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Lipases and Their Industrial Applications

An Overview

Alain Houde,* Ali Kademi, and Danielle Leblanc

Agriculture and Agri-Food Canada,
Food Research and Development Centre,
3600 Casavant Boulevard West, St-Hyacinthe, Quebec, Canada, J2S 8E3,
E-mail: houdea@agr.gc.ca

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Abstract

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. The physiologic role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. These enzymes are widely found throughout the animal and plant kingdoms, as well as in molds and bacteria. Of all known enzymes, lipases have attracted the most scientific attention. In addition to their natural function of hydrolyzing carboxylic ester bonds, lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media. This versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries. The most significant industrial applications of lipases have been mainly found in the food, detergent, and pharmaceutical sectors. Limitations of the industrial use of these enzymes have mainly been owing to their high production costs, which may be overcome by molecular technologies, enabling the production of these enzymes at high levels and in a virtually purified form.

Index Entries: Lipases; industrial applications; detergent; protein engineering; rational protein design; directed evolution.

Introduction

The activities of enzymes have been known and exploited since ancient times. Enzymes have found great uses in several industries such as food, dairy, pharmaceutical, detergent, textile, pulp and paper, animal feed, leather, and cosmetics. The number of enzymes commercially available

*Author to whom all correspondence and reprint requests should be addressed.

and the range of applications are gradually increasing. In 2000, the industrial market for enzymes reached US $1.5 billion *(1)*. There are many reasons for the growing interest in enzyme-mediated reactions compared to chemical processes, including high degree of specificity, mild reaction conditions, decrease in side reactions, and simplicity of postrecuperation. Furthermore, enzyme-mediated processes are energy saving and reduce the extent of thermal degradation *(2,3)*.

Among all enzymes, lipases are gaining more importance. They are used in most of the fields mentioned for enzyme applications. This great interest in lipases is mainly owing to their properties in terms of enantioselectivity, regioselectivity and broad substrate specificity.

This review presents the current and some potential industrial applications of lipases, including commercially available lipases, and the recent advances in molecular biology technologies for the production of lipases with genetically improved properties and in industrial amounts.

Definition of Lipases

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. The natural function of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Lipases are widely distributed throughout the plant and animal kingdoms, as well as in molds and bacteria. In addition to lipases, carboxylic esters bonds can be hydrolyzed by esterases. The distinction between lipases and esterases has been based for a long time on the interfacial activation and presence of a lid for the former enzyme. Interfacial activation is defined as a sharp increase in lipase activity observed in the presence of an interface when the triglyceride substrate forms an emulsion *(4)*, whereas the lid is an amphiphilic surface loop that covers the active site of lipase in solution and moves away on contact with the interface. However, lipases from *Pseudomonas aeruginosa*, *Candida anatarctica B*, and *Burkholderia glumae* did not show interfacial activation although they possessed a lid *(5)*. Interfacial activation and the presence of a lid are therefore unsuitable criteria to classify a true lipase, which can simply be defined as a carboxylesterase that catalyzes the hydrolysis and synthesis of long-chain acylglycerols *(5)*. Since the "long chain" of acylglycerols has not strictly been defined, Jaeger et al. *(6)* proposed that glycerol esters with an acyl chain length of 10 carbon atoms or more could be considered lipase substrates.

Kinetic Model of Lipolysis

Lipolysis occurs at the substrate/water interface and therefore cannot be described by the Michaelis-Menten model, which is valid only for biocatalysis in a homogeneous phase, in which the substrate and the enzyme are soluble. A simple model has been proposed to describe the kinetics of lipolysis at an interface *(7,8)* and consists of two successive equi-

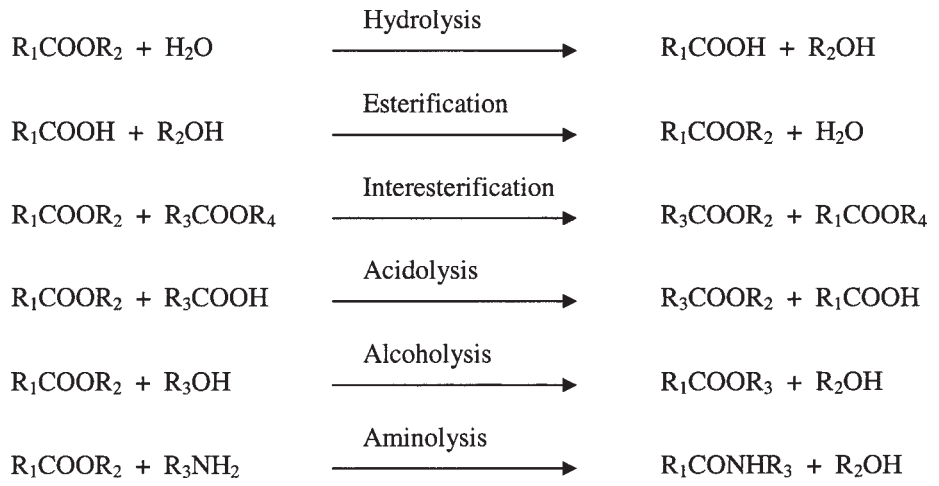

Fig. 1. Different reactions catalyzed by lipase.

libria. In the first equilibrium phase, reversible adsorption of the enzyme to the interface (E⇌E*) occurs, while in the second phase, the adsorbed enzyme (E*) binds a single substrate molecule (S), resulting in the formation of the (E*S) complex. This latter equilibrium is equivalent to the Michaelis-Menten equilibrium for the enzyme-substrate complex. Once the (E*S) complex is formed, subsequent catalytic steps take place, ending with the release of the products and regeneration of the enzyme in the (E*) form. This model takes into account the fact that the concentration of the substrate in the vicinity of the adsorbed lipase at the interface is the concentration at the surface (expressed in moles per unit of surface area) instead of volumetric concentration established in the environment. In this model, the regenerated lipase remains adsorbed to the interface and is only released after several catalytic cycles.

Applications of Lipases

Lipases are versatile biocatalysts. In addition to their hydrolytic activity on triglycerides, they can catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis (Fig. 1). As hydrolases, lipases do not require cofactors. Most regioselective lipases act preferentially on ester bonds at the *sn*-1 and *sn*-3 position of the triglyceride structure, whereas few lipases are active at the *sn*-2 position. Lipases can be found with optimum activities over a wide range of temperatures. Several three-dimensional structures of these enzymes have been resolved allowing the design of rational engineering strategies.

Lipases have potential applications in the detergent, food, leather, textile, oil and fat, cosmetic, paper, and pharmaceutical industries *(9)*. Many lipases are currently commercially available; Table 1 presents the trade names of these lipases from different suppliers. Because of their high per-

Table 1
Commercially Available Lipases and Their Industrial Applications

Industry	Application	Trade name[a]	Supplier
Dairy		Lipase A "Amano" 6 (*Aspergillus niger*)	Amano
		Lipase M "Amano" 10 (*Mucor javanicus*)	Amano
		Lipase F-AP15 (*Rhizopus oryzae*)	Amano
		Lipase AY "Amano" 30 (*Candida rugosa*)	Amano
		Lipase G "Amano" 50 (*Penicillium camembertii*)	Amano
		Piccnate (*Mucor miehei*)	Gist-Brocades
	EMC production (cheddar-type flavors)	Lipomod™ 187P-L187P (fungal lipases)	Biocatalysts
	EMC production (cheddar-type flavors)	Lipomod™ 224P-L224P (porcine pancreas)	Biocatalysts
	EMC production (blue-type flavors)	Lipomod™ 338P-L338P (*Penicillium roquefortii*)	Biocatalysts
	EMC production	Lipomod™ 34P-L034P (*Candida cylindracea* [*rugosa*])	Biocatalysts
	Cheese flavor (cheddar-type flavors)	Lipomod™ 621P-L621 (*Penicillium* sp./*Aspergillus* sp.)	Biocatalysts
	EMC production (cheddar-type flavors)	Lipomod™ 29P-L029P (*Candida cylindracea* + porcine pancreas)	Biocatalysts
	Cheese-flavor enhancement	**Palatase®** (*Rhizomucor miehei*)	Novozymes
Oil and fat		Lipase A "Amano" 6 (*Aspergillus niger*)	Amano
		Lipase M "Amano" 10 (*Mucor javanicus*)	Amano
		Lipase G "Amano" 50 (*Penicillium camembertii*)	Amano
		Lipase F-Ap15 (*Rhizopus oryzae*)	Amano
		Lipase AY "Amano" 30 (*Candida rugosa*)	Amano
		Newlase F (*Rhizopus niveus*)	Amano
	Interesterification of vegetable oil	**Lipozyme® TL IM**	Novozymes
Pharmaceutical	Pharmaceutical ingredient	Lipase MY (*Candida cylindracea* [*rugosa*])	Meito Sangyo
	Synthesis of chiral compounds	Lipase ALC, Lipase ALG (*Achromobacter* sp.)	Meito Sangyo
	Synthesis of chiral compounds	Lipase PLC, Lipase PLG, Lipase QLC, Lipase QLG (*Alcaligenes* sp.)	Meito Sangyo

		Lipase SL (*Burkholderia cepacia*)	Meito Sangyo
		Lipase TL (*Pseudomonas stutzeri*)	Meito Sangyo
		Lipase UL (*Rhizopus* sp.)	Meito Sangyo
	Chiral synthesis	Lipase AK "Amano" (*Pseudomonas fluorescens*)	Amano
	Chiral synthesis	Lipase AYS "Amano" (*Candida rugosa*)	Amano
		Lipase PS "Amano" (*Pseudomonas cepacia*)	Amano
Detergent		Lipolase®, Lipolase® Ultra, Lipo Prime™, Lipex® (*Thermomyces lanuginosus*)	Novozymes
Baking	Improvement of dough texture and color	Lipomod™ 627P-L627P (*Rhizopus oryzae*)	Biocatalysts
	Emulsifier	**Lipopan® F**	Novozymes
Leather	Liming	**NovoLime®** (with protease)	Novozymes
	Fat dispersion	**Greasex®**, **NovoCor® AD**	Novozymes
Cosmetics	Production of isopropyl myristate (cosmetic component)	**Novozym® 435** (*Candida antarctica* B)	Novozymes
Paper	Control of pitch	**Resinase®** (*Candida rugosa*)	Novozymes
Noodles/pasta	Improvement of quality of noodles and wheat-based pasta products	**Noopazyme®**	Novozymes
Miscellaneous	Dietary supplement	Lipase L036P-L036P (*Rhizopus oryzae*)	Biocatalysts
	Dietary supplement	Lipase F-DS (*Rhizopus oryzae*)	Amano
	Delipidation of egg white	Lipomod™ 34P-L034P (*Candida cylindracea*)	Biocatalysts
	Various uses	Lypolyve AN (*Aspergillus niger*)	Lyven
	Various uses	Lypolyve CC (*Candida cylindracea*)	Lyven

[a]Lipases in bold are recombinant.

formance, some lipases are commercialized for one specific application whereas others can be used in different industrial fields. However, despite the relatively high number of commercial lipases available, industrial applications remain limited owing to the high cost of some lipases, the low number of available lipases in industrial amounts, and the low performance of some lipase-mediated processes. Nevertheless, lipases are currently used mainly in the food, detergent, and pharmaceutical industries.

Food Industry

Infant formulas

Infant formula offers a good alternative to breast milk and ideally tends to mimic human milk as much as possible. Milk fat represents the main source of energy in human milk and provides the lipids required to build the structure of cell membranes. The major triglyceride present in human milk is unsaturated at the *sn*-1,3 positions and saturated at the *sn*-2 position. Palmitic acid (C16:0) represents 20–33% of the total fatty acids with one-third located at the *sn*-2 position. The location of palmitic acid on the glycerol backbone is important *(3)*. Higher amounts of palmitic acid were reported in blood samples taken from infants fed with human milk compared with that found in infants fed with formula containing the same total concentration of palmitic acid but not specifically at the *sn*-2 position of the triglyceride. During digestion, pancreatic lipases specifically hydrolyze fatty acids at the *sn*-1,3 positions, producing a monoacylglycerol with palmitic acid at the *sn*-2 position that is more readily absorbed than free palmitic acid. Free palmitic acid binds to calcium and forms poorly absorbed insoluble soaps that cause constipation.

The fat present in most infant formulas comes from vegetable sources and has an unsaturated fatty acid usually located at the *sn*-2 position of the triglyceride. Modification of the triglyceride by lipase to increase the proportion of palmitic acid at the *sn*-2 position leads to a fat with an improved absorption capability in infants *(3)*. Betapol™ (Loders Croklaan) was the first commercial product made by the 1,3-specific lipase treatment of tripalmitin with unsaturated fatty acids that resulted in 1,3-diunsaturated-2-saturated triglycerides intended for infant formula *(3,10)*.

Structured Lipids

Fats are generally divided into three categories with respect to the degree of saturation of their fatty acids: saturated, monounsaturated, and polyunsaturated fats. Saturated fats, such as butterfat, tallow, and lard, usually are of animal origins but can also be found in vegetables such as coconut oil and palm kernel oil. Most saturated fats appear as solids at room temperature. Monounsaturated fats such as olive oil and canola oil, are mostly found in plants and are generally liquid at room temperature, and are used in frying and seasoning. Polyunsaturated fats are also found in plants, including safflower, sunflower, soybean, and corn, as well as in fish. They are liquid at room temperature and are considered healthier

than saturated fats owing to their role in decreasing serum cholesterol and therefore the risk of coronary disease as well as tumor development *(11)*. *trans*-Fatty acids are generated during hydrogenation processes used for the conversion of vegetable oils into solid fats for margarines and shortenings. *trans*-Fatty acids have been suggested to raise blood low-density lipoprotein cholesterol and lower high-density lipoprotein cholesterol, thereby leading to coronary heart disease *(3)*.

Structured lipids are modified fats or oils modeled to be more nutritious and with specific functional and physical properties that make them more suitable for food applications. These modifications include changes in fatty acid content, changes in the position of the fatty acid on the glycerol backbone, and changes in the length of the fatty acid as well as its degree of saturation. Structured lipids may be the most effective approach to include the desired fatty acid in the diet for nutritive and therapeutic purposes. Lipids possessing these higher-added-value attributes can also be produced through lipase-catalyzed interesterification reactions and/or by acidolysis of a less desirable and cheaper lipid.

As structured lipids, plastic fats intended for food applications, such as the production of margarine, shortening, and modified butter, are solid in appearance and possess a low resistance to small stresses, thereby making them easy to spread and rapidly melt in the mouth. The proportion of solid to liquid crystals is the key factor that determines the hardness of the mixture. A solid fat content between 15 and 35% characterizes plastic fats that can be produced enzymatically or chemically. Enzymatic approaches present several advantages: (1) no modification of the chemical properties of the original fat by interesterification, (2) constant fatty acids unsaturation levels and, (3) no *cis-trans* isomerization *(3)*. Econa® oil or diacylglycerol (DAG) oil was produced enzymatically from natural oil and contains 80% or more DAG. DAG oil was introduced to the market by Novozymes and Kao and possesses virtually the same energy value as triacylglycerol oil but is not transformed into body neutral fat *(12)*. This oil represents the first industrial application to obtain a product using an immobilized lipase process. In Japan, the total sale of Econa oil and derived products reached annual sales exceeding $150 million in 2002 *(13)*.

Reduced Calorie Fats

Consumers are now more aware of the nutritional quality of food, the energy content of food, and the long-term effect of food on health. Although fats possess a high-calorie intake, their good taste and smoothness make them difficult to be circumvented. Reduced calorie and substituted fats are now available. Akoh and Yee *(14)* produced a low-calorie lipid by interesterification of tristearin (C18:0) with either tricaprin (C10:0) or tricaprylin (C8:0) using an immobilized lipase. Kanjilal et al. *(15)* performed an interesterification of sunflower oil with a lipase and incorporated behenic acid at the *sn*-1 and *sn*-3 positions. The resulting oil, Bohenin, produced by Fuji Oil, is a commercial triglyceride containing behenic acid at

the *sn*-1 and *sn*-3 positions with oleic acid at the *sn*-2 position and has 5.36 cal/g. Bohenin has a taste very close to sunflower oil *(3)*.

Cocoa Butter

Cocoa butter is a mixture of oil and fat composed of triglycerides possessing palmitic acid, stearic acid, and oleic acid as the major components. Cocoa butter is a fat with a high commercial value for the confectionery industry, in particular chocolate, owing to its useful properties such as gloss, snap, melting temperature, and bloom resistance. The high price of cocoa butter is the result of its low availability. Consequently, interesterification of abundant and less expensive fats, including illipe fat, shea butter, sal fat, and kokum butter, offers a good alternative for the production of cheaper cocoa butter substitutes. The introduction of palmitic or stearic acids at the *sn*-1 and *sn*-3 positions by a selective lipase produces cocoa butter substitutes with a cooling, melting sensation characteristic of chocolate and similar physical properties at a lower cost *(3)*. Newlase, an immobilized lipase from *Rhizopus niveus*, specifically incorporates stearic acid at the *sn*-1 and *sn*-3 positions of triglycerides in safflower oil or sunflower oil. Fuji Oil has exploited this process since 1993 to produce a cocoa butter substitute.

Acceleration of Cheese Ripening

Cheese ripening is composed of a complex sequence of events and is the result of many transformation processes such as proteolysis and lipolysis in milk by indigenous microflora. The attributes of texture, aroma, and visual appearance characterize the different types of cheeses. Cheese texture is related to the fat content, and aroma is generated by fat degradation leading to primary and secondary products. The right equilibrium between the specific primary and secondary products is reached with the help of numerous enzymes. In a regular cheese-making process, milk fat is hydrolyzed during lipolysis to liberate free fatty acids, which contribute directly to the aroma and also act as precursors for methyl ketones, secondary alcohols, and aliphatic and aromatic esters. The addition of exogenous lipase accelerates the ripening process. However, the addition of free lipases to the process can lead to excessive lipolysis resulting in texture and flavor defects, whereas lipase encapsulation regulates the enzyme/substrate ratio and overcomes this problem *(16)*. As potential industrial application, the use of an encapsulated enzyme cocktail containing a lipase (Palatase M from Novozymes) and a protease results in a full-flavored cheese without a bitter taste even after 90 d of ripening *(17)*.

Manufacture of Cheeselike Products

High-intensity cheeselike products find numerous applications in the food industry, where they can add cheese flavor to salad dressings, dips, soups, sauces, snacks, frozen foods, and so on. They include enzyme-modified cheeses (EMCs), cheese powders, and cheese flavors. The flavor intensity of EMCs can be increased up to 15–30 times that found in natural

cheese. EMCs do not increase the fat content in the intended application but provide rich mellow tones, a pleasant flavor-enhancing effect, a fatty feel in the mouth, reduced production costs, and enhanced product stability.

A wide variety of lipases are available and their contribution to the resulting EMC depends on the type or source of enzyme. Thus, knowledge of the metabolic pathways involved in flavor production and the specific activity of the enzyme are both essential for a reproducible cheese flavor. Porcine pancreatic lipase extracts contain trypsin and serine proteases that can cause bitterness, while microbial lipases preferably liberate fatty acids at the *sn*-1 and *sn*-3 positions on the glycerol backbone. Lipases can also be used individually or as a mixture *(18)*. Kraft Foods has patented a process to produce EMC flavoring using mammalian lipases *(19)*.

Detergent Industry

In the past, ground porcine or bovine pancreases, rich in lipases, were used in the fine chemical industry as detergent additives. Actually, the use of lipases in the detergent industry represents the main significant application of these enzymes. In 1995, detergent enzymes represented 30% of the total enzyme market, estimated at US $30 million *(20)*. In 2000, this market reached US $1.5 billion *(1)*. Novozymes, formerly Novo Nordisk, launched Lipolase® in 1988, the first commercial lipase developed for the detergent industry. Lipolase has an optimal pH of 10.5–11.0; is active over a broad range of temperatures, with an optimum at 40°C; is stable in proteolytic wash solutions; shows oxidation stability; and is stable toward several other detergent ingredients including surfactants. This enzyme is widely used in detergent formulations to remove fat-containing stains including fried fat, salad oils, butter, fat-based sauces, soups, human sebum, and lipstick. Lipolase also has a broad range of substrate specificity. This lipase is currently added to a significant number of major detergent brands throughout the world. Novozymes later introduced three variants of Lipolase: Lipolase® Ultra, LipoPrime™, and Lipex®.

Pharmaceutical Industry

In the pharmaceutical industry, biocatalysis offers numerous advantages over chemical synthesis, thereby justifying the growing demands for enzymes. These advantages include enantio- and regioselectivity; mild conditions that avoid isomerization, racemization, epimerization, and rearrangement reactions; overexpression of the enzymes; reuse of the immobilized biocatalysts; economy of the process; and mutagenesis of the enzymes for specific functions. The ability of lipases to resolve racemic mixtures by the synthesis of a single enantiomer is currently exploited for drug production by the pharmaceutical industry. In fact, only one enantiomer of a drug is responsible for the desired therapeutic effect, and milder or fewer side effects are observed when using optically pure drug products compared with those found with the use of racemic mixtures.

Some of the lipases listed in Table 1 are suitable to be used in the synthesis of various enantiopure molecules such as alcohols, amides, carboxylic acids, and esters. These molecules are used in antiinflammatory drugs (ibuprofen, naproxen) *(9)*, anticancer drugs (Taxol®, spergualin), an antiviral drug (lobucavir), an antihypertensive drug (captopril), anticholesterol drugs (squalene synthase inhibitor), an anti-Alzheimer disease drug ([*S*]-2-pentanol), and vitamin A *(21)*.

Resolution of Racemic Chemicals

The anticancer drug Taxol® (paclitaxel) is an antimitotic agent that inhibits the depolymerization process of microtubulin during mitosis. This drug is used in the treatment of ovarian cancer and metastatic breast cancer. Taxol® generates annually about US $1 billion in sales *(22)*. Paclitaxel was originally extracted and purified from the bark of the yew *Taxus brevifolia* in a very low yield. Paclitaxel was also be obtained by a semisynthetic process by coupling baccatin III (paclitaxel without the C-13 side chain) or 10-deacetylbaccatin II ([10-DAB], paclitaxel without the C-13 side chain and the C-10 acetate) to C-13 paclitaxel side chains. Baccatin III and 10-DAB can be extracted from renewable sources such as the extract of needles, shoots, and young *Taxus* cultivars, therefore eliminating the cutting of yew trees. The C-13 paclitaxel side chain was obtained from the enantioselective hydrolysis of racemic acetate-*cis*-3-(acetoxy)-4-phenyl-2-azetidione to the corresponding (3*S*)-alcohol and the intact desired (3*R*)-acetate. Hydrolysis was catalyzed by the lipase PS-30 from *Pseudomonas cepacia* or Bristol-Myers Squibb (BMS) lipase from *Pseudomonas* sp. SC 13856. Both lipases were immobilized on Accurel Polypropylene before use. An enantiomeric excess (ee) of >99.5% and reaction yields of >48% (maximum theoretical yield: 50%) were obtained for (3*R*)-acetate. The process was scaled up to 75 and 150 L using immobilized BMS lipase and Lipase PS-30, respectively. The (3*R*)-acetate was then converted chemically into (3*R*)-alcohol (C-13 paclitaxel side chain) *(23)*.

Resolution of Racemic Carboxylic Acids

Ibuprofen, a nonsteroidal antiinflammatory drug commercialized as Advil or Motrin with sales estimated to be about $290 million in the United States for Motrin alone *(24)*, represents another example of the applications of lipases. Ibuprofen, 2-(4-isobutylphenyl) propionic acid, inhibits the binding of arachidonic acid and prevents the synthesis of prostaglandins acting on the inflammatory response. Ibuprofen is a racemic mixture containing two enantiomers. The (*S*)-ibuprofen molecule is 160 times more potent in inhibiting the prostaglandin synthesis than the (*R*)-ibuprofen one *(25)*. As a potential application, resolution of racemic ibuprofen by esterification of (*S*)-ibuprofen with methanol or butanol in organic media using a specific lipase leads to synthesis of the corresponding (*S*)-ester. This ester is then completely separated from the (*R*)-ibuprofen and chemically transformed to (*S*)-ibuprofen *(9)*.

Resolution of Racemic Ester

With an annual market of about US $85 million in 1996 and 22.5 billion yen in 2002, diltiazem, a calcium channel blocker, constitutes another economical, important industrial lipase application. Tanabe manufactures 50 t of diltiazem annually. Resolution of racemic epoxyesters represents a key step in the production of an important intermediate essential for the synthesis of diltiazem. This enantiospecific hydrolysis is catalyzed by a lipase from *Serratia marcescens*. The product of the reaction, 2(*R*),3(*S*)-methyl-*p*-methoxyphenylglycidate, is found in an ee of >98% and is later converted into diltiazem *(6)*. Sepracor has successfully operated a multiphase membrane bioreactor at a multikilogram scale to produce the key diltiazem intermediate 2(*S*),3(*R*)-methoxyphenyl glycidate *(26)*.

Molecular Technologies

Most of the numerous lipases currently on the market exist in native form and are unavailable in large quantities. Consequently, prices are very high, which limits their potential industrial applications.

Recombinant DNA Technology

Recombinant DNA technology represents a very attractive feature that can be used to overcome the cost limitation of the industrial application of lipases. Briefly, a suitable lipase is selected for a specific application and cloned into an expression system to produce and purify this enzyme in large quantities. Recombinant DNA technology can therefore result in a 40% decrease in the cost of raw material, water, steam, and electricity compared with that for the production of native enzymes *(27)*. The production of recombinant lipase has recently been reviewed *(28,29)*. Many lipase genes, mainly from microbial origin, have been cloned and the sequences are available on several databases. However, recombinant enzyme production has been limited to a few microbial lipases. The first lipase produced by recombinant DNA technology was Lipolase, introduced into the market by Novozymes in 1988. This lipase, originating from *Thermomyces lanuginosus*, formerly *Humicola lanuginosa*, was expressed in *Aspergillus oryzae*. Palatase® was another commercial lipase, from *Rhizomucor miehei*, expressed in *A. oryzae*. Since then, 10 other recombinant lipases have been produced and commercialized (Table 1).

Molecular Pharming® technology can be used to produce active proteins that are economically more competitive and exhibit optimal biologic safety. Meristem®Therapeutic, in collaboration with Solvay Pharmaceuticals, used Molecular Pharming technology for the production of mammalian proteins in plants to manufacture new drugs. They have produced a recombinant mammalian lipase expressed in corn seeds and currently in phase IIa clinical trials in Germany and France. This product is intended for patients with exocrine pancreatitis deficiency in which the digestive

enzymes are unable to reach the digestive system. Consequently, food lipids cannot be digested and are found in excess in the feces *(30)*.

Protein Engineering

In industrial processes, biocatalysts must often exhibit properties that are different from those found in their physiologic environment mainly in terms of enantioselectivity, thermostability, and stability in organic media. In most cases, the enantioselectivity of a selected enzyme is too low for a desired reaction, which, in turn, contributes to the limitations of lipase applications. It is, therefore, a big challenge to isolate or develop the most suitable biocatalyst and to determine the best reaction conditions for efficient applications *(31)*.

The efficiency of some processes can be improved by optimization of the reaction medium or by using some physicochemical techniques such as immobilization, microencapsulation, or modification of amino acid residues of the enzyme *(32)*. However, in some cases, these optimizations are not sufficient to conduct the enzymatic process at the industrial scale because the catalytic properties of the enzyme must be improved. The traditional way to identify new biocatalysts consists of screening strain collections or soil samples by enrichment cultures. However, this technique is not always successful. The selection of suitable biocatalysts from wild-type enzymes by protein engineering technologies is an attractive approach to modify the properties of existing biocatalysts or to develop new enzymes *(33)*.

Rational Protein Design

Rational protein design is a protein-engineering technology that requires knowledge of both the three-dimensional structures of the enzymes and the relationships among sequence, structure, and mechanism/function. The human pancreatic lipase and the lipase from *R. miehei* were the first lipases whose X-ray structures were elucidated *(34,35)*. Since then, several microbial lipase structures have been reported and are available in the Lipase Engineering database *(36)*. Rational protein design starts with the development of a molecular model based on available knowledge of the protein structure and function of the desired enzyme *(37)*. This molecular model is built to predict, by planning mutations, how the activity, stability, or stereoselectivity of the enzyme can be improved. Mutants are then generated by site-directed mutagenesis, and vectors containing the mutant genes are transformed into the host organism. After expression, the recombinant variants are purified and analyzed for the desired properties *(33)*.

Based on a molecular modeling prediction, the Ser^{47} residue in the sequence of lipase B of *Candida antarctica* was changed to an Ala residue. This modification resulted in a significant increase in enantioselectivity from 14 to 28 toward the resolution of 1-chloro-2-octanol *(38)*. For the same

lipase, substitution of the Thr residue near the active site, responsible for transition-state stabilization, to a Val residue resulted in a loss in lipase activity. However, this activity was restored by using 2-hydroxypropanoate as substrate and the enantioselectivity (E) was improved from $E = 1.6$ to 22 *(39)*.

Novozymes launched two variants of Lipolase issued from rational protein design: Lipolase Ultra and LipoPrime. Lipolase Ultra is more effective than Lipolase at low wash temperatures, and LipoPrime produces, after one wash cycle, the same desirable results as those obtained with Lipolase after three washes in certain types of detergent. These variants were also expressed in *A. oryzae*.

Directed Evolution

Directed evolution represents an alternative to rational protein design when the structure and mechanism of the enzyme are not available. This technology was also known as molecular evolution, sexual polymerase chain reaction (PCR), and in vitro evolution. Directed evolution uses random mutagenesis and/or recombination of a target gene or a set of related genes to create molecular diversity. The variants generated must be expressed in a suitable overexpression system for screening and/or selection. The choice of the screening/selection method is crucial for isolating the best variant in a large library of potential candidates.

Libraries of variants can be generated by various methods. Site-specific saturation mutagenesis, error-prone PCR *(40)*, and cassette mutagenesis are nonrecombinative methods, whereas DNA shuffling *(41)*, heteroduplex recombination *(42)*, incremental truncation for the creation of hybrid enzymes *(43)*, random-priming recombination *(44)*, and the staggered extension process *(45)* are DNA recombination strategies. Jaeger et al. *(46)* described and discussed all these methods.

The evolution procedure is repeated several times until the desired catalytic properties are reached. Improved variants isolated from an evolution round are used as starting material for subsequent rounds. Selection of variants can be achieved by a growth-related assay, and screening can be performed using different high-throughput technologies such as the spectrophotometric assay, infrared-thermography, electrospray ionization mass spectrometry, and capillary array electrophoresis on chiral columns, depending on the selected property of the enzyme. Some applications of directed evolution technology to microbial lipases are given in Table 2.

Recently, Novozymes launched Lipex, a third variant of Lipolase. Lipex, obtained by directed evolution technology, can remove fatty stains in the first wash at temperatures as low as 20°C and acts on fat stains entrapped in cloth fibers. With its improved binding properties, Lipex breaks down fat into glycerol and fatty acids, which are subsequently removed with the wash solution.

Table 2
Microbial Lipases Improved by Directed Evolution Technologies

Microorganism	Desired property	Mutagenesis method	References
Pseudomonas aeruginosa PA01	Increase in enantioselectivity toward 2-methyldecanoate acid *p*-nitrophenylester	Error-prone PCR	*6*
Bacillus thermocatenulatus BTL2	Conversion of lipase to phospholipase activity	Random mutagenesis	*47*
Staphylococcus hyicus	Improvement in phospholipase activity	Error-prone PCR + DNA shuffling	*48*
Thermomyces lanuginosus	Improvement in properties in presence of detergents	Combinatorial protein engineering/ phage display approach	*49*
Bacillus subtilis	Enantioselectivity toward *meso*-1,4-diacetoxy-2-cyclopentene	Various mutagenesis methods	*50*

Conclusion

Although lipases have several interesting potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries, their industrial uses still remain limited by their high production costs, commercialization in small amounts, and low performance of some lipase-mediated processes. Recombinant DNA and protein engineering (rational protein design and directed evolution technologies) have already successfully been applied to produce some commercial lipases and represent very attractive features to overcome the main limitations of lipases in industrial applications. An increased availability of these enzymes is thus expected and should significantly contribute to an important expansion of this field in the near future.

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