge regarding their capabilities and requirements.

A superficial examination of the hydrolytic specificities of the lipases of *Rhizomucor* and *Rhizopus* suggests their potential utility in the production of 1,2- and 2,3-diglycerides, and 2-monoglycerides from triglycerides. Their use in this capacity for the production of glycerides and phosphoglycerides of defined structure has been known for some time (91, 155). These early publications appear to have arisen primarily from a concern for the synthesis of defined compounds of interest to the lipid biochemist. They employ lipases for the partial hydrolysis of glyceride bonds, using oil emulsions in primarily aqueous reaction mixtures. The past decade has witnessed an ever-increasing realization of the catalytic diversity of all enzymes, and in particular, the lipases. Research in this area is quite vigorous, and numerous symposia and convocations dedicated to lipase utilization have been held. The rate of publication of articles describing lipases and their applications exceeds 1,000 articles per year. The enzymes from the Mucorales are among the most frequently employed in these studies. A detailed discussion of all the applications that have been described is not feasible here. An effort has been made to examine general features of the use of these enzymes as catalysts, and to discuss representative examples of their applications.

### 15.6.1. General Principles

Three realizations have fostered the growth in the use of lipases as applied catalysts over the past decade.

1. Ester hydrolysis is a readily reversible reaction. Of its own accord, the ester bond is not particularly unstable. Hydrolysis is the predominant reaction in aqueous systems due to the high concentration of the cosubstrate, water. When water activity is low, as in organic solvents, the reverse reaction can be favorable, resulting in ester synthesis. Lipases have proven to be useful in the catalysis of such syntheses in hydrophobic organic solvents. Iwai's group was among the first to exploit this fact (168), achieving the synthesis of glycerides from oleic acid and glycerol using not only the RD lipase, but also enzymes from *Aspergillus niger*, *G. candidum*, and *Penicillium cyclopium*. The reaction mixtures contained only glycerol, enzyme, and fatty acid. In this, and a subsequent article (130), these researchers also documented some of the substrate flexibility of these enzymes, describing the synthesis of glycerides containing dibasic and aromatic acids in place of fatty acids, and various primary alcohols other than glycerol.

2. The second key observation underlying the contemporary development of lipases as applied catalysts is that these, and other enzymes, are active in organic solvents. This principle had been noted earlier, and was implemented for lipase-mediated gly-

ceride modification in the early 1980s (109). However, its enunciation by Klibanov's group in 1984, where the ability of pancreatic lipase to catalyze the transesterification of tributyrin with various primary and secondary alcohols in nearly anhydrous organic media was described (182, 183), initiated a widespread and continuing examination of this phenomenon (46, 47, 62, 181). The advantages of organic solvents for the conduct of reactions include the high solubilities of nonpolar molecules, the shifting of thermodynamic equilibria of reactions (such as hydrolysis) that involve water, enhanced thermostability of the enzymes, the absence of bacterial contamination, alterations in enzyme specificity, and the ease of product and catalyst recovery.

Although the notion that enzymes can retain their activities in organic solvents was foreign to most researchers, it has been shown to be valid and particularly useful in the case of lipase-catalyzed reactions. Some basic principles governing the use of enzymes in organic solvents are:

a. The enzymes are inactive in completely anhydrous systems. A small amount of water is required for activity. It has been postulated that this necessary water forms a layer over, and thereby imparts flexibility to, crucial regions of the protein (62, 180, 182). Excessive amounts of water, however, can lead to enzyme inactivation or reversal of the desired reaction. The optimal amount of water is a function of the catalyst, the nonprotein components in the catalyst preparation, the solvent, and the reaction being conducted. In practice it is necessary to experimentally identify the range of added water that provides optimal enzyme activity. One of the most precise approaches to the problem of water levels and their relation to enzyme activity is the work of Halling and co-workers, who have explored the use of salt hydrates to maintain optimal water levels during lipase catalyzed reactions in organic media (101, 170, 171).

b. The suitability of a solvent is a function of its polarity. The less polar solvents generally foster the most enzyme activity. This is thought to result from the increased water capacities of polar solvents, and their resulting abilities to strip the water of hydration from the catalyst. Various theories have been proposed in an attempt to correlate solvent polarity and enzyme activity, and to guide the optimization of activity through proper solvent choice. The most useful of these has been the approach of Laane et al. (102, 103), who found a good correlation between enzyme activity and  $\log P$ , where  $P$  is the partition coefficient of the solvent in an octanol/water biphasic system.

There is no one best solvent for all reactions. Rather, solvent suitability is a function of other parameters, such as the polarity of the reactants and products. Most investigations of lipase-directed catalysis do not include the examination of a wide range of solvents. In fact, the majority employ only one solvent, usually hexane. In those cases in which more than one solvent was investigated, isooctane has often emerged as the one that supports maximum lipase activity. Often it is unnecessary to employ any organic liquid, since many triglycerides are liquid at or slightly above room temperature and serve well as both solvent and reactant. These enzymes are also active in supercritical  $\text{CO}_2$  (49, 112).

3. The third realization that has been pivotal in the development of lipases as catalysts is that their substrate ranges are quite broad. They are catalytically active on a diversity of substrates, including not only the esters of glycerol, but also of many other hydroxyl-containing compounds and their analogs. Therefore, they have been

used in the synthesis and hydrolysis of various glyceride esters, glycoside esters, acyl esters, thioesters, and amides (Section 15.6.2).

Enzymes are insoluble in hydrophobic organic solvents. In many studies of the activities of lipases in solvents they have been added as lyophilized powders to the reaction mixture. A disadvantage of this method is that a large proportion of the catalyst will be included within enzyme particles, and not optimally in contact with the substrate. A more desirable approach is to immobilize the catalyst on an inert, insoluble carrier. Not only can this increase the apparent activity of the enzyme by maximizing its contact with the substrates, but it also facilitates recovery of the enzyme at the end of the incubation. A thorough review of the many efforts in this area has been provided by Malcata et al. (111).

Many approaches have been taken regarding enzyme immobilization on carriers. These range from the direct use of the dried mycelia of organisms containing cell-bound enzymes, through the growth of lipase-producing fungi on biomass support particles, to the entrapment of free enzymes in gels made from photopolymerizable precursors. No general rules for choosing a best immobilization method or support have yet become clear, although general features affecting the activity of immobilized enzymes have been identified (173). Nearly each enzyme and application appear to represent a unique case.

It is clear that, for reactions conducted in nonpolar solvents, it is not necessary that the enzyme be covalently bound to the support, although covalent linkage may enhance enzyme activity (38). It is often sufficient to simply deposit a lipase from aqueous solution onto an inert carrier, such as Celite, by evaporating the water with a stream of nitrogen. Bound in this fashion, lipases are generally active in both aqueous and organic solvents. Catalyst produced in this manner may not be suitable for use in aqueous systems, however, since the enzyme will desorb and be lost to the liquid phase. Few immobilized enzymes are commercially available. Lipozyme, Novo's preparation of RM lipase electrostatically bound to Duolite (88), for years was the only one. The attractiveness of the immobilized format has led to the use of this product in numerous investigations of lipases as applied catalysts.

## **15.6.2. Specific Applications**

### ***15.6.2.1. In Situ Flavor Production***

The lipolytic release of short and medium chain fatty acids (C:4–C:10) from milk fat triglycerides is responsible for the development of desirable flavors in dairy products and foods containing them (96, 148). Since longer fatty acids (C:12 and above) impart soapy and bitter flavor, their release is undesirable. Historically, the development of flavor in dairy products was due to the action of endogenous lipases that preferentially released short chain fatty acids. The fortification of dairy products with these lipases has been explored to speed aging and augment flavor development. However, fluctuations in cost and availability limit the application of this technology. Microbial lipases can substitute for their mammalian counterparts in some of these applications. The 1,3-specific enzymes are useful since in the short chain fatty acids of milk fat, triglycerides are predominantly located at the terminal positions. Mucorales enzymes have been used in various formats for flavor development, both within products and in the production of flavor concentrates such as "lipolyzed" milk fat (96). For example,

the use of RM lipase to stimulate flavor development in hard Italian cheeses has been described (77, 123). Chen and Yang reported that *Rhizopus arrhizus* lipase, as well as the nonspecific lipase of *Candida cylindracea*, released short chain fatty acids from milk fat, and that this effect was increased by immobilization in photopolymerized gels (37).

#### **15.6.2.2. Glyceride Hydrolysis**

The predominant contemporary industrial method of fat hydrolysis is based upon the Colgate-Emery process, which involves incubation of triglycerides at about 250°C and 750 lb of pressure/in<sup>2</sup>. Considerable effort has been expended, particularly over the past 15 years, in developing lipases as alternative, energy-efficient, catalysts for complete triglyceride hydrolysis. The Mucorales enzymes have been largely ignored in this work because of their inefficiency in directly hydrolyzing the secondary ester bonds of triglycerides. It has been demonstrated that, in combination with a positionally nonspecific enzyme, they are able to achieve greater than 95% hydrolysis of selected triacylglycerols (133). They have not, however, been the enzymes of choice for enzymatic fat splitting in aqueous systems.

The fact that the lipases retain their activities in organic solvents, in which triglycerides are readily soluble, has been exploited to achieve the hydrolysis of natural fats in organic solvents at room temperature (21). In this work, essentially quantitative hydrolysis was achieved using Lipozyme, probably due to the addition of secondary amines to the reaction. Such a process could reduce energy costs, facilitate recovery of the fatty acids, and yield a product of superior quality.

It has long been known that in aqueous solution, lipases, including those of the Mucorales, can hydrolyze the ester bonds of phospholipids (PLs) (43, 104, 154). This ability has been exploited for the synthesis of defined PLs (91, 155) and for the removal of PL contaminants from preparations of plasmalogens (42, 74). The limited water solubilities of PLs reduces the large-scale practicality of such systems for PL hydrolysis. Phospholipids are more soluble in organic solvents and their hydrolysis by immobilized RM lipase has been demonstrated (66). This could facilitate the industrial recovery of the fatty acids of PLs for use in other applications. Currently there is no route for this recovery.

Because they contain both polar and nonpolar groups, mono- and diglycerides are efficient emulsifiers. They are employed in this capacity in a variety of industrial sectors and are consumed in large amounts in these applications. Since the positional specificity of the Mucorales lipases, and other 1,3-specific enzymes, renders them incapable of hydrolyzing secondary esters, these enzymes are the biocatalysts of choice for the enzymatic production of mono- and diglycerides. Holmberg and Osterberg (76) employed the RD lipase in a microemulsion system to achieve an 80% yield of 2-monoglycerides from palm oil. Subsequent slow acyl migration resulted in the uncontrollable formation of 1-monoglycerides. These were susceptible to further enzymatic hydrolysis, yielding glycerol and fatty acids. Alternate routes for the production of partially substituted glycerides are described as follows.

*Enzymatic fractionation* is a term referring to the use of lipases in the selective recovery of particular fatty acids or groups of fatty acids from glycerides. In many naturally occurring lipids the distribution of fatty acids on the glycerides is not random. As mentioned previously, for milk fat, particular fatty acids are, in some cases,

preferentially located at the terminal positions of natural triglycerides. In such cases, the 1,3-specificity of the Mucorales lipases facilitates the recovery of enriched preparations of these fatty acids. Thus the RD lipase has been used in an aqueous-oil emulsion system for the recovery of high purity erucic acid (C22:1) from Crambe oil and of dimorphecolic acid (C18:2,OH) from Dimorphotheca oil (45). This enzyme can also be used for the production of enriched erucic acid preparations from rapeseed oil (54). Using the lipase from *Rhizopus arrhizus*, Hayes and Kleiman reported the recovery of lesquerolic (C20:1,OH) and auricollic (C20:2,OH) acids in 85–90% yield from *Lesquerella fendleri* seed oil (72). These acids constituted only 54% of the total fatty acids in the unfractionated oil.

The specificities of these enzymes can be used in other means to effect selective fatty acid recovery or enrichment. Mbayhoudel and Comeau (113) isolated a 97% pure preparation of petroselenic acid (C18:1,Δ6) in 70% yield from fennel oil by first incubating with *Rhizopus arrhizus* lipase. The enzyme was *inefficient* in releasing petroselenic acid from the oil, leaving the resulting partially substituted glycerides enriched for it. These glycerides were then successfully hydrolyzed by the nonspecific lipase from *Candida rugosa*, yielding the enriched preparation. Using a similar approach, docosahexaenoic acid (C22:6) was concentrated threefold in the monoglyceride fraction, resulting from the partial hydrolysis of cod liver oil with RN lipase (177). Other approaches to fatty acid enrichment exploit this inability of the Mucorales lipases to process particular fatty acids. For example, the enzymatic formation or hydrolysis of the ethyl or butyl esters of all but the desired fatty acids has been used in the isolation of gamma-linolenic (gamma 18:3) and docosahexaenoic (22:6) acids (73, 105). The use of these differential substrate selectivities for fatty acid enrichment via transesterification is described as follows.

### 15.6.2.3. Glycerolysis

The replacement of water by glycerol in the formal hydrolysis equation results in the synthesis of glycerol esters from a triglyceride, rather than the release of free fatty acids. This reaction is termed *glycerolysis*, the cleavage of fatty ester bonds with glycerol (Figure 15.4). Holmberg's group described the use of *Rhizopus* lipases in microemulsions for glycerolysis (75). In this report, the water necessary to achieve significant reaction rates also fostered hydrolysis, leading to the synthesis of both 2-monoglycerides (generated by hydrolysis of the terminal ester bonds of the triglycerides) and 1-monoglycerides (through glycerolysis). This increased the efficiency of monoglyceride production: when formed via partial hydrolysis alone, only one monoglyceride results per triglyceride substrate. Through combined glycerolysis/hydrolysis, three monoglycerides are produced per triglyceride.

The production of monoglycerides through glycerolysis has also been investigated in nonmicroemulsion systems and without the use of organic solvents. Thus, McNeill et al. found that in reactions containing a triglyceride, a twofold molar excess of glycerol, and 3.5–4.6% water, beef tallow was converted to monoglyceride with an approximately 80% yield by RM lipase (114). This high yield was largely the result of a dual-temperature incubation profile. An initial incubation at 42°C was followed by an extended period at 5°C. The authors postulated that the precipitation of the monoglycerides as the low temperature pulled the reaction in the direction of monoglycerides. Even higher yields were obtained with a *Pseudomonas* lipase, resulting

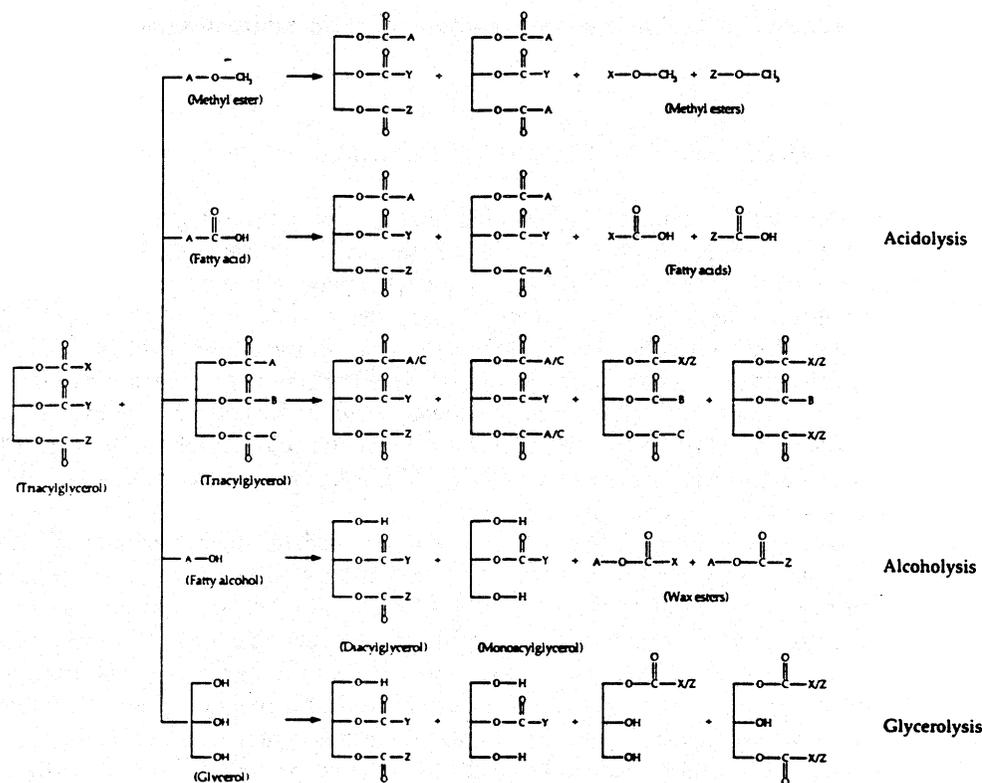


Figure 15.4. Interestification reactions of 1,3-specific lipases, using triacylglycerol as a substrate. The occurrence and effects of acyl migration are not considered. Source: Adapted from Schuch and Mukherjee (146).

in a preparation whose purity was comparable with that of commercially available material. The latter is produced via nonenzymatic glycerolysis at elevated temperatures and requires a molecular distillation step to remove undesirable side products. Enzymatic glycerolysis offers a less complicated and less energy intensive alternate route for the production of monoglycerides.

#### 15.6.2.4. Triglyceride Restructuring

Glycerolysis is but one of a variety of transformations made possible by the reversibility, under appropriate conditions, of the ester hydrolysis catalyzed by lipases. In addition to the synthesis of triglycerides from fatty acids and glycerol, the fatty acid and alcohol constituents of a glyceride ester can be exchanged with their counterparts in other molecules. These processes, collectively referred to as *trans-* or *interestification*, are illustrated in Figure 15.4. Thus, the incubation of a glyceride with a fatty acid results in fatty acid exchange, a process termed *acidolysis*. Simple esters (e.g., methyl, ethyl) of fatty acids can also serve as donors in the exchange of a fatty acid into a glyceride, resulting in the formation of esters containing the acid formerly residing in the glyceride. In the process of alcoholysis, a free alcohol exchanges a

proton for the fatty acid of a glyceride, forming a new ester and a diglyceride. When this alcohol is glycerol, the process is termed *glycerolysis*. Finally, the fatty acids within two glycerides can be exchanged with one another.

These reactions have practical significance. Fats and oils are used in numerous applications. The applications for which a lipase is suitable are dependent upon its physical properties. These are a function of the fatty acid composition of the glycerides comprising that lipid. Thus, it is possible to alter the physical properties of a glyceride through alteration of its fatty acid content. This can be achieved by chemically catalyzed interesterification, wherein a chemical catalyst is employed to exchange the fatty acids of the lipid with other fatty acids. In this case, ester exchange occurs at all three glyceride bonds of the triglyceride. However, the physical properties of a glyceride are also dependent upon the positioning of the constituent fatty acids within the molecule. Since chemical interesterification acts randomly at all positions of a glyceride, it is ineffective at addressing those physical properties that result from fatty acid position. On the other hand, enzyme-catalyzed interchange with a 1,3-specific lipase offers the opportunity for such a positionally specific alteration in composition.

These facts have stimulated extensive research on the use of 1,3-specific lipases, including those produced by Mucorales, as interesterification catalysts. Among the earliest successful efforts was the work of Tanaka et al., which employed the RD lipase to introduce stearic acid into olive oil in a hexane solution (164). These authors demonstrated that small amounts of water were required to confer enzyme activity, but that larger amounts caused the reaction to shift in favor of ester hydrolysis. Posorske et al., using Lipozyme in a continuous operation column format, investigated the use of enzymatic interesterification to exchange the fatty acids of two triglycerides, palm stearin and coconut oil (138). These workers established the need for water in the system to confer and maintain enzyme activity, and investigated several variables (e.g., catalyst age, substrate flow rate, substrate identity, degree of substrate conversion) related to optimum operation of the reaction in a continuous mode. Other researchers have documented various features of the use of lipases as interesterification catalysts, including the identity of the lipase (24), the retention of activity upon immobilization (124), the effects of the immobilization support on the recovery of activity (24), the impact of double-bond position on suitability as a substrate (132), the recyclability of the enzymes (124), their susceptibility to poisoning by crude oil preparations (175), the positive effect of precoating the immobilization medium with protein (175), the saturability of the resin with the enzyme (175), and the effects of the presence and amount of solvent (1). In investigating the abilities of various substrates to participate in the interesterification of the triacylglycerols of ucuhuba oil (isolated from *Virola surinamensis*, which contains almost exclusively lauroyl and myristoyl fatty acids) by Lipozyme, Schuch and Mukherjee found the order of interesterification activity to be long chain alcohol > fatty acid > triacylglycerol > methyl ester > glycerol (146).

The most significant and frequently investigated application of this principle is in the search for a synthetic analog of cocoa butter. Cocoa butter is a naturally occurring fat that exhibits several desirable properties in the areas of melting point, melting range, and organoleptic performance upon melting. It is extensively and preferentially used in many confectionary and cosmetic applications, and is among the most expensive of all fats. The desirable properties of cocoa butter result not only from its fatty acid composition (approximately one each of stearic [18:0], oleic [18:1], and

palmitic [16:0] acids per molecule), but also from the fact that the oleic acid primarily occupies the inner, or secondary, ester positions within the glycerides. The triglycerides of many abundant fats and oils contain oleic acid in their secondary ester positions. However, because they differ from cocoa butter with regard to their total fatty acid content, they exhibit less desirable physical properties. Random chemical interesterification of these fats could be used to adjust the fatty acid content to that of cocoa butter. However, due to the lack of positional specificity on the part of the chemically catalyzed reaction, the predominance of oleic acid at the secondary positions of the resulting glycerides would be lost, and the product would not exhibit properties like those of cocoa butter.

The use of a 1,3-specific lipase as an interesterification catalyst allows the adjustment of overall fatty acid content of a lipid to that of cocoa butter without reducing the high content of oleic acid at the secondary positions of the glycerides. This concept has been explored in many laboratories and with many feedstocks over the past 15 years. The Mucorales lipases have been the enzymes of choice in the majority of these endeavors. The synthesis of cocoa butter analogs by the introduction of saturated fatty acids into relatively unsaturated oils, such as olive oil and palm oil, using immobilized 1,3-specific lipases in the presence (109, 158, 180) or the absence (39) of organic solvents, has been described. This method has also been applied in the case of fats containing more saturated fatty acids than cocoa butter. Bloomer et al. investigated the ability of 12 commercially available lipases to introduce stearic acid, from either the free acid itself or from ethyl stearate, into palm oil midfraction (24). Heptane was the solvent in these reactions. They found that several *Rhizopus* and *Rhizomucor* enzymes successfully catalyzed this reaction. Interestingly, not all enzyme preparations from *Rhizopus* were able to catalyze this reaction. These authors also investigated the effects of immobilization on the enzymes, and found that there was no one support that consistently gave superior enzyme activity for all the enzymes examined. Cho and Rhee (38) have increased the efficiency of synthesis of a cocoa butter analog by covalently immobilizing the enzyme, through a hydrophobic arm, to the insoluble support.

Another example of the application of enzymatic interesterification is the use of Lipozyme to replace the long chain saturated fatty acids of peanut oil (which lie at the terminal positions in the glycerides and have been implicated in atherogenesis) with oleic acid (159). These authors also employed interesterification to replace the polyunsaturated fatty acids of several plant oils, which contribute to oxidative rancidity, with oleic acid. Similarly, the essentially quantitative introduction of polyunsaturated fatty acids (20:5, 22:6, associated with reduced coronary heart disease when consumed in the human diet) into the terminal positions of cod liver oil triglycerides via Lipozyme-mediated interchange has been described (69). The reaction proceeded slightly faster when ethyl esters rather than free acids were used as cosubstrate. A similar enrichment of groundnut (peanut) oil, though to a much lower degree of completion, has been reported (157). These studies employed enzymes that had been passively immobilized on inert carrier. It has been reported that the interesterification activity of the RM lipase can be substantially increased by covalent immobilization through a hydrophobic spacer (38).

Enzymatic interesterification has also been used to exchange the fatty acids in two populations of triglycerides. For example, Forssel et al. employed Lipozyme to interesterify tallow and rapeseed oil in a solvent-free system (56). A product was obtained

that had improved melting properties. A similar exchange of fatty acids between sunflower oil and either tallow or butterfat using Lipozyme, RD, or *G. candidum* lipases has also been reported (55).

As a complement to their abilities to hydrolyze the ester bonds of PLs, lipases have been found to be able to interesterify these bonds as well. Phospholipids are important constituents of membranes, and also are good emulsifiers. Their performance in these roles is a function of their fatty acid content. The availability of PLs with defined fatty acid composition could be useful in several areas. Several preparations of *Rhizopus* and *Rhizomucor* lipases have been found to be able to introduce free fatty acids into PLs by interesterification in hexane (160, 178). Reaction occurred only at the 1-position of the PL. Lipozyme achieved about a 50% substitution at this position, with approximately fivefold higher interesterification rates using free fatty acids, as opposed to their esters, as acyl donors (161). Using RD lipase, Totani and Hara employed this method to create PLs enriched in polyunsaturated fatty acids at the 1-position (166).

#### 15.6.2.5. Glyceride Ester Synthesis

Under limiting water conditions, lipases are able to reverse the hydrolytic reaction, causing the synthesis of glyceride ester bonds. Since glycerides of defined composition are potentially useful in several applications, this reaction has been extensively studied. The lipases retain the same positional specificity as they exhibited in hydrolysis. Studies of the stereoselectivity of ester synthesis have indicated that in at least one case, the *Rhizopus* lipases preferentially esterify the *sn*-3 position first, while Lipozyme, which contains the RM lipase, demonstrates a slight stereoselectivity for the *sn*-1 position (115). The origin of such a difference in selectivity between two groups of closely related enzymes has not been examined.

In most cases, water is generated by glyceride synthesis. (The exceptions are in reactions in which enol esters or anhydrides serve as the acyl donors.) The removal of this water is essential to avoid shifting the reaction to hydrolysis or inactivating the catalyst. The approaches taken in this regard include the inclusion of molecular sieves in the reaction, incubation at elevated temperature or under a vacuum, bubbling dry air through the reaction, and the use of refluxing systems with water traps (25).

Using glycerol as a starting alcohol, it is possible by this reaction to synthesize homogeneous triglycerides of defined fatty acid composition. The reaction can be conducted in the absence of an organic solvent, eliminating concerns about residual traces of the solvent in products intended for dietary use (51, 52, 106, 168). Even using Lipozyme, it has been possible to achieve glyceride populations more than 90% trisubstituted (51, 52). This rather puzzling synthesis of triglycerides by a nominally 1,3-specific lipase has not been adequately explained. Evidently, under some conditions this positional specificity is relaxed.

The production of mono- and diglycerides from glycerol and various fatty acyl donors in aprotic solvents by RD, RM (Lipozyme), and other lipases have been investigated by Berger et al. (17, 20). The reaction was facilitated by the adsorption of glycerol, which is hydrophilic, onto a solid support to increase its contact with the organic solvent. Lipozyme catalyzes this reaction in reverse micelles, in which case the production of triglycerides is greatly slowed, mono- and diglycerides are the predominant products, and the extent of the reaction is reduced (70). Partial glycerides also accumulate when the water generated by ester formation is not removed (147).

Interest in the production of mono- and diglycerides stems from the fact that their superior emulsification properties lend them to a variety of applications, primarily as surfactants.

Another approach to the synthesis of defined monoglycerides involves: (1) esterification of two adjacent hydroxyl groups of glycerol with a single molecule, such as through the formation of the acetonide or isopropylidene; (2) enzymatic esterification of the remaining free-hydroxyl group with a fatty acid; and (3) mild hydrolysis to remove the protecting group (131, 135).

The elevated fatty acid levels resulting from unavoidable hydrolysis of the triglycerides in natural fats and oils can lead to difficulties in refining the oils, due to emulsion formation by the soaps of these fatty acids. By adding a lipase (Lipozyme) to such a hyperacid oil, Ducret et al. achieved resynthesis of the triglycerides, reducing the level of free fatty acids sevenfold to within the range acceptable for processing the oil (48). The synthesis of triglycerides from fatty acids and diglycerides in reverse micelles has also been described (126).

### ***Synthesis and Hydrolysis of Nonglyceride Esters***

Lipases are not confined to the use of glycerol alone as an alcohol donor in ester synthesis. The enzymes have a broad substrate range and a wide array of hydroxyl-containing molecules are able to play this role. Virtually any primary hydroxyl group, or analogous functionality not containing oxygen, may be considered a likely substrate for these enzymes. This has led to the widespread use of lipases in organic synthesis (28). The Mucorales enzymes have been frequently employed in simple ester synthesis, where the enzymes from *C. cylindracea* (recently renamed *C. rugosa*), porcine pancreas, and various species of *Pseudomonas* have been more often used in the synthesis and hydrolysis of more complicated esters. In the following paragraphs an attempt is made to highlight some of the applications of the Mucorales lipases. This is necessarily limited in scope, though an attempt has been made to present representative cases.

Simple esters play a variety of roles, from flavor and aroma compounds to compounding agents in the food, cosmetic, and pharmaceutical industries. Their synthesis by lipases has been investigated in many laboratories. Lipases have been found to be active in reverse micellar, biphasic, and low-water ("microaqueous") environments. For Lipozyme, the synthesis of butyl butyrate has been found to be highest in the latter two systems (30).

The production of the oleic acid esters of simple glycols by RD and other lipases, and of the oleyl esters of various fatty acids and dibasic acids, in some cases, with high efficiency, has been described in the past (130). These reactions were conducted in biphasic solutions with no added organic solvent other than the liquid organic reactants. Since then, numerous other studies have been reported. One of the most exhaustive is that of Miller et al., who conducted a thorough examination of the substrate range of Lipozyme (116). The enzyme was active in solvents too nonpolar to strip it of its essential water layer. It esterified fatty acids five or more carbons in length, and most of those containing cyclohexyl and aromatic residues. Branching and unsaturation within the acyl chain of the fatty acid decreased, but did not eliminate, its ability to serve as a substrate. Interestingly, branching at the carbon lying beta to the carboxylic acid caused the greatest reduction in suitability as a substrate.

Only slight enantioselectivity (favoring the *trans* form) and little stereoselectivity (toward the *S*-isomer) were exhibited toward the acid. The range of alcohols that served as substrates was quite large, and included such compounds as benzyl alcohol, geraniol, pyridine methanol, and dimethylamino ethanol. The RD lipase catalyzed the esterification of ethylene glycol by fatty acids in reverse micellar media, with a yield of approximately 50% (71). The addition of powdered insoluble material, for example, silica, facilitated quantitative acylation of larger, hydrophilic diols, such as diethylene glycol and hexane diol in organic solvents (18). It was postulated that the reaction rate accelerated due to adsorption of the diols to the silica, increasing the area of contact between the substrate, catalyst, and fatty acid. Long chain alcohols serve as substrates for these enzymes. The products of their esterification with long chain fatty acids, known as wax esters, are efficiently synthesized by Lipozyme (61, 167).

The alcohol groups of sugars can also be acylated by lipases. In general, the enzymes retain their characteristic specificities, and therefore they have been used in both the regioselective acylation and deacylation of the hydroxyl groups of carbohydrates. Interest in the production of such compounds arises from their potential uses as nondigestible fat substitutes, artificial sweeteners, and biosurfactants, and as starting materials for organic synthesis. The 1,3-selective enzymes are largely active on the primary alcohols of carbohydrates. Porcine pancreatic lipase has been most frequently used in this application (165). However, the lipases of the Mucorales are also suitable.

Activity at secondary hydroxyl groups in carbohydrates has also been reported and exploited, particularly in compounds lacking unsubstituted primary hydroxyl groups. For example, the regioselective hydrolysis of carbohydrate secondary acyl esters by lipases, including that from RM, has been reported (98). In this case, the enzyme preferentially hydrolyzed the C-4 acylester of a glucose derivative esterified at carbons-2, -3, and -4. Lipases also catalyze the reverse reaction, the regioselective acylation of secondary hydroxyl groups. For example, positionally selective lipase-mediated acylation of the hydroxyl groups of an arabinotol derivative has been reported (128). Interestingly, in this report enzyme preparations from HL and *Rhizopus japonicus*, two lipases that probably exhibit substantial sequence homology, exhibited different regioselectivities in esterification.

The actions of lipases are not limited to carboxylic acid ester synthesis and hydrolysis. Structurally analogous functionalities can also serve as substrates. For example, the use of Lipozyme in organic solvents to synthesize fatty amides (RCONHX) from primary amines and either free fatty acids or their esters has been reported (22, 120). Hydroxamic acids, whose general formula is RCONHOH, are metal chelating agents that are used in analytical chemistry, therapeutics, agronomy, and the nuclear industry. Although chemical routes for their synthesis using water soluble organic acids are in use, the production of hydroxyamic acids using long chain fatty acids has been more difficult. However, it has been shown that Lipozyme is able to catalyze this synthesis, condensing hydroxylamine with either long chain free fatty acids (e.g., oleic acid) or fatty acid esters (151). The reaction proceeded well in water, and was slowed by the addition of an organic solvent, an effect that was postulated to be due to the dilution of the hydrophobic substrate by the added solvent. Other examples of lipase activity towards analogs of carboxylic acid ester are presented as follows.

In contrast to their behavior in aqueous systems, the Mucorales lipases exhibit stereoselectivity in organic solvent media. This trait has been applied to the resolution

of enantiomeric mixtures of compounds containing esters, hydroxyl groups, or ester analogs. Because many such compounds exist, and many of these are precursors for high-value pharmaceuticals or agrochemicals whose activity is stereospecific, the use of lipases as stereoselective biocatalysts has been an active topic lately. This area has been thoroughly reviewed (36).

Alcohols have been resolved by lipase-catalyzed acylations of chiral mixtures with achiral acids or anhydrides, or through transesterification of ester derivatives. The breadth of the substrate range toward alcohols is substantial and includes molecules that are clearly not glyceride-like in structure. For example, RM lipase hydrolyzes, with very high enantioselectivity, the acetate esters of bicyclic alcohols (41, 107). In an interesting investigation of the activity of a Mucorales lipase toward secondary hydroxyl groups, Sonnet described the resolution of aliphatic alcohols via esterification by RM lipase (156). The reaction strongly favored the *R* enantiomer. Stereoselection was dependent upon the chain length of the acid being employed as the esterifying agent, with best selectivity associated with even-numbered molecules between 5 and 9, as well as 16 and 18, carbons in length. Currently the state of knowledge regarding these enzymes, and the relationship of structure to activity, is insufficient to provide insight into the basis of this observation, or into the origin of lipase stereoselectivity itself.

Lipase-mediated stereoselection has also been employed in the resolution of racemic mixtures of carboxylic acids. For example, Miyazawa et al. found the lipases of *Rhizopus javanicus* and *Pseudomonas cepacia* to exhibit high enantioselectivities and reaction rates in the resolution of amino acids through transesterification of the trifluoroethyl esters of their *N*-benzyloxycarbonyl derivatives with achiral alcohols in anhydrous organic solvents (118). The L-enantiomers reacted preferentially. The Mucorales enzymes displayed little enantioselectivity in the resolution of these mixtures by hydrolysis of the esters, although *A. niger* lipase was quite selective in this reaction mode (119).

A commercially available *Rhizopus* lipase successfully resolved racemic diethyl malate, producing optically pure (*R*)-(+)-malate, a useful synthetic precursor (169). Interestingly, a preparation of RN lipase from another supplier did not display enantioselectivity. The impact of the host organism, growth conditions, lipase multiplicity, or nonlipolytic components of the enzyme preparation on the observed stereoselectivity has not been adequately investigated.

Another representative use of the stereoselectivities of these enzymes is the resolution, by differential ester hydrolysis, of racemic mixtures of the methyl esters of cyclopentane carboxylic acids. One of the racemates is a precursor in the synthesis of carbocyclic analogs of purine and pyrimidine nucleosides, which are of great interest as potential antiviral and antitumor agents. *Rhizopus arrhizus* lipase enantioselectively hydrolyzed 4-(benzoylamino)-2-cyclopentenecarboxylic acid methyl ester, leaving the (-)-1*R*,4*S* enantiomer unhydrolyzed (110). The enantioselectivity, although not high, was opposite that exhibited by pig liver esterase, which could allow complementary use of these enzymes in a synthetic scheme. In addition to defining the existence of stereospecific hydrolysis by several lipases, the authors of this report investigated the effect of substrate structure, pH, solvent type, and substrate concentration in improving the enantioselectivity.

The activity and stereoselectivity of lipases is not limited to carboxylic acid esters. Related functionalities can also be hydrolyzed, in some cases, stereospecifically. For

example, sulfhydryl-containing carboxylic acids are precursors in the synthesis of the potent and high-value endopeptidase inhibitors captopril and the related family of compounds. These substances are widely used as antihypertensives. Their efficacy is dependent upon their stereochemistry. In a thorough study, Patel et al. examined the abilities of several lipases to stereoselectively hydrolyze thioesters of the precursor mercaptocarboxylic acids (134). Despite good selectivity on the part of some commercial lipase preparations, that from RN showed little stereospecificity in reactions conducted in CFC-113 (1,1,2-trichloro-1,2,2-trifluoroethane). However, heat-dried whole mycelia of *Rhizopus arrhizus* exhibited acceptable stereoselectivity in both toluene and CFC-113, although not in water. This observation raises several issues, among them the impact of the solvent upon stereoselectivity (and thus probably upon enzyme conformation), the possibility that mycelial lipases exhibit selectivities not seen with the extracellular enzymes, and the role of nonlipase components in modulating stereoselectivity.

## 15.7. Prospects for the Future

Maximum utilization of an enzyme as an applied catalyst requires: (1) knowledge of its capabilities; (2) structural and mechanistic models of the catalytic cycle; and (3) the availability of cloned genes, which serve as the starting material for rational mutagenesis to customize and improve the enzyme. The years 1984–1994 have brought unprecedented achievement in these areas as they relate to lipases. Klivanov's demonstration (97), that enzymes, particularly lipases, retain their activities in organic solvents, has fostered a vigorous and widespread investigation of this phenomenon, and it has highlighted numerous new applications of lipases as efficient and selective catalysts. The recent accumulation of structural information for these enzymes has provided great insight into the details of their binding to substrate micelles and the subsequent hydrolysis of glycerides. At the same time, genetic engineering has provided cloned, expressed lipase genes, the raw materials for enzyme improvement through site-directed mutagenesis. It can be anticipated that the coordinated application of molecular modeling and rational mutagenesis will result in lipases with unique physical and catalytic properties, customized for specific applications. Thus, lipases may be produced that display enhanced selectivity for particular types of fatty acids or alcohols, elevated thermostability or catalytic rate, increased stability in various organic solvents, or an increased activity in ester synthesis. Since 1985 there has been a tremendous increase in the rate of research and publication on the characteristics and applications of lipases. There is little doubt that this trend will continue for these versatile and readily available biocatalysts.

## Acknowledgments

The authors thank Ms. Karen Scott for her assistance in composing this chapter, and Drs. Robert Moreau and Gerald McNeill for critically reviewing it.

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