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Review

Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches

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Abstract

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol. Besides this, they are also efficient in various reactions such as esterification, transesterification and aminolysis in organic solvents. Therefore, those enzymes are nowadays extensively studied for their potential industrial applications. Examples in the literature are numerous concerning their use in different fields such as resolution of racemic mixtures, synthesis of new surfactants and pharmaceuticals, oils and fats bioconversion and detergency applications. However, the drawbacks of the extensive use of lipases (and biocatalysts in general) compared to classical chemical catalysts can be found in the relatively low stability of enzyme in their native state as well as their prohibitive cost. Consequently, there is a great interest in methods trying to develop competitive biocatalysts for industrial applications by improvement of their catalytic properties such as activity, stability (pH or temperature range) or recycling capacity. Such improvement can be carried out by chemical, physical or genetical modifications of the native enzyme. The present review will survey the different procedures that have been developed to enhance the properties of lipases. It will first focus on the physical modifications of the biocatalysts by adsorption on a carrier material, entrapment or microencapsulation. Chemical modifications and methods such as modification of amino acids residues, covalent coupling to a water-insoluble material, or formation of cross-linked lipase matrix, will also be reviewed. Finally, new and promising methods of lipases modifications by genetic engineering will be discussed.

Keywords: Lipases; Biocatalysis; Genetic engineering

1. Introduction

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. These enzymes are distributed among higher animals, microorganisms and plants in which they fulfil a key role in the biological turnover of lipids. They are required as digestive enzymes to facilitate not only the transfer of lipid from one organism to another, but also the deposition and the mobilization of fat that is used as an energy

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reservoir within the organism. They are also involved in the metabolism of intracellular lipids, and, therefore, in the functioning of biological membranes. Lipases have been extensively investigated with respect to their biochemical and physiological properties, and lately for their industrial applications.

The increasing interest in lipase research over the past decades has likely occurred for three reasons [1,2]. The first is related to the molecular basis of the enzyme catalytic function or the lipase paradigm. Indeed, lipases, though water soluble, catalyze reactions involving insoluble lipid substrates at the lipid–water interface. This capability is due to the unique structural characteristic of lipases. These latter indeed contain a helical oligopeptide unit that covers the entrance to the active site. This so-called lid only moves upon access to a hydrophobic interface such as a lipid droplet. Thus, it is not surprising that lipases as well as phospholipases have, for many years, served as models for studying the regulation of interfacial, enzyme-catalyzed reactions [3]. The second reason is linked to the enzyme's medical relevance, particularly to atherosclerosis and hyperlipidemia [4], and its importance in regulation and metabolism, since products of lipolysis such as free fatty acids and diacylglycerols play many critical roles, especially as mediators in cell activation and signal transduction [5]. Lastly, it was discovered that lipases are powerful tools for catalyzing not only hydrolysis, but also various reverse reactions, such as esterification, transesterification, and aminolysis, in organic solvents. Such biocatalysts present some important advantages over classical catalysts. Indeed, their specificity, regioselectivity and enantioselectivity allow them to catalyze reactions with reduced side products, lowered waste treatment costs and under conditions of mild temperature and pressure. Accordingly, considerable attention has been given lately to the commercial use of lipases.

Because of their capability to preserve their catalytic activity in organic solvents [6], the activities of lipases as catalysts have been investigated to determine their potential for the conversion of surplus fats and oils into higher value products for food and industrial uses. Further examples of their applications are numerous and are found in the resolution of racemic mixtures, the synthesis of pharmaceuticals and new surfactants, the bioconversion of oils, fats, etc. However, the low stability, low activity or selectivity encountered occasionally with a number of these enzymes, and the relatively prohibitive cost of native enzyme have been the chief obstacle hindering more rapid expansion of industrial lipase technology on a large scale. Therefore, customization of lipases by chemical and physical modifications has more recently been attempted to improve their catalytic properties in hydrolysis and synthesis involving aqueous and non-aqueous solvents. In addition, the cost of enzyme can be reduced by the application of molecular biological tools, such as recombinant DNA technology and protein engineering, which may allow the production of lipases in large quantities and with genetically enhanced properties.

The present review, which mainly covers the literature until 1997, will survey the procedures of chemical, physical and genetical modifications of lipases to enhance their catalytic activities. Each of these procedures involves a different degree of complexity and efficiency. Chemical methods feature the formation of covalent bonds between the lipase and the modifier, while physical methods are characterized by weaker interactions of the enzyme with the support material, or mechanical containment of the lipase within the support. Additionally, genetic engineering of lipase involves modification of the gene encoding the enzyme. Features of this technology include the ability to isolate and express genes of interest and the ability to change the amino acid occupying a single, or multiple, sites in a protein. This technology also allows the insertion or deletion of single or multiple amino acids, and the fusion of segments from different genes and different organ-

isms. The selection of an immobilization strategy or a modification procedure is based on process specifications for the biocatalyst, which include such parameters as overall enzymatic activity, effectiveness of lipase utilization, deactivation and regeneration characteristics, cost of the immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized lipase. Obviously, a broad subject such as this cannot be covered completely in this review. However, the sections that follow will explore each of these concepts in greater detail and will comment on relevant examples dealing with these modifications.

2. Lipases as applied catalysts

The biological function of lipases is to catalyze the hydrolysis of esters, especially long-chain triacylglycerols, to yield free fatty acids, di- and mono-acylglycerols, and glycerol. Lipases are also capable of catalyzing the reverse reaction, achieving esterification, transesterification (acidolysis, interesterification, alcoholysis), aminolysis, oximolysis and thiotransesterification in anhydrous organic solvents [6,7], biphasic systems [8] and in micellar solution [9,10] with chiral specificity. The equilibrium between the forward (hydrolysis) and the reverse (synthesis) reactions is controlled by the water activity of the reaction mixture. The applications of lipases are of course numerous and have already been extensively reviewed (for a recent review see, Ref. [11]). Most industrial applications are found in racemic mixture resolutions, textile detergency, pharmaceuticals synthesis, or in oils and fats bioconversion. Basically, the following four operations can be considered.

2.1. Hydrolysis

Total hydrolysis of ester bonds in triacylglycerols may be accomplished at high temperatures and pressure in the presence of steam. Fatty acids can alternatively be produced by ambient pressure saponification or chemically catalyzed hydrolysis. However, the use of lipases for enzymatic splitting of fats in the presence of excess water (Fig. 1) is more appealing since the reaction proceeds under mild conditions of pressure and temperature with specificity and reduced waste [12]. This technology is currently employed in the production of fatty acids, diglycerides, monoglycerides, flavoring agents for dairy products and detergents for laundry and household uses (Table 1).

2.2. Esterification

Esterification reactions between polyhydric alcohols and free fatty acids are catalyzed by lipases in water-poor organic solvents under conditions of low water activity or even solvent free systems (Fig. 1). Although ester synthesis can be done chemically with acid or base catal-

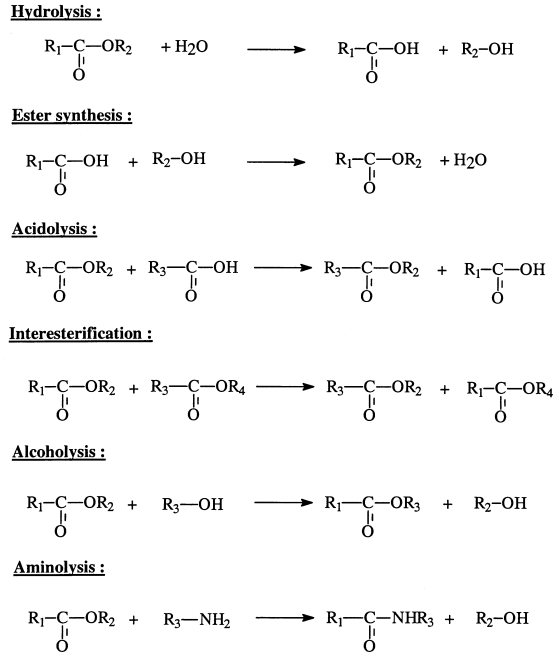

Fig. 1. Different reactions catalyzed by lipase in aqueous and non-aqueous solutions.

Table 1
Examples of industrial applications of lipases

Field of industry	Application	Product
Hydrolysis		
Food (dairy)	Hydrolysis of milk fat	Flavoring agents for dairy products
Chemical (oil processing)	Hydrolysis of oils and fats	Fatty acids, diglycerides, and monoglycerides
		Reagents for lipid analysis
	Analysis of fatty acid distribution in triglycerides	
Chemical (detergent)	Removal of oil stains, spots and lipids	Detergents for laundry and household uses
Medical	Blood triglyceride assay	Diagnostic kits
Esterification		
Chemical (fine chemical)	Synthesis of esters	Chiral intermediates
		Esters, emulsifiers
Food (chemical and pharmaceutical)	Transesterification of natural oils	Oils or fats (e.g., cocoa butter equivalent)

ysis, the use of enzyme technology offers the advantages of mild conditions, reduced side reactions, and specificity. Examples of high-value chemicals obtained by lipase-catalyzed ester synthesis (Table 1) include the production of oleic acid esters of primary and secondary aliphatic and terpenic alcohols [13], and the production of geranyl and menthyl esters from butyric acid and geranol, or lauric acid and menthol, respectively [14].

2.3. Transesterification

The term transesterification refers to the process of exchanging acyl radicals between an ester and an acid (acidolysis), an ester and another ester (interesterification), or an ester and an alcohol (alcoholysis) (Fig. 1). Transesterification is accomplished industrially by heating a mixture of the anhydrous ester and another reactant species at relatively high temperatures. Alternatively, alkali metals or alkali alkylates may be used at lower temperatures. However, the application of lipases for the modification of fats and oils by transesterification offers again the advantages of mild conditions, reduced side reactions, and specificity. One example is the production of cocoa butter analogues from cheaper feedstocks. This reaction is accomplished via lipase-catalyzed transesterification reactions involving palm oil mid fraction and stearic acid [15], or palm oil mid fraction and tristearoylglycerol [16–18].

2.4. Catalysis on ‘‘unnatural’’ substrates

Lipases are not limited to catalysis of the synthesis and hydrolysis of carboxylic acid esters. They can utilize compounds other than water and alcohols as nucleophiles. Lipases are thus capable of catalyzing different reactions such as aminolysis, thiotransesterification, and oximolysis in organic solvents with selectivity [6]. The selectivity of lipase in the aminolysis of esters in anhydrous media has been successfully used for peptide and fatty amide syntheses [19]. These results hold promise for using lipase technology in the synthesis of optically active peptides, polymers, surfactants and new detergents at low cost (Table 1).

3. Modifications of lipases

In order to use lipases more economically and efficiently in aqueous and non-aqueous solvents, techniques for their modification have been the subject of increased interest. These techniques can conveniently be classified into three main categories: chemical modification, physical modification, and genetic engineering. Modification is performed to increase their ac-

tivity, selectivity or stability and to improve their solubility in organic solvents.

3.1. Physical modification

The physical modification that is currently most applied to lipases is ''immobilization''. This process involves attaching the enzyme onto an insoluble solid support. The use of such a modified lipase is very advantageous since, besides a generally improved operational and temperature stability, it can be easily recovered at the end of the reaction and continuously recycled [20]. Methods to immobilize enzymes are numerous and varied and can be classified in three groups: (i) adsorption on a carrier material; (ii) inclusion or encapsulation; and (iii) covalent attachment to an activated matrix (discussed in Section 3.2).

3.1.1. Physical adsorption on a carrier material

The procedures involved in physical adsorption are quite simple, making it one of the most widely used methods of enzyme immobilization. The enzyme is immobilized onto a solid support by low energy binding forces, e.g., Van der Waals interactions, hydrophobic interactions, hydrogen bonds, ionic bonds... Many carrier material exist, the choice of one often depending on properties that are important for potential industrial applications: mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme load capacity and cost. Initially, mineral supports such as porous glass beads [14,21], diatomaceous earth [15], silica [22,23] and alumina [24,25] were used. More recently, the most used supports are ion exchange resins, celite [26–28] and biopolymers [29–31].

The success and efficiency of the physical adsorption of the enzyme on a solid support is dependent of several parameters. The size of the protein to be adsorbed, the specific area of the carrier and the nature of its surface (porosity, pore size) are crucial. Typically, the use of a porous support is advantageous since the enzyme will be adsorbed at the outer surface of the material and within the pores as well. An efficient immobilization is also dependent on the enzyme concentration. The amount of adsorbed enzyme per amount of support increases with the enzyme concentration reaching a plateau at the saturation of the carrier. This operation is usually carried out at constant temperature, and, consequently, adsorption isotherms are obtained which follow Langmuir or Freundlich equations [32]. The pH at which the adsorption is conducted is equally important since ionic interactions are crucial in such an immobilization. Usually, the maximum adsorption is observed for pHs close to the isoelectric point of the enzyme. Finally, addition of water miscible solvents during the immobilization process favors the adsorption by reducing the solubility of the enzyme in the aqueous phase.

Regeneration of the immobilized biocatalyst is often possible. Once the enzyme has lost a significant amount of its initial activity, its desorption is sometimes possible by modification of the pH [33], followed by binding of new active enzyme. However, ready desorption would also be a major drawback of this immobilization technique if it occurs during the catalyzed reaction. Unfortunately, there are no empirical rules to predetermine the strength of adsorption. In some cases, the simple addition of the substrate will be sufficient to induce a significant desorption, whereas, in other cases, no desorption will be observed under drastic conditions.

There is a great number of publications dealing with the applications of lipases immobilized by physical adsorption. Most of them were excellently reviewed by Balcao et al. [34]. Therefore, we will try to discuss herein some of the recent and relevant publications dealing with the properties and uses of such enzymes.

The main concern with enzyme immobilization is to obtain a biocatalyst with a stability and activity that have not been affected during the immobilization process in comparison with the enzyme in its free form. Ideally, the immo-

bilized enzyme will even exhibit improved catalytic performances. There is no rule to predict the obtained activity and stability of an enzyme upon immobilization. Immobilization may inhibit or activate enzyme activity [34]. Most immobilized lipases exhibit higher optimum temperature values than their free counterparts. This observation is attributed to the fact that immobilized enzymes are less sensitive to thermal deactivation since their structure is more rigid after the immobilization. For example, Montero et al. [35] compared the thermal stability of *Candida rugosa* lipase when immobilized on commercially available microporous polypropylene with the free enzyme in solution. Above 40°C, both forms were less active but the immobilized form retained a larger part of its initial activity. Moreover, the soluble lipase showed its maximal activity at 37°C, while that of the adsorbed lipase was at around 45°C. Concerning the pH range of immobilized lipases, it is generally observed that the optimum pH is generally slightly shifted toward more alkaline values.

Interestingly, the immobilization of a lipase can sometimes preserve it from deactivation by chemicals produced during the enzyme-catalyzed reaction. The deactivation effect of acetaldehyde toward some lipases is a well-known example. This co-product is formed when vinyl esters are used as irreversible acyl donors in (trans)esterification reactions. The mechanism of deactivation by acetaldehyde is attributed to a Schiff-base formation in a Maillard-type reaction with lysine residues. The sensitivity of several microbial lipases was recently tested and a certain correlation was found with the relative lysine content of a lipase and its sensitivity toward acetaldehyde [36]. Nevertheless, the authors showed that this was true only to some extent and believe that the position of lysine residues (for instance, near the active site) is important as well. However, it was found that for *C. rugosa*, lipase deactivation by such an aldehyde can be overcome by immobilization of the enzyme [37].

The nature of the support is obviously very important for the efficiency of the immobilization. Work has been recently undertaken by Bosley [38] to establish the ideal physical and surface chemistry requirements of support material for the physical adsorption of lipases. The author observed that large pores (>100 nm) are required for the efficiency (activity/loading) to become independent of pore diameter. Below this, the enzyme activity was highly dependent on pore diameter, presumably, as a result of limitations in the rate of diffusion of substrates. The surface chemistry was also found to be important since supports with long alkyl groups lead to higher activities than surfaces containing high degrees of unsaturation. The effect of the enzyme loading was also studied using lipase immobilized on Accurel EP100. A ''bell-shaped'' curve was obtained with a maximum efficiency at a loading of approximately 100,000 lipase units/g (10% of its saturation value). The author explained this phenomenon by splitting the curve into two regions. Below the maximum, it is suggested that at low loadings, the lipase molecule attempts to maximize its contact with the excess surface area available and therefore suffers important conformational changes leading to a loss of activity. Above the maximum, the efficiency drops as a result of diffusion limitation. Interestingly, the author mentions that the reduction of activity can be overcome by the addition of an inert protein, e.g., ovalbumin, which may act by blocking all the remaining adsorption sites, therefore preventing the lipase from spreading on the support surface.

Generally, hydrophobic materials are better supports for enzyme immobilization. The amount of enzyme adsorbed on such supports are usually larger and higher enzyme activities are obtained. Hydrophilic supports tend to compete with the enzyme for the available water in the reaction. When the lipase and the support are fully hydrated, the hydrophilic supports lead to a higher water concentration in the environment of the enzyme favoring hydrolytic reac-

tions. A few examples of lipases immobilized on hydrophilic supports can be found in the literature [14,39,40].

The effect of the internal structure of the support was recently studied by Al-Duri et al. [41] with *Candida cylindracea* lipase. The surface area, porosity and particle size distribution were compared in six hydrophobic polymers. Although the support with the largest surface area gave the most active immobilized enzyme, the results with the five other supports showed that surface area, though important, is not the prime factor that controls enzyme activity. Parameters, such as particle size distribution and chemical composition, are other factors to be considered. For example, the authors showed that the best support was the one presenting the freest amino groups at its surface, groups that are known to have high affinity for lipases. Moreover, on a support particle, which can be divided in two areas, namely macropore region (pore size > 100 nm) and micropore region (pore size < 100 nm), it was demonstrated that the localization of the surface area is a crucial factor. Indeed, if much of this area is located in the micropore region, the accessibility of the support for adsorption is considerably reduced. However, this disadvantage can be overcome by carrying out the immobilization process over times longer than 24 h to allow the lipase to move into the support pores. Shaw et al. [42] evaluated the lipase-coupling efficiency of six different types of materials, namely: PVC, chitosan, chitin, agarose, Sepharose and Trisacryl. The authors showed that the amount of immobilized lipase was affected by the length of the hydrocarbon chain attached to the matrix. It appeared that a spacer with chain length longer than six carbons favors the immobilization of enzyme.

Numerous publications have appeared describing the investigation of new supports for lipase immobilization. For example, Ruckenstein and Wang [43] studied the immobilization by adsorption of *C. cylindracea* lipase on microporous poly(styrene–divinylbenzene) supports. The porous carrier material was in fact prepared by co-polymerisation of styrene and cross-linkage with divinylbenzene. The effect of the ratio of divinylbenzene/styrene, was studied. This parameter determines the cross-linking, and, consequently, the diffusion of the substrate and products through the biocatalyst. As expected, an increase of this ratio leads to higher specific surface area and a larger number of small pores, but in this case, the lipase adsorption is weak since small pores are inaccessible to the enzyme. Recently, the ''interfacial adsorption'' of different bacterial lipases on strongly hydrophobic supports, namely, octyl-agarose gels, was tested [44,45]. The immobilization process used by the authors allows the preparation derivatives containing very large amount of lipase. It was observed, contrary to typical results for the adsorption of proteins, that maximum immobilization was obtained with low ionic strength. Moreover, there was selectivity of immobilization with all lipase activity, regardless of source, being adsorbed very rapidly, whereas other proteins contained in the extracts remained in the supernatant. Interestingly, the obtained immobilized lipases showed an improvement of enantioselectivity and a dramatic hyperactivation. For example, lipases from *Mucor miehei* and *Humicola lanuginosa* were, respectively, 7- and 20-fold more active than the corresponding free enzyme in the hydrolysis of *p*-nitrophenyl esters. The authors suggest that lipases recognize these strongly hydrophobic supports as solid interfaces, and they become adsorbed through the external area of the large hydrophobic activity centers of their open and hyperactivated structure. On the other hand, they explain the increase of enantioselectivity by the fact that the connection between open lipase and the support is very intense and leads therefore to additional hindrances of some enantiomers.

Recently, rice hull ash was used as a support material for the immobilization of *C. cylindracea* lipase [46]. This virtually cost-free material showed interesting properties. Although re-

tained activity following immobilization was not good (30%), the thermal stability of the enzyme was significantly improved.

Some publications describe the use of additives or solvents during the immobilization procedure to improve its efficiency. For example, Kogusi et al. [47] pretreated the carrier material with a polar solvent to immobilize a lipase from *Pseudomonas fluorescens*. The support used was a weak anion exchange resin, Dowex 66. When the immobilization was directly carried out without pretreatment of the support the adsorption was very poor. On the contrary, addition of 50% of a polar solvent, such as ethanol or isopropanol, led to an increase of the immobilization ratio to 96–97%. Montero et al. [35] also showed that pretreatment of supports with solvents strongly affected both the adsorption of the soluble protein and the activity of the resulting immobilized lipase. It is believed that polar organic solvents ''absorb'' water molecules from the support therefore favoring the adsorption of the proteins. The most commonly used additive to improve immobilization efficiency is probably glutaraldehyde, which is said to stabilize the interaction of the enzyme with the support. An interesting example of the effect of additions of various compounds on the stabilization of enzymes during immobilization on Celite was recently given [48]. A large number of different additives was tested. Proteins such as albumin gelatin, and casein, as well as polyethylene glycol (PEG), were effective additives, whereas, amino acids, carbohydrates and polysaccharides were ineffective. It was clearly demonstrated that stabilization occurred during the immobilization step. The amount of additive required was dependent on the additive used. For example, more tryptone (protein hydrolysate) was needed for the enzyme to retain full activity compared to albumin or gelatin, but usually, the variations were small. It was also found that the molecular size of the additive was a factor influencing the stabilization. Ruckenstein and Wang [49] have studied the effect of the addition of a surfactant during the immobilization of *C. cylindracea* lipase [49]. In this work, the porous material, styrene co-polymerized and cross-linked with divinylbenzene was obtained by the concentrated emulsion polymerization method [50] in the presence of Span 80. The authors showed that the specific area of the support and, consequently, the amount of adsorbed lipase, increased with increasing surfactant concentration.

Bosley and Peilow [51] studied recently the effect of enzyme loading during the immobilization of several lipases on porous propylene. An apparent suppression of esterification efficiency at low lipase loading was observed for *M. miehei* and *Humicola* sp. lipases. This result was explained by the fact that at low loading, only a small portion of the surface area was occupied, which gave the lipase the opportunity to spread. The authors hypothesized that the reduction in efficiency at low loading was due to a distortion of the active molecular conformation caused by the lipase maximizing its contact with the support as a result of its high affinity for the support surface. The relationship between affinity and loading was different for each of the lipases studied.

Lipase immobilization by physical adsorption finds applications in oils and fats bioconversion. Their unique specificities [52] allow the design of synthetic routes that predetermine product structure and distribution whereas chemical catalysts generally lead to random reaction product mixtures. Thus, the use of lipases makes it feasible to obtain new products with predetermined physical and chemical properties. Dozens of publications dealing with immobilized lipase-catalyzed structured triglyceride synthesis appear annually. It is not the purpose of this article to review them. One should cite, however, an excellent study concerning the different parameters that may influence acyl migration, an intramolecular phenomenon that must be avoided during lipase-catalyzed process [53]. Among them, the nature of the enzyme support was found to be of paramount importance. Different support materials were tested and the

authors showed that supports with surface charges (e.g., Duolite 568 or silica gel 60) could catalyze the acyl migration. On the contrary, neutral supports such as EP100 (polypropylene) or acid-washed Celite had no such effect. It was believed that the charged groups on the surfaces may induce acid or base catalyzed acyl migration.

Finally, one interesting case is that of some plant lipases that may offer, as crude catalytic powders, an alternative to the immobilization of their microbial counterparts. A relevant example is *Carica papaya* latex, which, besides its well-known proteolytic (papain) activity, also exhibits a lipase activity. It was demonstrated [54] that in this plant extract, the lipase can be considered a ''self-immobilized'' enzymes since it is naturally linked to polysaccharides. The lipase specificity of *C. papaya* latex was studied [55,56] and this very low cost plant material was used in the enzyme-catalyzed synthesis of structured triglycerides [57].

3.1.2. Entrapment and microencapsulation

The other physical immobilization of a lipase is its inclusion in an insoluble polymer or in a micro-capsule. The lipase is brought into solution in a monomeric phase, which, upon polymerization, leads to its entrapment. Polyacrylamide gels are the most commonly used matrixes. Gels formed upon contact of sodium alginates or careegenans (both are polysaccharides found in marine algae) with, respectively, calcium or potassium chloride, are also widely used [58]. Urethane prepolymers and photo-cross-linkable resins are also used and, in this case, the immobilization occurs with the introduction of a photosensitizer in the prepolymer and initiation of the polymerization reaction by UV radiation.

Microencapsulation, which is probably the less developed immobilization technique, is very similar to entrapment, although in this case, it is the enzyme and its whole environment that are immobilized. Microencapsulation creates artificial cells delimited by a membrane. Large molecules such as enzymes are not able to diffuse across the synthetic membrane whereas small molecules, e.g., substrates and products, can pass through it.

The advantage of such an immobilization technique is that the enzyme does not chemically interact with the polymer; therefore, denaturation is usually avoided. However, mass transfer phenomena around the membrane are problematic. The diffusion rate across the membrane of the substrate and the product is often the limiting parameter. Generally, high substrate concentrations are necessary in order to limit its influence. Finally, entrapped enzymes are better used with small substrates since larger ones may not be able to pass the membrane and reach the active site of the biocatalyst.

A number of very relevant publications dealing with entrapment of lipases have recently appeared. For example, Jenta et al. [59] immobilized the lipase from *Chromobacterium viscosum* in gelatin-containing Aerosol-OT (dioctyl sodium sulphosuccinte AOT) microemulsion-based organogels (MBGs). Reetz et al. [60] described the immobilization of several lipases by entrapment in chemically inert hydrophobic silica gels, which are prepared by hydrolysis of alkyl-substituted silanes in the presence of the enzyme. A significant influence of the nature of the silane precursor on the activity of the entrapped lipase was observed. Lipase activity in gels from a mixture of tetramethoxysilane and alkyltrimethoxysilanes was dramatically enhanced with changes in the alkyl chain length of the silanes. The effect of additives was also investigated. The lipase activity was enhanced in the presence of additive the best result being obtained with macromolecular additives such as PEG, polyhydroxy compounds or with proteins like albumin or gelatin. On the other hand, the addition of low molecular weight additives such as sorbitol, glycerol or triacylglycerols had nearly no effect on lipases activities. The authors proposed that the resulting increase of activity when using macromolecular additives

could be due to a protection of the enzyme from aggregation or from denaturing effects due to the silanes employed during the formation of the matrix. Several lipases from different sources were immobilized with this method. In every case, the activity of the sol–gel immobilized lipase was better than its free equivalent giving esterification activities enhanced by a factor of up to 88 compared to the commercial enzyme powders under identical conditions. Finally, the authors showed that the stability of such immobilized lipases was excellent with retaining more than 85% of their initial activities after 30 successive cycles.

Recently, a team from Novo-Nordisk developed a new and simple method to immobilize lipases via granulation [61]. Granulation is a well-established method for making larger particles by agglomeration of finer ones. In the case of detergent enzymes, the granulates are typically built by agglomerating different salts and carbohydrates polymers in the presence of the enzyme concentrate. Detergent enzyme granulates are not active as such but the enzyme is liberated upon dissolution of the particles. The authors have investigated whether granulation can be used to produce granulates of lipases having activity in organic media in which the granulates are stable and do not permit the release of the enzyme in the solution. Immobilization of lipase B from *Candida antarctica* using silica-base granulation was studied and compared to the same commercially available lipase immobilized on poly-acrylic resin, namely Novozym 435. A number of different hydrophilic or hydrophobic silicas were tested and many gave granulated enzymes with activities comparable to Novozym 435, the best results being obtained with Syloid 244 FP. In order to investigate whether a combination of different types of silicas could produce a granulate of even higher activity to Clarcel CBL-3 (Ceca) was added 7% of either Microtalcum (DEC) or hydrophobic Aerosil 974 (Degussa). Results showed that both additives improved the esterification activity. Addition of the hydrophobic silica was particularly effective as the activity was increased by 46.

3.2. Chemical modifications

Chemical modification of lipases using various types of modifiers, which interact with specific amino acids, has two major objectives. The first is to elucidate protein structure such as the active sites [62–65] and the second is to alter and improve the native properties of lipases, and to endow them with useful new functions. The procedure involves either chemical modification of amino acid residues, covalently coupling the enzyme to a water insoluble materials, lipase attachment to a matrix by covalent bonds, or formation of a cross-linked lipase matrix using various bi-functional reagents.

3.2.1. Modification of amino acid residues

Reductive alkylation of the amino group via the Schiff base formed by reaction of the amino group with an aldehyde or ketone was first described by Means and Feeney [66]. This procedure was recently used by Kaimal and Saroja [67] to enhance the catalytic activity of porcine pancreatic lipase. Chemical modification of the enzyme with *n*-butyraldehyde, isobutyraldehyde and acetone resulted in about 50% increase in the V_{max} value. Hydrolytic and interesterification activities were both enhanced without affecting the 1,3-specificity of the lipase (Table 2).

Likewise, the esterification activity of *C. cylindracea* lipase was markedly enhanced by modification of lysine residues with pyridoxal 5′-phosphate or by chemical reduction of disulfide bonds with dithiothreitol without loss of the hydrolytic activity (Table 2) [68].

3.2.2. Covalent coupling to a water insoluble material

In this approach, the lipase is covalently bound to a water insoluble material via reaction between functional groups of the protein and

Table 2
Examples of properties of chemically modified lipases

Source of lipase	Name of the modified lipase	Modifier	Method	Properties of the modified lipase	Reference
Pancreatic lipase	–	Aldehyde/ketone	Reductive alkylation	50% increase in V_{max}, but 40% decrease in E_{act}; enhanced hydrolysis and transesterification; 1,3-specificity.	[67]
C. cylindracea	–	Pyridoxal 5-phosphate and dithiothreitol	Modification of amino acid residues	Increase of esterification activity; hydrolytic activity unaffected.	[68]
Lipoprotein lipase	PEG-lipase	Activated-PEG triazine	Covalent	Increased solubility and activity in organic solvents.	[69]
C. cylindracea	PEG-lipase	Activated-PEG triazine	Covalent	Increased solubility and activity in organic solvents.	[70]
C. rugosa	PEG-lipase	Activated-PEG triazine	Covalent	Stability increased in water; less stable in benzene; selectivity increased to favor oleic than stearic acid.	[71]
Ps. fragi	Magnetite PEG-lipase	Fe^{2+}, Fe^{3+} activated-PEG triazine	Covalent	Increased solubility and activity in organic solvents; ease recovery.	[72]
Ph. nites	FA-modified lipase	DSP ester	Acylation	Efficient esterification of TGs; specificity depends on the nature of FA.	[73]
C. rugosa	Amidinated-lipase	amidoester hydrochlorides	Acylation	Thermostability; solvent solubility; efficient synthesis; lower hydrolysis.	[74]
Rhizopus delemar	Detergent-modified lipase	didodecyl glycosyl glutamate	Covalent	Specificity for hydrolysis of long-chain TGs; inhibition by calcium ions.	[75]

DGs: diglycerides; DSP: dimethylsulfoniophenyl; FA: fatty acid; N.A.; information not available; PEG: polyethylene glycol; TGs: triglycerides.

reactive groups on the activated material. Examples of derivatized enzymes obtained through this procedure include PEG-modified lipase [69–72], fatty acid-modified lipase [73], amidinated lipase [74], and detergent-modified lipase [75].

Covalent attachment of PEG to free amino groups in the protein bovine serum albumin was first reported by Abuchowski et al. [76,77]. Later, Inada et al. [69,70], and Baillargeon and Sonnet [71] prepared a series of amphipathic enzymes by conjugating with PEG. Derivatized enzymes, including lipoprotein lipase, *C. cylindracea* lipase and *C. rugosa* lipase, were made soluble and more active in organic solvents (Table 2) by chemical modification with 2,4-bis-(*o*-methoxypolyethylene glycol)-6-chloro-*s*-triazine, i.e., activated PEG2 [69–71]. This polymer as a modifier has the advantage of amphipathic nature. Its hydrophilic part makes it possible to modify enzymes in aqueous solution and its hydrophobic part should make modified enzymes function in hydrophobic environment. In addition, a ferromagnetic-modified lipase was prepared by including ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions with PEG-modified lipase from *Pseudomonas fragi* [72] (Table 2). This ferromagnetic-modified lipase can be easily recovered from the reaction mixture by magnetic separation. Fatty acid-modified lipase was prepared by reacting amino groups in the enzyme using the water-soluble acylating reagent, dimethylsulfoniophenyl (DSP) ester under mild conditions with less damage to the enzyme [73]. The modified lipase from *Phycomyces nites* was able to catalyze more efficiently the esterification of glycerides in *n*-hexane. However, the specificity and selectivity of the modified lipase was dependent on the nature of the attached fatty acid: lipase modified by attachment of saturated fatty acid transesterified saturated fatty acid and triglyceride more efficiently than unsaturated fatty acid and vice versa (Table 2).

Amidinated lipase [74] and detergent-modified lipase [75] were also highly soluble in organic solvents. The former, i.e., the modified lipase from *C. rugosa*, was prepared chemically by amidination with imidoester hydrochlorides of different hydrophobicity [74], and the latter, i.e., the detergent-modified lipase from *Rhizopus delemar*, was manufactured using a synthetic detergent, didodecyl glucosyl glutamate [75]. The amidinated lipase was more thermostable and solvent-stable than the native enzyme and showed a higher ester synthesis activity and a lower ester hydrolysis activity [74]. The detergent-modified lipase was also thermostable in organic solvent up to 50°C and showed a higher specificity toward the hydrolysis of longer chain triglycerides, but was inhibited by calcium ions compared to the native enzyme [75] (Table 2).

3.2.3. Lipase attachment to a matrix by covalent bonds

This immobilization technique is not as common as the physical adsorption, but presents the advantage of avoiding the desorption phenomenon. The main concern is to carry out the covalent attachment on amino acids that are not in the catalytic machinery. This may be difficult to achieve and, usually, enzymes immobilized with this technique lose a part of their initial activity. Concerning the protein, covalent attachment to the matrix proceeds on side chain groups of the amino acids (e.g., primary amines for lysine, carboxylic functions for aspartic and glutamic acids, hydroxyl groups for tyrosine, serine and threonine, thiol for cysteine). Many organic or mineral supports can be used, but prior to the covalent attachment this support must be ‘‘activated’’. This activation corresponds actually to the incorporation of a chemical group that is capable of reacting with the side groups of the protein. Different methods of activation are available : the most common is the use of glutaraldehyde. This compound easily polymerize to give polymers containing ω unsaturated aldehyde functions which can react with amino groups of the support. The excess of glutaraldehyde is first eliminated with buffer

washing and then the activated support is suspended in the aqueous lipase solution.

As examples, the *C. cylindracea* and *Rhizopus oryzae* lipases were covalently attached to porous glass beads and alumina [78], respectively, by treatment of these supports with γ-aminopropyltriethoxysilane in acetone followed by reaction with glutaraldehyde solution. The resulting preparations were washed with buffer to avoid cross-linking prior to mixing with buffered lipase solutions. The *Rhizopus oryzae* lipase modified retained only 23% of its original activity. Alternatively, covalently modified pancreatic lipase was manufactured by partially hydrolyzing polyamide with hydrochloric acid at high temperature followed by treatment with glutaraldehyde [79]. The resulting preparation was then washed and mixed with the lipase solution. The modified enzyme retained 9% to 150% of its hydrolytic activity with 3 to 1030 μmol product/min $g_{support}$ (Table 2).

The cyanogen bromide method is also largely used. It is applied on supports containing ω glycol groups such as agarose beads [80], cellulose or Sephadex. Glycols react with the cyanogen bromide to give imidocarbonates that react with side amino groups of the enzyme [32]. For example, *C. cylindracea* lipase was immobilized to cyanogen bromide-activated Sepharose using ethanolamine [78]. The resulting modified lipase retained 24.1% to 29.2% of its hydrolytic activity with 7 to 25 μmol product/min $g_{support}$.(Table 2). Other activating agents are carbodiimides, which are used to create a link between carboxylic functions of the support and amino group of the protein. This procedure has been attempted with such supports as aminoalkyl derivatives of kieselguhr and glass, carboxymethylcellulose, carboxylagarose, and CH- and AH-Sepharose with the lipase of *Geotrichum candidum* [81].

As we have already mentioned, the main advantage of the covalent attachment is that such an immobilization is very solid. Unlike physical adsorption, any desorption of the immobilized enzyme is therefore impossible. Moreover, the obtained immobilized enzymes are usually very stable and resistant to extreme conditions (pH range, temperature). Finally, a large number of different supports and methods to activate them are available. However, experimental procedures are obviously more difficult to carry out than for physical adsorption. The 3-D structure of the protein is considerably modified after the attachment to the support. This modification generally leads to a significant loss of the initial activity of the biocatalyst.

Studies dealing with covalent immobilization of lipases are less numerous than the ones concerning physical adsorption. Among the relevant publications, one can cite Manjon et al. [82], who compared the behavior of *M. miehei* lipase adsorbed on Celite and covalently bond to nylon. The latter has been chosen since, in contrast to porous supports, the immobilization takes place only in the more external areas of the support allowing, according to the authors, a better expression of the enzymatic activity. It was, however, observed that covalent binding of the lipase to nylon resulted in an immobilized lipase with a much lower activity than the lipase adsorbed onto Celite. To explain such a result, they suggest that the chemical modification of the lipase is of crucial importance given the changes in activity observed when changing the aminoacyl residue involved in the linkage to the support. Stark and Holmberg [83] studied the covalent immobilization of *Rhizopus* sp. on activated silica. Three different coupling media varying in their polarity were tested. The reaction medium is in fact believed to influence both the conformation of the enzyme to be immobilized and the relative reactivity of the functional groups of the protein, thus influencing the immobilization pattern. First, two immobilization supports, namely tresyl- and epoxy-activated silica particles, were evaluated in terms of amount of lipase bound as a function of time. It was observed that immobilization with the former was fast and almost completed after an hour, whereas, attachment to the latter was much slower. The amount of bound lipase was satis-

factory for both carrier materials being, respectively, 47 mg/mg dry support for the tresyl, and 34 mg/mg dry support for the epoxy. Further experiments were exclusively carried out with the tresyl activated silica. The role of the coupling medium was then investigated, comparing aqueous buffer, hexane and a microemulsion based on hexane and aqueous buffer. A good discussion was given about the potential influence of such a parameter. It was indeed anticipated that in an aqueous medium the protein tends to fold to present its more hydrophilic residues to the solvent whereas in low polarity medium a different conformation is obtained with the more hydrophobic amino acids at the surface. Moreover, since the coupling reactions are SN2, the rate is greatly dependent on the solvent polarity. The results showed that the rate of hydrolysis for reactions catalyzed by enzymes immobilized from the three different media was slightly higher with the microemulsion medium. However, differences were not very significant and it seemed that in this case, the polarity of the reaction medium did not greatly influence the enzyme performance. The study was then further extended to transesterification, palm oil being reacted with stearic acid as a model reaction. Contrary to the hydrolysis, the solvent used during the immobilization was of paramount importance. The lipase immobilized in aqueous medium was completely inactive, whereas, the preparation from hexane showed 22% and from the microemulsion, 43% of the activity of the free enzyme. The authors suggested that the more pronounced effect of the immobilization medium in transesterification may be due to the fact that in this case, the steric requirement on the active site is larger since two bulky reactants are involved. Therefore, coupling to amino acids residues near the active side would affect this reaction more than hydrolysis, which only involves one large molecule. The effect of introducing a very long hydrophilic spacer arm, namely PEG 1500 on the carrier surface was also investigated. It was found that such a spacer had an important effect on the activity in hydrolysis. Direct coupling gave an activity of immobilized lipase of 28–34% of that of the free enzyme whereas coupling with the spacer exhibited 56–57% activity. This phenomenon is probably due to the fact that the spacer minimizes the interactions between the protein and the support by suppressing both hydrophobic interactions and electrostatic attractions. It is worth noting that the addition of the spacer had no effect on the activity of the enzyme in transesterification. Such a reaction being carried out in an almost non-aqueous medium, in which the PEG is not soluble, it is believed that the hydrophilic spacer arm, which in an aqueous medium stretches out of the bulk phase, will here adhere to the particle. The authors suggest that for such a reaction, the ideal spacer would therefore be a non-charged hydrophobic arm. Finally, they studied and compared the stability of the covalently immobilized lipase with that of its adsorbed counterpart. It was shown that when the acitivities of both the free and the adsorbed lipases deteriorated rapidly within three weeks, the covalently bound enzyme retained its full initial activity.

3.2.4. Formation of a cross-linked lipase matrix using various bi-functional reagents

During cross-linking, the lipase molecules are chemically linked with one another using various bi-functional reagents. The procedure includes a preliminary step involving immobilization on an ion exchange resin, such as Amberlite or Dowex, followed by treatment with a buffered solution of glutaraldehyde to form a Schiff base as a product of reaction with amine residues. The resulting mixture is then either treated with hydrogen sulfide solution to reduce the excess Schiff base and glutaraldehyde or washed with a buffered solution. Using this procedure, Omar et al. [21] immobilized the lipase from *H. lanuginosa* on Amberlite by cross-linking with hexamethylenediamine and glutaraldehyde. The modified-lipase adsorbed on

resin was characterized by a retained hydrolytic activity of 35.4–66.5%.

Lipases have also been successfully immobilized on polyacrylamide using a diazonium intermediate, on a polyaminopolystyrene resin by azo linkages, and to the alkylamine derivatives of controlled pore size glass and Spherosyl via either glutaraldehyde or 1-cyclohexyl-(2-morpholinoethyl) carbodiimide [84]. In the above studies, the immobilized pancreatic lipase on polyacrylamide showed an hydrolytic activity of 0.564 μmol product/min $g_{support}$, while the modified *C. cylindracea* lipase retained 1.1%, 3.7%, and 12.5% of its hydrolytic activity on controlled pore glass-glutaraldehyde, spherosyl-glutaraldehyde, and spherosyl-carbodiimide supports, respectively (Table 3).

The diazonium intermediate has also been used to couple lipase to *p*-aminobenzylcellulose, carboxymethylcellulose, carboxymethyl-Sephadex, poly-*p*-aminostyrol, and aminoacryl derivatives of Kieselguhr [81]. Similar methods involving adsorption to the arylamine derivatives of glass and Spherosil by diazobinding have also been described [26,81]. The immobilized *G. candidum* lipase used in these studies exhibited K_m values of 0.48 and 0.50 μmol/ml on *p*-amonobenzylcellulose and carboxymethylcellulose supports, respectively, and retained 18%, 66%, 12%, and 22% of its hydrolytic activity on azide carboxymethyl Sephadex, azide carboxymethylcellulose, azopoly-*p*-aminostyrol and azo-Kieselguhr derivatives, respectively (Table 3).

Cross-linked enzymes catalysts (CLECs) have recently been described and two lipases from *Pseudomonas cepacia* and *C. rugosa* are commercially available from Altus. The latter shows significantly enhanced enantioselectivity in the resolution of ibuprofen ($E = 60$ for CLEC-CR vs. $E = 5$ for native lipase) [85].

3.2.5. Conclusion

Chemical modifications of lipases by either reduction of amino acid residues, covalent coupling to an insoluble carrier, attachment to a matrix by covalent bonds, or by formation of cross-linked lipase matrix are certainly excellent tools for increasing the stability and improving the performance of these enzymes in hydrophobic environment. These procedures should produce enzymes capable of catalyzing not only the reverse reaction of hydrolysis, ester synthesis, but also reactions of ester exchange and aminol-

Table 3
Lipases immobilized by covalent bonding and chemical cross-linking

Source of lipase	Support	Method of binding	Activity after immobilization	% Activity retained	Reference
Rhizopus oryzae	Alumina	Covalent	N.A.	23	[78]
Pancreatic lipase	Polyamide	Covalent	3–1030[a]	9–150	[79]
C. cylindracea	Spherosyl-glutaraldehyde	Covalent	N.A.	1.1	[26]
C. cylindracea	CPG-glutaraldehyde	Covalent	N.A.	3.7	[26]
C. cylindracea	Spherosyl-carbodiimide	Covalent	N.A.	12.5	[26]
Pancreatic lipase	PA	Cross-linking	0.564[a]	N.A.	[84]
G. candidum	CMC	Covalent	0.50[b]	N.A.	[81]
H. lanuginosa	Amberlite	Cross-linking	N.A.	35.4–66.5	[21]
G. candidum	AEPA	Covalent	22[a]	6.24	[81]
G. candidum	APPAS	Covalent	12[a]	3.42	[81]
Geotrichum candidum	ACMS	Covalent	18[a]	5.04	[81]
G. candidum	ACMC	Covalent	66[a]	18.5	[81]

CMC: azide-carboxymethylcellulose; ACMS: azide-carboxymethyl-Sephadex; AEPA: aldehyde-enzacrylpolyacetal; APPAS: azoply-*p*-aminostyrol; CMC: carboxymethylcellulose; N.A.: information not available; PA: polyacrylamide.

[a]Activity in μmol product/min $g_{support}$.

[b]Activity in μmol product/ml.

ysis in organic solvents. The immobilized lipases can easily be recovered and reused, or alternatively reused in a continuous process involving packed bed reactors. To render these enzyme very active in organic solvents, the procedures of immobilization should preserve the hydration shell around the lipases and keep the enzymes physically separated from the external organic environment. This strategy enables a micro-heterogeneous environment with a high interfacial area. Thus, chemical modifications should be performed under mild conditions with less damage to the tertiary structure of the lipases. It should also be emphasized that medium engineering, i.e., modification of immediate surroundings of the biocatalyst in nonaqueous solvents to provide a better microenvironment for the enzyme, may further increase the performance of a given modified biocatalyst.

The data compiled in Tables 2 and 3 show that the activity of the different modified lipases vary tremendously depending upon the binding procedure applied and the support selected. Values of the percent activity retained range from 1 to 150. In some cases, transesterification and hydrolytic activities increase concomitantly, but in other cases, hydrolysis is favored, while the transesterification activity decreases, and vice versa. The same observation applies also to solvent solubility and specificity. These observations may reflect either enzyme denaturation, the contribution of the testing parameters, such as water content, nature of the solvent, temperature, ions concentration, or other unknown phenomenon. Thus, one can conclude that the immobilization of lipase by chemical means is still a trail and error procedure.

3.3. The use of genetic engineering to modify the properties of lipases

3.3.1. Introduction

Recombinant DNA and genetic engineering technologies allow the isolation and manipulation of nucleic acids and the alteration of the amino acid sequence, and thus the properties, of an enzyme. As opposed to more traditional forms of mutagenesis (e.g., chemically induced), these changes in the genetic material can be localized to precise sites or regions within the protein. This ability to focus genetic change, and, in the case of amino acid substitution, to direct the identity of the new amino acid, gives genetic engineering great power for productive protein alteration.

However, in the absence of models for enzyme structure and function, the power of directed mutagenesis cannot be fully exploited. Fortunately, advances in knowledge of the structural and catalytic chemistry of lipases have come rapidly within the past decade. Since publication of the first high-resolution 3-D lipase models in 1990 [86], at least a dozen additional structures have been published [87,88]. Determination of the structures of lipases co-crystallized with substrates, or covalently modified with active site-directed substrate analogues, provided further insight into the mechanics and geometry of substrate binding [89–92]. In turn, molecular genetic technologies facilitated crystallographic studies and the development of mechanistic models, not only by providing large enzyme quantities through the overexpression of cloned genes, but also by providing amino acid sequence data derived by sequencing cloned lipase genes, and by allowing alteration of the occupancy of amino acid sites in order to investigate their roles in stability, substrate binding, and catalysis.

As the applications of genetic engineering to lipases are discussed below, it should be noted that these enzymes are employed on many different substrates, in numerous types of reactions. One lab will monitor esterase activity using nitrophenyl esters as substrates, another will determine the activity of mutated enzymes against one or two triglycerides of interest, and a third will ignore alterations of substrate range in its search for thermostability. Thus, unified pictures of the contributions of regions or amino acid sites to activity and stability are slow to

emerge. It is prudent to consider the properties that are described here to be minimum achieved alterations. Other changes may also have resulted, but gone unnoticed.

3.3.2. Conceptual framework: common features displayed by virtually all lipases

Due to contributions from solution biochemistry, crystallography, and genetic engineering, the following concepts presently constitute the core of understanding of lipase structure and function, and guide efforts to improve lipases through genetic modification. Detailed discussions of these concepts have recently appeared [87].

3.3.2.1. Lipases belong to a single structural superfamily. Lipases are ubiquitous, being produced by virtually every living organism. Despite differences in size, sequence homology, substrates, activators, inhibitors, and other properties, most of them adopt a similar core topology, known as the α/β hydrolase fold [93, 94]. The interior topology of α/β hydrolase fold proteins is composed largely of parallel β-pleated strands (at least five in lipases), separated by stretches of α-helix, and forming, overall, a superhelically twisted–pleated sheet. Helical peptide sections packed on both faces of this sheet form much of the outer surface of the protein.

3.3.2.2. Lipases are catalytic triad serine hydrolases. Despite widely varying degrees of sequence homology between the members of this family, one sequence is exceptionally highly conserved: the pentapeptide Gly–X–Ser–X–Gly. The conservation of this serine, and the loss of catalytic activity upon its modification or replacement, argue that this amino acid is crucial to catalysis [86,95–99]. Its topographic location is also conserved and significant: it sits at the apex of a tight bend (''nucleophilic elbow'') in the protein chain, a bend that can be formed only when the amino acids at the −2 and +2 positions relative to the serine have small sidechain groups — hence, the predominance of glycine at these locations [93,94].

In addition to the catalytic serine, the active centers of most lipases consist of a histidine and an acidic amino acid (Asp or Glu), the three residues forming a catalytic triad whose topology is highly conserved among the lipases and is in many ways analogous to that of the serine proteases [86,93,95,100].

3.3.2.3. The catalytic triad sits in a hydrophobic cleft or cavity in the enzyme. This may be a relatively shallow groove in the surface of the enzyme. The fatty acyl chain of the substrate ester docks into this groove, aligning its ester bond with the catalytic triad sitting at the bottom of one end of the cleft. *Rhizomucor miehei* lipase (RML), and members of its homology family, have active sites of this type [100]. In other cases, such as the *C. rugosa* lipases [92], the active site sits at the mouth of a deep hydrophobic cavity. The hydrocarbon chain of the substrate fatty acid enters the cavity, leading with its terminal methyl end and ultimately aligning the scissile ester bond with the catalytic residues.

3.3.2.4. Interfacial activation is required for the demonstration of significant enzyme activity, and is mediated by a conformational change in the enzyme. With but few exceptions, lipases exhibit low activity against water-soluble substrates, and much higher activity against the micelles formed when a substrate exceeds its solubility limit. This ''interfacial activation'' results from a conformational change in the enzyme at the surface of the insoluble substrate. In the inactive state, the substrate-binding region and the active site are covered by peptide loops of the enzyme, termed ''lids'', of which there is generally one. In this state, the active site is not accessible from the bulk solvent. The lid is able to pivot away from its location over the active site, exposing the substrate-binding and catalytic sites, increasing the hydrophobicity of the surface in the region surrounding the

substrate-binding cleft, and thereby facilitating interaction between the enzyme and hydrophobic substrates [87,89].

In the genetic alteration of lipases, efforts to impart detergent resistance have focused on altering the polarity and hydrophobicity of the hydrophobic surface near the active site. Efforts to modify substrate specificity have focused on alteration of the amino acid sequence within the cleft/cavity region. Attempts to increase enzyme activity have involved mutagenic substitution of the amino acids in the lid peptide. There have also been efforts to alter thermal-, pH-, and protease-sensitivity, since these restrict the utility of lipases as applied catalysts and in their most frequent applied role, as components of laundry detergents.

3.3.3. Applications of genetic engineering to lipase improvement

3.3.3.1. Gene separation. In many cases, an organism produces multiple lipase isozymes. The lipolytic properties of a crude enzyme preparation from such an organism will be the average of the traits of all lipases present. By separating the genes for isozymes from one another and establishing them in individual cell lines, genetic engineering offers a means of resolving these mixed lipases. Results will be most substantial in situations where the isozymes differ significantly from one another in a biochemical property of interest. This use of genetic technologies to manifest useful lipolytic activities has been most strikingly applied in the case of the *G. candidum* lipase isozymes. Because these enzymes are very similar in their physical properties, biochemical separation is challenging and time consuming, and does not facilitate the preparation of large amounts of the enzymes. Cloning and expression of the individual lipase isogenes allowed the creation of microbial cultures that each synthesized only one of the isozymes [99,101]. Since one of these isozymes displayed a unique and desirable selectivity for 9–10 unsaturated fatty acids [101], genetic methods were essentially able to generate a strain producing a highly selective lipase from an organism that demonstrated low selectivity.

3.3.3.2. Alterations of activity and substrate selectivity through directed mutagenesis. The modification of lipase substrate selectivities through rational mutagenesis has been reported only recently, and advances in this area have been made slowly. This is largely due to the fact that researchers have an imperfect sense of the parameters guiding enzyme–substrate interactions, and also because such features as activity, specificity and stability are usually determined by multiple, not single, sites in a protein. Substantial progress will be required in these areas before rational mutagenesis can be employed in a truly directed manner to alter lipase specificity. Nonetheless, some advances have been made to date in efforts to tailor the substrate ranges of lipases. These are grouped here according to the structural and sequence homology families to which the subject lipases belong, since general principles are best highlighted in this fashion.

The Mucorales lipases. In all cases studied to date, lipases produced by fungi of the order Mucorales (including *Rhizomucor miehei* and all species of *Rhizopus*), as well as enzymes from related organisms such as *H. lanuginosa*, and *Penicillium* form a highly homologous group [102–104]. These enzymes are active on primary, but not secondary, esters. Therefore, they are regioselective in triglyceride synthesis and hydrolysis, acting primarily at the 1- and 3-positions of the substrate. This unique substrate specificity, and the early appreciation of its potential commercial utility [23], triggered extensive studies of this group of enzymes. This resulted in the accumulation of a substantial body of biochemical and structural information concerning these enzymes, and the cloning and expression of the genes for several of them. This information, in turn, facilitated the applica-

tion of rational mutagenesis in an effort to improve these enzymes for use as applied catalysts. The results of this work are discussed below. Although a substantial amount of progress has been made, this discussion will illustrate that the understanding of the role of structure–function relationships in determining the substrate specificity of lipases is far from complete.

The Mucorales lipases have molecular weights between about 30 and 35 MDa. They exhibit generally comparable hydrolytic activities toward fatty acid esters, irrespective of the degree of unsaturation or the chain length of the fatty acid [103]. (Activities are greatly reduced toward esters whose fatty acyl component is polyunsaturated and 20 or more carbons in length [104].) Crystallographic studies of the complex formed between RML and the active site-directed covalent inhibitor *n*-hexylphosphonate ethyl ester suggested that in the binding and hydrolysis of glycerides the acyl chain of the scissile fatty acid occupied a shallow groove on the surface of the enzyme [89]. This groove was lined with predominantly hydrophobic amino acids and appeared potentially able to accommodate acyl chains at least 12 carbons in length. Visualization of the geometry of interaction between the enzyme and the acyl chain of its substrate fostered a series of studies designed to explore the relationship between binding groove architecture and chain length preference in this family of lipases, and to use a coordinated approach involving molecular modelling and site-directed mutagenesis to generate lipolytic enzymes with chain length selectivities different than that of the parent enzyme [105–108].

This work was conducted using lipase genes cloned from *Rhizopus delemar* and *Rhizopus oryzae*, two fungi that are now known to be actually the same species [109]. The amino acid sequences of these lipases differ at only two of their 269 positions. In addition, these enzymes are sufficiently homologous to RML that initial studies could use the available 3-D structure of the latter enzyme to guide the choice of targets for mutagenesis [86,100]. Subsequently, the structure of the *Rhizopus delemar* lipase (RDL) became available [110] and was used to guide mutagenesis.

In the earliest of these studies [105], the approach taken was to perform directed mutagenesis at selected single sites on the sidewalls of the putative substrate-binding pocket, replacing target wild-type amino acids with each of the 20 possible amino acids. Given the lack of sophistication of knowledge concerning the interactions that dictate enzyme activity and substrate binding and release, this approach seemed to offer a greater potential for the generation of useful mutants than did the a priori selection of any one particular amino acid for introduction. The mutated DNAs were introduced back into bacteria, and members of the resulting population were scanned with a visual assay that allowed the detection of organisms with altered lipolytic activity toward tributyrin (C4:0), tricaprylin (C8:0), or olive oil (predominantly C18:1 fatty acids). All the mutant lipase preparations identified in this manner had lower specific activities than the wild-type enzyme. Given the ability to produce large amounts of modified enzyme through the overexpression of cloned genes, this should not be a major barrier to the use of modified enzymes as applied catalysts.

Phe95 in RDL appeared to be appropriately positioned to be capable of sterically hindering the entry of a fatty acyl chain longer than approximately 10 carbons into the putative substrate-binding groove (Fig. 2). Indeed, following mutagenesis of this site, a modified lipase was isolated whose activity toward TC relative to that toward TO was between two- and six-fold greater than that of the parent enzyme. DNA sequence analysis indicated that in the mutant enzyme, Phe95 had been replaced by aspartic acid. In other studies, this time using the cloned *Rhizopus oryzae* lipase gene [108], replacement of this Phe95 with tyrosine again resulted in an enzyme with less activity than the parent lipase. However, the hydrolytic activity of the mutant

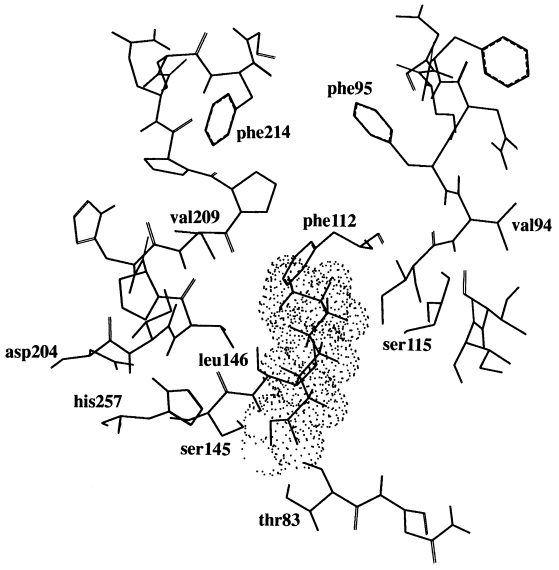

Fig. 2. Model of the structure of the active site region of the *Rhizopus delemar* lipase with the sn3 acyl chain of tricaprylin (dotted surface) docked into the catalytic center [107]. Protein structure derived by X -ray crystallography [111]. The catalytic triad residues are Ser145, His257, and Asp204. Selected residues that form part of the probable acyl binding site are displayed.

toward the methyl esters of a mid-size fatty acid (caproic acid, C6) was less reduced than that against methyl oleate [108]. The net result was a 1.5-fold increase, compared to the wild-type enzyme, in relative activity toward esters of caproic acid vs. oleic acid. These data again indicate a role for position 95 in chain length discrimination by this enzyme and demonstrate that this trait pertains not only in the case of triglycerides but for simpler esters as well. Clearly, other interactions also play a role in chain length discrimination, as evidenced by the fact that Atomi et al. [108] found that the activity of the mutant enzyme toward stearic acid [18:0] esters, although reduced by mutagenesis, remained nearly three times higher than the activity toward caproic acid esters. The reduction in relative activity toward long-chain unsaturated fatty acids (e.g., oleic) may result from the introduction of a polar residue into the binding pocket. The greater acyl chain flexibility of saturated substrates, such as stearic acid, may allow them to bind to the enzyme without entry of the more distal portions of the acyl chain into the pocket, avoiding to some extent the inhibitory effects of the Phe95 mutations discussed above. That this phenomenon may occur is suggested by the crystal structures of the *H. lanuginosa* lipase (HLL) complexed with a 12-carbon substrate analog. The first eight carbons of the chain appear to interact with the enzyme, and the remaining ones extend out from the enzyme surface [111]. The pronounced reduction in activity in the mutant enzymes toward all substrates tested, regardless of the lengths of their fatty acyl chains, is less easily explained, and suggests that the relative polarization of the binding pocket in the mutant enzyme reduces the overall efficiency of binding of hydrophobic substrates.

Phe112 of RDL is another residue that appeared, based on the crystal structure of the enzyme, to be a component of the substrate-binding pocket in the enzyme (Fig. 2). Since this amino acid lies closer to the catalytic triad than Phe95 discussed above, it was postulated that mutations at this site, particularly those that introduced bulky or polar amino acids, might reduce the binding of both C8 and C18 fatty acids, with little effect on shorter chains. In agreement with this proposal, random mutagenesis of position 112 generated a lipase whose activities toward C8 and C18 fatty acids were so reduced that the relative activity toward C4 increased almost eight-fold [105]. DNA sequence analysis indicated a Phe112Trp mutation in this lipase, suggesting that steric hindrance may underlie the change in substrate specificity. The lipase recovered upon mutagenesis of Val209, which lies across the putative substrate-binding channel from Phe112, also displayed increased activity toward C4 fatty acids relative to C8 and C18 [105]. The amino acid at this location in the mutant lipase was found to be a tryptophan also. By combining these two mutations into a single lipase gene, it was possible to achieve an 80-fold increase in

the hydrolysis of tributyrin relative to tricaprylin, and virtually eliminate triolein hydrolysis [106].

However, it cannot be considered dogmatic that amino acid sites closest to the active site regulate only specificity toward short-chain fatty acids: replacement of Val206 in RDL with the polar amino acid threonine caused an increase in the relative activity toward mid-chain rather than short-chain fatty acids, while mutations at Val209, which is more distal to the catalytic triad (Fig. 2), elevated relative activity toward both tricaprylin and tributyrin [105]. As in other mutants examined in this study, the changes in selectivity were more pronounced when single substrates were assayed than when a mixture of triglycerides was employed. This suggests that some aspects of the substrate emulsion also play a role in enzyme specificity.

Another approach to the alteration of chain length specificity in the Mucorales lipases involved the postulate that formation of a salt bridge across the substrate-binding trough might block the entry and hydrolysis of esters of long-chain fatty acids. It was found that when complementary charged amino acids were introduced into RDL at positions 95 and 214, which sit across from one another in the substrate-binding trough (Fig. 2), the hydrolysis of TC increased three-fold relative to TO [106]. However, the theory that polarization of the active site or the formation of salt bridges within it can confer substrate selectivity has its limits: introduction of complementary pairs of charged amino acids close to the catalytic center, e.g., at positions 146 and 115 (Fig. 2) reduced activity toward all substrates to nearly zero [106]. The introduction of even a single charged residue into the trough can cause substantial perturbations to the enzyme, as evidenced by the fact that the replacement of Phe112 (Fig. 5) with glutamine abolished activity [106].

Under some conditions, changes in substrate selectivity have been amplified by combining single mutations with one another in the same lipase molecule. An example of the utility of this approach is shown by the attempt to augment the mid-chain selectivity of RDL by combining the Val206Thr and Phe95Asp mutations [107]. Each of these mutations had given a four- to six-fold preference for tricaprylin over triolein when assayed against single substrates. In the corresponding double mutant lipase, the relative activity toward tricaprylin was about 10-fold greater than that toward olive oil, indicating the additivity of effects of these two mutations. The increase in activity toward tricaprylin relative to that toward tributyrin was even greater, by about an order of magnitude. The selectivity of the double mutant enzyme encompassed fatty acid chain lengths from 8 to 14 carbons. This work also illustrated that fact that mutations can have unexpected pleiotropic effects: The double mutant enzyme displayed a pronounced pH-sensitivity that was not shown by the wild-type lipase. Activity and selectivity were optimal at pH 7.0. Above pH 7.5, the enzyme was inactive, and below pH 6.5, its selectivity was reduced. This pH sensitivity does not rule out the use of the double mutant enzyme as an applied catalyst, since pH control is a well-developed technology. However, it does emphasize the value of a detailed characterization of enzymes generated by rational mutagenesis.

Another region of the *Mucorales* and related lipases that has been postulated to play a role in substrate binding is the peptide ‘‘lid’’. Structural models derived from crystallographic analyses of both RML and HLL complexed with active site directed inhibitors indicated that the acyl portion of the inhibitor was sandwiched between the active site and Trp89, a lid residue [90,111]. A crucial role for Trp89 was also suggested by its high degree of conservation in this family of enzymes and in the pancreatic lipases. Site-directed mutagenesis of Trp89 in the lid of the HLL indicated that this residue is important for effective tributyrin hydrolysis [112]. Martinelle et al. [113] found that replacement of the Trp with a Phe reduced activity toward tripropionin, tributyrin, trihexanoin, tri-

octanoin and triolein by three- to five-fold, while activity toward triacetin was reduced by less than two-fold. Thus, there was a net relative increase in the activity toward triacetin. This work suggests a role of the lid residues in substrate discrimination/binding, but will have to be expanded before practically useful selectivity differences are realized: The activity of the wild-type enzyme toward triacetin was 20- to 100-fold less than that toward the other substrates tested, and thus, even with the augmentation in triacetin selectivity that accompanied the Trp89Phe mutation, the activity toward triacetin was no better than 10% that toward other substrates. The importance of this position for catalytic activity is also indicated by work with *C. antarctica* A lipase. Svendsen et al. [114] reported that replacing the Phe normally found in the analogous position of the lid of this enzyme with Trp resulted in a four-fold increase in tributyrin hydrolysis.

Replacement of Trp89 in HLL with other smaller amino acids was found to affect the selectivity of the enzyme for the alcohol leaving group of its substrates [113]. The hydrolytic activity of the enzyme was reduced in all cases. However, the magnitude of the decrease varied for different alcohol groups, with the relative activities of the mutant enzymes toward vinyl vs. ethyl esters being about twice that of the wild-type enzyme. However, since the wild-type enzyme itself was already nearly 50-fold more active against vinyl vs. ethyl esters, it is not clear that the increased specificity conferred by the Trp89 mutations will make the enzyme a more practically useful catalyst. It was also found that reducing the bulk of the amino acid at position 89 in HLL, such as by replacing the wild-type Trp with Phe, doubled or tripled the activity of the enzyme toward esters of bulkier fatty acids, such as 2-methyldecanoate [113]. Presumably, this results from alleviation of steric conflicts between these substrates and the binding pocket. Overall, these studies suggest that Trp89 plays a role in aligning the substrate in the active site, an observation that might be exploited in the future to refine the selectivity or enlarge the substrate range of this group of lipases.

Glu87 lies on the surface of the body of the HLL structure and appears able to stabilize the open configuration of the enzyme through formation of electrostatic interactions with the lid [115]. Directed mutations at this site caused comparable reductions in enzyme activity toward all substrates tested regardless of the chain lengths of their fatty acids [113], indicating that the mutations did not alter the position of the lid in a manner that fosters substrate discrimination. However, the enantiospecificity of the enzyme for *S*- vs. *R*-heptyl 2-methyldecanoate was nearly doubled by the replacement of Glu87 with an alanine [115].

Presently, the greatest potential and actual opportunity for the use of lipases in industry lies in their addition to detergent formulations to remove lipid stains from fabrics. A number of approaches have been taken to increase lipase activity in laundry applications. The assays used to assess activity in these cases measure the ability of the modified lipase to remove lipid stains from fabric swatches during a simulated laundry cycle. However, the modified lipases generated in these programs may display enhanced activity in other reaction settings as well. Svendson et al. [116] described three general approaches, using (but not restricted to) the RML and HLL genes, to produce lipases with improved laundry performance. Their efforts relied on knowledge of the 3-D structure of the enzymes, and employed directed mutagenesis to achieve single or multiple deletion, substitution or insertion events. In the first approach, negatively charged, neutral or hydrophilic amino acids within the primarily hydrophobic substrate-binding region of the enzyme were replaced with neutral, positively charged, or hydrophobic residues, respectively. The theory underlying this approach was to maximize the affinity of the modified enzyme for substrate micelles, which have a negative surface charge due to the fact that fatty acids generated by

hydrolysis orient themselves with their carboxylate ends facing the outer environment. A second approach involved the replacement of bulky amino acids in this ''lipid contact zone'' with smaller amino acids, or the total deletion of such amino acids, to more fully expose the active site. The third approach focused on directed mutation of the lid of the enzyme, and of the region of the body of the enzyme that interacts with the lid when it is in the open configuration, to increase the propensity of the lid to open. A preferred route in this latter approach was the joint introduction of cysteine residues into the lid and body of the enzyme in positions where disulfide bond formation would be expected in the recombinant lipase. Another strategy was to replace hydrophilic residues in the binding pocket of the enzyme with less hydrophilic residues, which was postulated to dispose the lid to be in the open configuration in aqueous media. The authors largely presented data for single-site mutants, and reported activity increases of as much as four-fold over the wild-type enzyme in model wash systems. Some of these mutant enzymes also displayed relative activities as much as 1.7-fold greater than that of the wild-type enzyme in more conventional assays of hydrolytic activity using an emulsified substrate. Small changes (1°C) in thermostability were also achieved, and some of the mutants exhibited up to two-fold increased protease- and alkalai-resistance. No postulates were made as to the structural bases of these improvements. The results presented do not show particularly large improvements in lipase activity. Perhaps substantially more active mutant enzymes were obtained but not disclosed in the Patent report [116].

The Geotrichum candidum / C. rugosa lipases. A second class of esterases and lipases [117], distinguishable from those of the Mucorales group of enzymes on the basis of size, structure, and specificity, are represented by enzymes produced by *G. candidum* [101,118] and *C. rugosa* [92,119,120]. These enzymes are composed of single peptide chains with molecular weights of about 60,000. The acidic residue of their catalytic triads is a Glu rather than an Asp. The acyl chain of the substrate is bound in a cavity that extends from the surface into the central portion of the enzyme, rather than in a shallow surface groove.

The lipolytic activity of *G. candidum* attracted early attention because it exhibited catalytic selectivity for fatty acids containing a double bond between the carbons 9 and 10 residues removed from the carboxylate end of the molecule [121]. Thus, these enzymes are selective for the ubiquitous fatty acids oleic, linoleic and linolenic. Crude *G. candidum* lipase preparations display specificity for these fatty acids, the degree varying for different fungal isolates, and generally being too low to be practically useful. However, the great potential utility of this selectivity for applications such as the isolation of unsaturated fatty acids, their derivitization, and the preparation of unsaturate-depleted samples, was appreciated early in the history of lipase research. This triggered efforts to characterize the lipolytic activity, to apply it in hydrolysis and esterification reactions, and to improve the specificity for unsaturated fatty acids. A substantial advance in the latter area was achieved by researchers at Henkel [122], who employed ultraviolet mutagenesis to increase the oleic/palmitic selectivity ratio from a reported 1.5 to greater than 20, a value that should be sufficiently high to facilitate practical fatty-acid selective catalysis.

Biochemical investigations identified the presence of multiple isozymes in lipase preparations from *G. candidum* (and *C. rugosa* as well) and determined that the degree of selectivity for unsaturated fatty acids varies among these isozymes. The genes encoding the isozymes were subsequently separated by molecular cloning, allowing characterization of both the genes and the isozymes they encode [99, 101,123,124].

The two prominent lipase isozymes of *G. candidum* strain ATCC34614, GCL I and II, are both active toward fatty acids containing a *cis*-

Δ9 acyl moiety [101]. GCL I is relatively selective for this functionality, exhibiting 4–10-fold lower activities toward saturated substrates. GCL II lacks this selectivity, displaying marked activity against saturated as well as Δ9-unsaturated substrates [101]. Investigation of the active site residues of the GCL II isozyme resulted in the development of a more selective enzyme. Vernet et al. [99] employed site-directed mutagenesis to confirm the catalytic importance of the Ser217, His463 and Glu354 residues that constitute the catalytic triad of the GCL II isozyme of *G. candidum* ATCC34614. Replacement of either the Ser or His inactivated the enzyme when using either triolein or tributyrin as substrate. The mutant enzyme containing an Asp in place of Glu354 displayed an approximately two-fold reduction in its activity toward triolein. However, activity toward tributyrin was diminished 10-fold. As a result, the specificity of the mutant enzyme for triolein relative to tributyrin increased substantially, from 24:1 to 80:1. Unfortunately, the activities of the mutant enzyme toward substrates with acyl chain lengths between 4 and 18 were not reported, and the structural basis for the augmentation in substrate specificity was not identified.

However, subsequent work by the same research team very successfully dissected the contributions of primary sequence to substrate specificity in the *G. candidum* lipases. By creating hybrid lipases composed of regions from the GCL I and II genes, Holmquist et al. [125] identified a 57-residue stretch of amino acid sequence that was largely responsible for determining activity toward short-chain fatty acyl substrates. Hydrolytic activity toward triolein was essentially constant regardless of whether the GCL I or II portion of this segment was present in the lipase. Further, directed mutagenesis within the 57-residue stretch identified four amino acid sites, two at the entrance of the substrate-binding cavity and two lying at its bottom, which were largely responsible for determining whether or not GCL was active against esters of mid-chain fatty acids [127]. This thorough and exemplary work has provided some of the most precise structure –function information concerning the determinants of substrate specificity in the lipases. However, there remains a much-unexplored territory. Even in these thorough studies, only one amino acid substitution at each of the locations of interest has been reported, and the activity of the resulting lipase was determined toward only one substrate, trioctanoin. Perhaps in the future, the basic knowledge generated by this work, coupled with an examination of the effects of other mutations at these four crucial sites, and an expanded substrate range study, will result in recombinant enzymes with refined abilities to discriminate between saturated acyl chains.

The Pseudomonas lipases. With the *Pseudomonas* lipases, changes in substrate specificity have been achieved through mutagenic alteration of amino acids in the immediate vicinity of the catalytic triad. In the *Pseudomonas mendocina* enzyme, this triad consists of Ser126, Asp 176 and His 206. Single mutations at residues Gln127, Ser205 and Phe207 generated a group of mutant lipases whose k_{cat}/K_m values against short- and mid-chainlength *p*-nitrophenyl esters varied over a 100-fold range spanning the activity of the wild-type enzyme [126]. These mutant lipases were also examined for changes in their affinity for hydroxyl-containing nucleophiles, the agents that deacylate the fatty acid–acyl enzyme intermediate formed during ester hydrolysis. Derivatives were identified that exhibited an increased preference for methanol vs. water in the deacylation step, thus favoring transesterification over hydrolysis [127]. Other mutations in these residues near the active site increased the affinity of the enzyme for hydrogen peroxide as a nucleophile relative to water. This resulted in as much as a four-fold increase in the perhydrolysis vs. the hydrolysis of ester bonds, a feature that was exploited to produce a laundry bleaching system [128].

Additional work on the *Ps. mendocina* lipase focused on improving its activity in a laundry setting. Thus, Boston et al. [129] used a muta-

tion-generating polymerase chain reaction technology (poisoning) to introduce mutations near the catalytic His206 residue. A plate assay was employed to identify mutant enzymes that hydrolyzed olive oil more effectively than did the wild-type enzyme. The activities of the resulting mutant lipases were further characterized by measuring the release, during a mock wash cycle, of radioactivity from cloth swatches soiled with radioactive triolein. Of over 200 mutant lipases detected with these methods, the replacement of tyrosine at position 203 by the small, non-polar residues leucine or valine resulted in the greatest activity increases, as measured by the radioactive cloth wash assay. Activities of the mutant enzymes were nearly 2.5 times greater than that of the wild type. Similarly, increases were also seen when activities were determined using emulsified olive oil as the substrate.

3.3.3.3. Alteration of substrate range: lipid-type specificity

Changes in the substrate specificity of a phospholipase. In an early and novel approach to increasing enzyme activity, an examination of sequence homologies among the phospholipases A2 led to the deletion of five contiguous amino acids in a surface loop of porcine pancreatic phospholipase A2 [130]. Compared to the parent enzyme, the resulting mutant enzyme displayed up to a 16-fold increased activity toward zwitterionic substrates, and a greater than four-fold reduction in activity toward negatively charged phospholipids [131]. Structural studies disclosed that the deletion had smoothed out the surface of the enzyme in the region where it binds to lipid aggregates [133], perhaps leading to the higher activity. Although this work dealt with a phospholipase rather than a lipase, it is mentioned here because of the unique rationale that guided the work, and because it is one of the few reports to detail the use of a deletion approach to improve lipolytic activity.

Changes in the phospholipase / lipase ratio. It has been known for some time that lipases are active on some types of phospholipids [132]. The levels of activity are generally low compared to those against glycerides [132–134]. (An exception to this is a *Staphylococcus hyicus* lipase, which displays activities as much as 100 times greater toward phospholipids containing acyl chains between 6 and 10 carbons in length than toward triglycerides [135–137].) Recent reports describe the use of lipases in the modification and hydrolysis of phospholipids [38,135,137–143] and the production of lysophospholipids [144]. In the fungal lipases, it has been shown that a propeptide that is normally removed from the lipase during its maturation eliminates the phospholipolytic activity of the proenzyme while increasing the lipolytic activity [145]. In contrast, studies with the mammalian enzymes hepatic lipase (HL) and lipoprotein lipase (LPL) have achieved substantial increases in phospholipolytic activity through the fusion of portions of these enzymes [134]. The 3-D structure of pancreatic lipase, a representative of a conserved family of mammalian lipases, shows that the enzymes are composed of two well-resolved topological domains. The amino-terminal is composed of approximately 308 amino acids and contains the lipolytically active portion of the enzyme. The carboxyl-terminal domain consists of 141 amino acids and is the site of interaction of the protein with the activating protein colipase [146]. In their attempts to dissect the functions of these domains in HL and LPL, Davis et al. [134] conducted a domain exchange experiment, generating LPL/HL and HL/LPL hybrid proteins. Compared to the parent enzymes, the hybrids displayed elevations in phospholipase activity and reductions in lipase activity that resulted in final increases of two- to four-fold in the [phospholipase/lipase] ratio. This work appears to suggest a route for further substantial modifications in the relative preference of lipases for phospholipids vs. neutral lipids. However, advances will be needed before these gains achieve practical significance: even in the best case reported to date, the phospholipase activity of

the hybrid lipase was only one-tenth that of the lipolytic activity.

Alterations in the ratio of activities toward cholesterol esters and triglyceride esters. Human lysosomal acid lipase/cholesterol ester hydrolase (HLAL) belongs to the mammalian acid lipase gene family, all of whose members share prominent sequence homology, including the strict conservation of three of the seven cysteines in HLAL. Despite this conservation, the enzymes in this family are serine-, not cysteine, hydrolases, and the reason for the conservation of the cysteines is unclear. Replacement by alanine of one or both of two of the conserved cysteines in HLAL virtually eliminated activity toward cholesterol esters while reducing triglyceride hydrolysis between three- and eight-fold [147]. (Human gastric lipase is a homologous lipase that hydrolyzes triglycerides but not cholesterol esters. These same investigators found that when the corresponding mutations were introduced into this enzyme, its hydrolytic activity toward triolein was virtually eliminated. Activity toward tributyrin at submicellar concentrations was reduced only four-fold [147]. It is not clear if this represents an alteration of the fatty acid substrate specificity of the enzyme or the elimination of lipolytic activity with partial retention of esterase activity.)

3.3.3.4. Alteration of lipase stability through molecular engineering. Because the ester bond is ubiquitous in organic chemistry and biochemistry, because esters are found in a diverse range of molecules of commercial interest, and because lipases are active in water and in a host of organic solvents, there is interest in the use of these enzymes to catalyze a variety of reactions under a variety of reaction conditions. However, natural selection has optimized lipases for fat hydrolysis in largely aqueous, biological environments, not for these varied applications. Molecular engineering has been applied to modify the biochemical properties of lipases in order to increase their suitability in novel applications. In some cases, advances have been made by extrapolation of basic biochemical knowledge about lipases and proteins. Advances of this type will continue as the base of biochemical knowledge grows. In other instances, desirable improvements appear to have been discovered almost serendipitously. At any rate, this is an active area of research from which substantial progress can be anticipated in the future.

Laundry applications. Since many types of fabric stains are lipids, lipases have substantial potential for, and are presently being used as, components of laundry detergents. There has been considerable effort to increase the stability and activity of these enzymes under the harsh conditions of the laundry environment. Progress in this area is largely documented in the Patent literature rather than in the formal scientific research literature. In addition to the increases in activity in a laundry setting that were discussed above (Section 3.2.1), areas in which genetic engineering has been applied to improve the utility of lipases as laundry additives include the following.

Protease resistance. Many detergents now contain proteases, and can reduce the effectiveness of lipases as laundry agents through proteolysis. Directed mutagenesis was employed to reduce the protease-sensitivity of a *Pseudomonas* lipase [148]. In this work, the sites within the enzyme that were cleaved by laundry proteases were first determined. (These generally lie in surface loops.) These were then mutagenically modified to reduce proteolytic susceptibility, using approaches such as (i) deletion of up to three residues at the cleavage site, (ii) conversion of the amino-donating component of the scissile peptide bond to a proline, (iii) changing the polarity or the charge of the region around the potential scissile bond to one opposite the polarity favored by the protease, and (iv) introducing sequence alterations that modified the glycosylation patterns of the lipase.

Resistance to oxidation. Another component of laundry detergents that could reduce the effectiveness of lipases is bleach, which primarily inactivates enzymes through the oxidation of

methionines. Accordingly, success in engineering bleach resistance into lipases has been achieved by the replacement of methionine groups with other amino acids by site-directed mutagenesis [148,149].

Thermostability. In some cases, alterations in biochemical properties can accompany changes in protein sequence that are undertaken for other reasons. For example, rather than the typical (highly conserved) sequence Gly–X–Ser–X–Gly around its active site serine, the B lipase of *C. antarctica* possesses a threonine at the amino-terminal position of this pentapeptide. To explore the effects of this sequence on the activity of the lipase, Patkar et al. [150] mutagenically replaced the Thr with a Gly. The change had little impact on the ester synthesis capability of the enzyme, as monitored by the synthesis of decanol esters of three different fatty acids. Tributyrin hydrolysis was reduced in the mutant, but only two-fold. Such a reduction in activity upon alteration of a residue in close proximity to the active-site serine is perhaps not surprising. What was notable, however, was a substantial increase in the thermostability of the mutant lipase. Compared to the wild-type enzyme, an approximately five-fold increase in the length of incubation at 60°C was required to reduce enzymatic activity by 50%. The authors speculated that internal rearrangements in the tertiary structure of the enzyme in response to the mutation, or binding of water molecules in the vacant space created by replacing the threonine side chain with the side chain hydrogen of glycine, were responsible for the altered thermostability of the mutant.

Up to this point, this review has largely examined cases where a single or a few amino acids were mutagenically modified, and where the identify of the new amino acid introduced by mutagenesis was predetermined by the researcher. An alternative method is to conduct mutagenesis across all, or a region, of a protein, allowing a variety of amino acid replacements at multiple locations, and then to identify from within the resulting population of mutant enzymes those that display desirable alterations in biochemical properties. This approach is potentially more powerful than that of introducing single mutations at chosen single sites, because it requires neither advance knowledge regarding which sites contribute to the trait of interest, nor knowledge concerning what type of amino acid replacement is required to effect a change in that trait. In the vast number of cases, such information is lacking. Furthermore, since the properties of an enzyme are generally determined by more than one of its amino acids, the ability to make multiple mutations has greater potential for creating a change in a property. What such an approach does require, however, is an efficient means of screening the large number of mutant enzymes generated by this approach. The error-prone polymerase chain reaction [151] is one method for the introduction of multiple random mutations into a DNA fragment. By screening 2600 mutant *Pseudomonas aeruginosa* lipases derived by this method, Shinkai et al. [152] isolated four enzymes that displayed slight increases in their thermostabilities. Some of these mutants also displayed increased pH stabilities, especially at alkaline pH values [152]. The k_{cat}/K_m values of the mutant lipases were reduced by 14–53% compared to the wild-type enzyme, and specific activities were reduced by 10–50%. Asn-to-Ser and Leu-to-Pro mutations at residues lying in the vicinity of the catalytic triad of the enzyme were identified as the causes of these changes. The authors postulated that the increased thermostability was due to reduced backbone flexibility as a result of mutation. The increase in pH stability was displayed by all mutants wherein mutation had replaced the asparagine. It was not determined if stabilization resulted from the reduction of a destabilizing effect caused by the Asn, or a stabilizing influence imparted by the Ser introduced in its place.

Improved single wash performance. It has been generally observed that in using lipases in laundry applications, two wash cycles are required to achieve effective stain removal. This

is believed to be due to the fact that the lipases do not catalyze appreciable amounts of hydrolysis during the wash cycle itself, but do so as the water content of the garment is reduced during drying. During a subsequent wash, the products of lipolysis are removed, resulting in removal of the stain. Frenken et al. [153] postulated that by increasing the size of the hydrophobic lipid contact zone they could increase the affinity of lipases for their lipid substrates and thereby achieve superior first-wash activity. One caveat to this work was that the degree of increase in hydrophobicity must be limited, to avoid aggregation of the enzyme. Using computer-assisted molecular modelling, these workers examined 3-D models of the *Ps. glumae* and *Ps. alcaligenes* lipases to identify sites within and near the lipid contact zone. Rational mutagenesis was then employed to increase the number of hydrophobic amino acids in this region. Interestingly, in addition to the amino acids generally accepted as having hydrophobic side chains (e.g., Ala, Val, Leu, Ile, Pro, Phe, Trp, Tyr), the authors also advocated the introduction of lysine and arginine, postulating that these residues increase surface hydrophobicity through exposure of the methylene portions of their side chains. Methionine was also reported to function successfully in this capacity. Using these principles, it was possible to generate mutant lipases with significantly enhanced single-wash cleansing capabilities.

A recent report describes a two-step approach to enzyme modification [154]. In the first step, any chemical or molecular biological method is used to randomly mutagenize the lipase DNA. Following expression in an appropriate host, the population is screened to identify mutant lipases exhibiting desired biochemical changes. The DNAs of two or more such mutant lipases are then introduced into a recombination-proficient host. Recombinant lipases generated in vivo during subsequent growth of this strain are screened to identify new enzymes with desirable properties. In this manner, multiple improvements can be readily introduced into a single lipase molecule. Using this technique, and a screening method that mimicked a single wash of a stained fabric, a lipase was produced that removed stains effectively on the first wash [154]. This is but a single example of the power of the two-step mutagenesis protocol, which should generally be useful in improving the catalytic performance of lipases.

Improved surfactant resistance. In laundry applications, lipases are faced with denaturation by surfactants, a standard component of laundry detergents. Surfactants are amphiphilic molecules possessing both hydrophobic aliphatic regions and polar or charged (negative or positive) regions. They are general protein denaturants, presumably acting via penetration of the hydrophobic portion of the surfactant into the hydrophobic interior of the protein and causing it to unfold.

Aehle et al. [155] and Frenken et al. [156] increased the surfactant resistance of *Pseudomonas* spp. lipases by examining 3-D models of enzymes and introducing mutations at those sites most likely to interact with surfactants. The modified sites included (i) hydrophobic patches on the enzyme surface, which were perturbed through the introduction of polar and charged amino acids to reduce surfactant affinity for these sites, (ii) hydrophobic cavities that could represent sites for surfactant penetration, which were filled through the mutagenic introduction of bulky amino acids into their walls, and (iii) charged amino acids lying adjacent to hydrophobic patches and thus forming potential first sites for surfactant–enzyme interaction via electrostatic interaction. These were modified by mutagenic conversion to polar but uncharged amino acids. At least 20 mutant enzymes with increased stability in a laundry setting were described [155,156], most of them containing a single mutation. These enzymes displayed up to ten-fold increases in their half-lives in the presence of anionic surfactants, and up to 60% increases in their abilities to remove lipid stains from fabric under laundry conditions. Furthermore, although it had not been a stated goal to

boost lipolytic activity, increases of up to three-fold over that of the parent enzyme were noted.

An increase in surfactant resistance has also been reported following random, or restricted random, mutagenesis [157]. An advantage of this approach is that it does not require that a 3-D model of the lipase be available.

Reduced calcium dependence. It has been often observed that lipase activity in aqueous solutions is stimulated in the presence of calcium, a phenomenon that is attributed to the ability of calcium to form electrostatic interactions with the fatty acids generated by hydrolysis, removing them from the surface of lipid micelles and allowing continued access by the lipase to triglycerides within the micelles. Since the wash water is the source of the necessary calcium in laundry situations, optimal lipase activity will not be attained in geographic regions where the water is naturally low in calcium or is treated to remove calcium. To address this issue, Svendsen et al. [157] used random chemical mutagenesis, and PCR-based mutagenesis localized by the choice of PCR primers, to modify the HLL gene. The authors indicate that the mutagenesis was best conducted on residues in the lid or hydrophobic cleft region near the active site. A filter-based, solid-phase assay was employed to identify mutant lipases that were active under conditions where the free calcium concentration had been reduced by the addition of a chelator. To detect mutant lipases that had also gained surfactant resistance, a similar screening protocol was employed, with the exception that an alcohol ethoxylate was added to the assay medium. Both single- and multiple-site mutants were identified that displayed the desired changes in calcium and detergent sensitivities. These enzymes displayed activities as much as five-fold greater than the parent lipase when measured with an assay that mimicked laundry conditions.

Improvements in the calcium independence and detergent resistance of lipases have also been recently achieved by the addition of peptide extensions to the amino- and carboxy-termini of these enzymes [158]. Earlier work had shown that the proenzyme forms of some lipases display biochemical properties different from those of the corresponding mature enzyme [145,159]. Evidently, a peptide extension, such as is present in a prolipase, is capable of interacting with an enzyme and may therefore be able to stabilize or activate it. In the application of this principle to lipase improvement, it was found that a peptide addition of between 1 and 15 amino acids gave optimal results [158]. The extension is added not by chemical fusion of a peptide to the mature protein but through extension of the lipase gene. Examples [158] of the use of this approach include the addition of terminal peptides that:

– form secondary structures, e.g., helices or sheets, thereby stabilizing the enzyme.
– contain one or more cysteine groups appropriately placed to form disulfide bridges between the extension and the body of the protein, thereby stabilizing the enzyme.
– contain basic and hydrophobic amino acids, to increase the affinity of the lipase for the negatively charged surface of its lipid micelle substrate.
– are themselves protease resistant due to the presence of proline residues.

In addition, it has been reported that alterations of lipase properties can be achieved by making similar changes in those portions of the mature enzyme that lie N-terminal proximal or C-terminal distal, respectively, to the first or last structural elements of the enzyme [158]. A further useful approach is to subject these regions to random mutagenesis and examine the resulting mutant population for individuals with increased stability or activity [158]. These efforts were made in an attempt to stabilize lipases for laundry applications, and to produce new enzymes with enhanced laundry performance. However, they appear to be generally applicable for modifying a lipase, or any protein, to opti-

mize its performance in a particular application or environment.

3.3.4. Future prospects for the modification of lipolytic enzymes by genetic engineering

Since laundry applications will continue to constitute a high-volume use for lipases, it is expected that efforts to improve the activities and stabilities of these enzymes in such settings will continue. Despite the fact that lipases are also used as catalysts in many essentially non-aqueous formats, there has been little reported progress on the alteration of these enzymes to increase their activities under these conditions. Also, although lipases can catalyze ester and amide bond formation and transformation using a wide variety of substrates, the main focus of efforts to date has been on improving their performance as glyceride hydrolases. With the continued and expanding use of these enzymes in non-traditional solvents, and for the catalysis of reactions other than lipid hydrolysis, future research should see efforts to increase lipase activities and specificities in these types of applications and conditions. This will be particularly true in cases where the products of the reaction have high economic values.

The use of directed gene modification for the improvement of lipases as applied catalysts has been hampered to date by a scarcity of knowledge regarding which regions or individual amino acids in these enzymes are involved in such phenomena as substrate binding, catalysis, and stability. As structure–function relationships become known, for example, through the development of methods and databases for more powerful interpretation of the structure–function implications of specific mutations [160], substantial improvements in enzyme performance through more efficient application of rational mutagenesis should occur.

Perhaps more important to the future direction of enzyme modification will be the development of alternate approaches to gene modification that do not depend for their success on the availability of structure–function information. To date, the majority of attempts to alter lipases by genetic engineering have involved single modifications localized to sites or regions of the protein predetermined by the investigator to play a role in determining one or another of its properties. This is a restrictive method of enzyme improvement because it relies on a preconceived decision about what part(s) of the enzyme determine the property of interest. The accumulation of the experimental data on which to base such a decision can be a slow and arduous process. Furthermore, many lipase mutagenesis projects to date have modified only a single amino acid residue at a time, whereas an enzyme property is most likely the sum of the contributions of multiple sites in that protein.

An alternate and very promising new approach is the introduction of single or multiple random mutations throughout a population of genes, particularly by means of a modified polymerase chain reaction, followed by screening of the resulting mutant population to detect enzymes with improved properties. Arnold [161] has pioneered the use of this strategy, augmenting its power by using sequential cycles of mutagenesis and screening to create successive generations of improved enzymes. In this manner, the enantioselectivity of a *Ps. aeruginosa* lipase in the hydrolysis of racemic *p*-nitrophenyl 2-methyldecanoate was increased 40-fold in just four rounds of mutagenesis [162]. By employing in vitro recombination in conjunction with this approach, hybrid enzymes can be produced that combine beneficial mutations that were originally generated independently from one another. Using this technique, which continues to improve [163–165], the activity of a bacterial *p*-nitrobenzyl esterase in aqueous-dimethylformamide for the deprotection of an ester intermediate formed during synthesis of the antibiotic loracarbef was increased about 60-fold [166]. Mapping the mutations onto the 3-D structure of the enzyme indicated that none of them was at a site that appeared to interact directly with the antibiotic substrate [166], further indicating the power of random mutagene-

sis for enzyme improvement. The use of this type of approach for the improvement of a lipase for laundry applications was discussed above [154]. One suspects that in the near future, profound advances in the production of custom lipases will be achieved through application of new mutagenesis technologies such as this.

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