Chapter 1

General Introduction

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1.1. Enzymes

Enzymes (gr. ἐνζυμο – in yeast) are biomolecular-based substances that catalyze chemical reactions. Nearly all known enzymes are proteins, although certain nucleic acids, called messenger ribonucleic acid (mRNA), also possess catalytic abilities. Enzymes are found in every living cell of every living plant and animal, including the human body, and almost all processes in a biological cell need enzymes to occur at significant rates. Although enzymes are generally large, only a small portion (around 3 to 4 amino acids) is near or in direct contact with its substrate and directly involved in the catalysis. The part of an enzyme that contains the catalytic residues, on which the substrate attaches, and then chemical change occurs is known as the active site (in the case of 3 amino acids being involved, the active site is called a catalytic triad).

The enzyme’s amino acid sequence determines the characteristic folding patterns of the protein’s structure, which is essential to enzyme activity and specificity. If the enzyme is exposed to changes, such as fluctuations in temperature or pH, the
protein structure may alter (denature) and lose its catalytic ability. Denaturation is sometimes, but not always, reversible.

In order to perform catalytic activity, most of the enzymes require an additional non-protein component bound to enzyme called a cofactor (vitamins, metal ions, etc). Most cofactors are not covalently attached to an enzyme, but are very tightly bound. If the enzyme and cofactor are tightly attached, such an enzyme is referred to as a holoenzyme, while the cofactor is called a prosthetic group.

Enzymes are promising biocatalysts with excellent features such as high activity, specificity and selectivity, and can catalyze under mild and environmental friendly conditions. However, enzymes have been optimized, via natural evolution, to fulfil their biological function: to catalyze reactions in complex metabolic pathways exposed to many levels of regulation. Therefore they can catalyze reactions with an accuracy that cannot be obtained via conventional methods.

Enzymes are classified into several broad categories, depending on the type of reaction they control:

(i) oxidoreductases (dehydrogenases, reductases) - catalyze the transfer of electrons from one molecule (the reductant) to another (the oxidant);
(ii) transferases (kinases, aminotransferases, thiolases) - catalyze the transfer of a functional group from one molecule (the donor) to another (the acceptor);
(iii) hydrolases (peptidases, glycosidases, lipases, phosphatases) - catalyze the hydrolysis of a chemical bond;
(iv) lyases (synthases, decarboxylases, dehydratases) - catalyze the breaking of various chemical bonds by means other than hydrolysis and oxidation, often forming a new double bond or a new ring structure;
(v) isomerases (isomerases, mutases) - catalyze the structural rearrangement of isomers;
(vi) ligases (synthetases, carboxylases, polymerases) - catalyze the joining of two large molecules by forming a new chemical bond, usually with accompanying hydrolysis of a small chemical group pendant to one of the larger molecules.

1.2. Lipases

Lipases or triacylglycerol acylhydrolases are water-soluble enzymes that catalyze the hydrolysis of ester bonds in water–insoluble, lipid substrates, and therefore comprise a subclass of the esterases. No bonds other than carboxyl ester bonds have been found to be catalyzed by lipases. In this respect, lipases must be considered as carboxylesterases and they constitute a distinct group in the large esterase family.

Lipases are ubiquitous enzymes of considerable physiological significance and perform crucial roles in the digestion, transport and processing of dietary lipids in
most of living organisms. Thus, lipases can be found in diverse sources, such as plants, animals, and microorganisms. More abundantly, they are found in bacteria, fungi and yeasts (Table 1.1).

Table 1.1. Some examples of the lipases produced by microorganisms.[1]

<table>
<thead>
<tr>
<th>Source</th>
<th>Genus</th>
<th>Species</th>
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<tbody>
<tr>
<td>Bacteria</td>
<td>Bacillus</td>
<td><em>B. megaterium</em></td>
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<td><em>B. cereus</em></td>
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<td></td>
<td>Staphylococcus</td>
<td><em>S. canosus</em></td>
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<td></td>
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<td><em>S. aereus</em></td>
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<td></td>
<td>Micrococcus</td>
<td><em>M. luteus</em></td>
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<td></td>
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<td><em>M. fredenreichii</em></td>
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<td></td>
<td>Pseudomonas</td>
<td><em>P. glomae</em></td>
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<td></td>
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<td><em>P. fluorescens</em></td>
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<td>Fungi</td>
<td>Rhizopus</td>
<td><em>Rhizop. Delemar</em></td>
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<td></td>
<td>Aspergillus</td>
<td><em>A. flavus</em></td>
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<td><em>A. niger</em></td>
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<td></td>
<td>Penicillium</td>
<td><em>Pc. Cyclopium</em></td>
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<td><em>Pc. Camamberti</em></td>
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<td></td>
<td>Mucor</td>
<td><em>Mu. miehei</em></td>
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<td></td>
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<td><em>Mu. Javanicus</em></td>
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<tr>
<td>Yeasts</td>
<td>Candida</td>
<td><em>C. rugosa</em></td>
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<tr>
<td></td>
<td>Pichia</td>
<td><em>Pt. Bispora</em></td>
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<td></td>
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<td><em>Pt. Sivicola</em></td>
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<td></td>
<td>Actinomycetes</td>
<td><em>Str. Cinnamomeus</em></td>
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<td><em>Str. Coelicolor</em></td>
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Lipases catalyze the hydrolysis of relatively long chain triglycerides (with acyl chain lengths of over ten carbon atoms) to the corresponding diacylglyceride, monoacylglyceride, glycerol and fatty acids. Since the water insoluble lipid interferes with the water soluble lipase, digestion of these triglycerides takes place at the water-oil interface.[2,3] The surface area of the interface therefore determines the rate of the hydrolysis. On the other hand, it is well known that the reaction is reversible and lipases can catalyze ester synthesis and transesterification in the reaction containing low water concentrations.[4,5] The hydrolytic and synthetic action of lipase is as shown Figure 1.1.

For usage in chemistry lipases from microbial origin are commonly used and commercially available.
1.2.1. Applications of lipases

Biocatalysts, especially lipases, are increasingly used in organic chemistry due to their excellent properties. First of all, the availability of lipases makes them very attractive. Lipases can be obtained by isolation from several microorganisms (Table 1.1) and can nowadays be bought from several commercial suppliers. With recombinant DNA techniques, lipases can even be obtained in very high purities, which make them ideal for use in organic chemistry.

A second advantage of lipases is their high stability. Many lipases tolerate elevated temperatures and exposure to several organic solvents. For example, high activities for the lipase from *Candida antarctica* at high temperature, and in hydrophobic solvents have been reported.[6-9]

Another very important property of lipases is the broad range of unnatural substrates that are accepted. Substrates different from the natural ones can be used, which increases the applicability of lipases.[10]

Furthermore, lipases catalyze in a regio- and stereoselective fashion. Lipases from different sources have different enantioselective properties and different substrate specificities. Therefore, it is usually possible to choose the right enzyme for a specific synthesis from a large database of lipases.

A final advantage of lipases is their ability to catalyze several different reaction types. Ester hydrolysis, esterification, interesterification, polymerization, and amidation reactions are reported in literature.[11,12]

These advantages, including the great variety of unnatural substrates, the different reaction types that can be catalyzed, the stability at elevated temperatures and in organic solvents, their enantioselectivity and their availability, make lipases very promising for use in organic chemistry.

Lipases already find a lot of applications for example as detergents, in food processing or in the synthesis of cosmetics, pharmaceuticals and chemicals.[1]

However, to be added to a laundry detergent, the lipase has to tolerate washing conditions (30-60 °C), proteases and surfactants (which are ingredients of many detergents) and they need to have a low substrate specificity in order to remove fats of different compositions.[1] In food processing, among other things, lipases have been used to replace undesirable fat with a high-value fat. For example, Undurraga
et al. have used Novo-Nordisk's lipozyme™ for a transesterification reaction which replaces the palmitic acid in palm oil with stearic acid to produce a cocoa butter substitute. The cosmetic, pharmaceutical and chemical industry use lipases among other things as catalysts to make enantioselective products.

1.2.2. Structure and the function of lipases

Lipases, like almost all enzymes, are generally globular proteins. Most lipases are very large in comparison with the substrate with which they interact. Like all proteins and enzymes, lipases are long, linear chains of amino acids that fold to produce a three-dimensional product. The three-dimensional structure of all lipases follows a common motif, the αβ-fold of hydrolase, where lipase consists of eight parallel β-sheets surrounded on both sides by α-helices (Figure 1.2). Though lipases differ widely in the number of amino acids in their primary sequences, the common feature of all lipases is that their active site is composed of the three amino acids serine (Ser), aspartate (Asp) or glutamate (Glu) and histidine (His). Most of the lipases have a lid, i.e. a surface helix covering the active site of the lipase. In order to perform full catalytic activity, lipases also need additional non-protein components called procolipases.

![Figure 1.2. Schematic diagram of αβ-hydrolase folding.](image)

The structure and function of lipases have been thoroughly studied. Already in 1990 Winkler et al. elucidated the structure of the human pancreatic lipase. Three years later van Tilbeurgh et al. revealed the mechanism involved in the interfacial activation (Figure 1.3). Namely, during the catalysis a short α-helix (the lid) adopts a totally different conformation and folds back leading to activation of the lipase (open form), which allows the substrate to bind to the enzyme's active site. Otherwise, in the absence of an aqueous/lipid interface, lipase resides in its inactive state and a lid covers the active site (closed form).
The functional consequences of the structural reorganization of the lid are as follows: (i) the substrate has access to the active site; (ii) a second loop (β5-loop, red in Figure 1.3), which makes van der Waals contacts with the closed lid, now changes its conformation and folds back upon the core of the protein when the lid is open; (iii) since both the lid and β5-loop are folded away from the active site, which is stabilized by many hydrogen bonds with the exterior of the molecule, hydrophobic side chains become exposed, whereas hydrophilic ones are buried, and therefore the hydrophobicity around the active site is considerably increased (a major contributor to this increase is the hydrophobic site of the amphiphatic main helix of the lid); (iv) interaction of the catalytic N-terminal domain with procolipase via the open lid occurs and is stabilized by hydrogen bonds.

![Fig. 1.3. Mechanism of interfacial activation of triacylglyceryl lipase.][17]

The position and conformation of amino acids forming the catalytic triad are not significantly different in the open and closed form. However, the combined conformational changes of the lid and the β5-loop, change the environment of the catalytic triad dramatically. The active site becomes totally accessible to solvent and sits at the bottom of a hydrophobic region which is well adapted for the binding of a lipid substrate.

1.2.3. *Candida antarctica* lipase B

*Candida antarctica* lipase B (Cal-B) is a versatile enzyme for enantio- and regioselective transformations on many low molar mass and polymer substrates and has been found to possess a broad range of catalytic activities for chemical synthesis. [18-22]
This enzyme has numerous other advantages: (i) stability in acidic pH range, (ii) quality of end product, (iii) less side products and (iv) performing at high temperatures.

The structure of Cal-B was elucidated by Uppenberg et al.\cite{23} in 1994. Cal-B is constituted of 317 amino acid residues with a formula weight of 33,273 Da. Cal-B is a globular α/β type protein with approximate dimensions of 30Å x 40Å x 50Å, measured by X-ray crystallography (Figure 1.4 shows the secondary structure and the surface of Cal-B). The amino acid sequence shows no significant homology to other lipase sequences and deviates from the consensus sequence around the active site serine that is found in other lipases. From the structures determined for other lipases, Cal-B would most likely contain a Ser-His-Asp/Glu catalytic triad (active site is emphasized in Figure 1.4).

In the structure of Cal-B, the active site is accessible to external solvent through a narrow channel. It is approximately 10Å x 4Å wide and 12Å deep. The channel walls are very hydrophobic and are lined with mostly aliphatic residues. The accessible active site suggests that the enzyme might have no lid to control entry to the active site. Cal-B has a large hydrophobic surface surrounding the entrance of the active site channel and is probably in close contact with a lipid surface during hydrolysis. The surface is dominated by side chains from aliphatic residues, oriented towards the solvent. Although Cal-B has no lid and did not display any interfacial activation\cite{24} (the catalytic behaviour of Cal-B is more typical of an esterase than of a lipase), a small helix (α5) in Cal-B has been suggested to act as a lid because of its observed disorder in some crystal structures, indicating a region
of high mobility.\textsuperscript{[23]} The proposed lid is a 5 residue long hydrophobic helix in which Glu-145 makes hydrogen bonds to Ser-150 and Thr-158, respectively, in the ordered structures (red arrow in Figure 1.4). Proposed \( \alpha_5 \) helix in Cal-B is not involved in any conformational change regulating the access to the active site. Instead this mobile helix may be seen as part of the lipid binding surface, anchoring the lipase to a water/lipid interface.

Recently this biocatalyst has been the subject of several structural modifications using genetic engineering to improve its selectivity\textsuperscript{[25]} and to create novel synthetic applications.\textsuperscript{[26,27]}

Due to the many benefits of using immobilized enzymes rather than their soluble counterparts, many researchers have focused on the improvement of Cal-B performance using enzyme immobilization.\textsuperscript{[28-38]} In fact Cal-B immobilized onto a macroporous acrylic polymer resin (VP OC 1600, Bayer) is the most widely used lipase for specialty chemical manufacture. Commercial utilizations of Cal-B are limited to high-priced commercially available Cal-B preparations: Novozyme 435 (Novozymes A/S) and Chirazyme (Roche Molecular Biochemicals).

1.3. Enzyme immobilization

As already discussed above, natural enzymes possess excellent features. However, most of them show none of their profound characteristics in organic solvents and can easily denature under industrial conditions (high temperature, solvent effects, mechanical shear, etc). Recovery of enzymes from reaction solutions and separation of the enzymes from substrates and products are generally difficult. All together this causes the relatively seldom use of biocatalysts in industry. An important route in improving enzyme performances in non-natural environments is to immobilize them. Enzyme immobilization is the localization of an enzyme, i.e. restricting the enzyme mobility. Enzyme immobilization was first introduced to enable the reuse of costly enzymes. Some of the initial attempts to do this were described during the early parts of the last century,\textsuperscript{[39]} but the enzymes when adsorbed to charcoal proved to be very unstable. Around the 1950s, several groups began to immobilize enzymes onto other supports.\textsuperscript{[40-42]} Georg Manecke was one of the first to succeed in making relatively stable immobilized systems of proteins on polymer carriers.\textsuperscript{[42]} The first industrial applications of immobilized enzymes were in the production of optically pure amino acids\textsuperscript{[44]} and the hydrolysis of penicillin G.\textsuperscript{[44]} Since then a lot of research has been conducted.\textsuperscript{[11,45,46]}

The main advantage of immobilization is the ease with which the enzyme can be recovered after the reaction. This also enables reuse of the enzymes even in another type of reaction. Another advantage can be the increased activity and increased stability of the enzyme. After immobilization some enzymes show an increased temperature resistance and an increased tolerance towards organic solvents.\textsuperscript{[47,48]}
There are different ways to establish immobilization:
- Physical adsorption: the enzyme is bound to a support by hydrophobic, van der Waals or ionic interactions.
- Covalent binding: the enzyme is covalently bound to a support.
- Entrapment via inclusion: the enzyme is trapped in a polymer network or a membrane device, such as a microcapsule.
- Crosslinking of enzyme aggregates: the enzyme molecules are cross-linked by a bifunctional reactant to create carrierless macroparticles.

### 1.3.1. Properties of immobilized enzymes

The properties of immobilized enzymes are determined by the properties of the enzyme and of the support. This is schematically represented by Figure 1.5.

![Properties of enzyme and carrier determine the properties of the immobilized enzyme.](image)

The biochemical properties of the enzyme, such as its molecular mass, functional groups on the surface and its purity are important factors for immobilization. The functional groups on the surface of the enzyme for instance give information on which kind of interactions between the support and the enzyme can take place. Also the purity of the enzyme is important as if for instance an impure enzyme is used the impurities can interfere with the substrates.
Other features of the enzyme that determine the parameters of the immobilized enzyme are the reaction type and the kinetics of the reaction that is catalyzed by the enzyme. Specific activities, kinetic parameters for activation and inhibition and stability against pH, temperature, solvents and impurities have impact on the immobilized enzyme as well.

The characteristics of the carrier material also influence the properties of the immobilized enzyme. One of the most important features of the carrier is the chemical structure that will determine interaction with enzymes. If the support material is highly porous, pore size and pore size distribution will play an important role in determining the immobilized enzyme properties. A small pore size can cause diffusion limitation resulting in structural rearrangement of the enzymes and subsequent inactivity. However, for very large pore sizes enzymes can cluster together and thus lose activity.

The mechanical properties of the carrier material are crucial in the application of the immobilized enzyme. When an immobilized enzyme is applied in a stirred tank, it has to have other properties than when it is applied in a column. In a stirred tank, the carrier material must be resistant to abrasion, while in a column it has to have some flow resistance.

Carrier particle size is also significant. An example of the influence of particle size on the distribution of enzyme throughout the carrier bead is given by Chen and coworkers.\textsuperscript{[50]} They examined enzyme concentration inside a carrier (methyl methacrylate resins) with infrared microspectroscopy and found that the carrier with the smallest particle size had an almost uniform distribution of enzyme in the carrier (Figure 1.6).

![Infrared microspectroscopy images to analyze Cal-B distribution within a series of MMA resins that differ in particle size (diameters: \#1=600μm, \#2=120μm, \#3=75μm, \#4=35μm).\textsuperscript{[50]}](image)

When the characteristics of the enzyme and carrier are combined, some other features also influence the properties of the immobilized enzyme. The
immobilization method, as was discussed above, is also crucial for biocatalyst properties (pH, temperature, time of immobilization, etc). The rate of the enzyme-catalyzed reaction is greatly influenced by mass-transfer effects. Due to mass transfer to, from and inside the immobilized enzyme, micro and nano environments occur with different pH, concentration, etc. This effect arises from the fact that an immobilized enzyme is bound to the support and has a deliberate restricted mobility. It can affect the mobility of the solutes as well. The solutes can be adsorbed to the support, resulting in a reduced mobility which causes a decreased reaction rate compared with soluble enzymes. Not only does this reduced mobility of the solutes cause a reduced reaction rate, but also diffusion restrictions on the carrier surface or effects from internal or porous diffusion can also lower the reaction rate.

1.3.2. Supports

Three of the above mentioned immobilization techniques require a support, physical adsorption, covalent binding and entrapment via inclusion. These three are also the most commonly used immobilization techniques. As can be seen from Figure 1.5, the properties of an enzyme immobilized on a carrier greatly depend on the properties of the support. A support is therefore important and should be chosen with care.

Frequently used carriers are categorized into:

- inorganic supports
- organic supports from natural sources
- synthetic organic supports

Inorganic supports often obtain a greater stability than organic supports, due to the higher inertness to the reaction conditions such as high pressure and temperature. On the other hand, abrasion can occur in stirred vessels. Frequently used inorganic carriers are silica and silica derivatives, celite and aluminum based carriers. All these materials have been extensively studied and developments have lead to the application on both laboratory and industrial scale.

On the other hand, organic supports from natural sources, mostly polysaccharides, have the advantage of enhanced compatibility with the enzymes. Due to their hydrophilicity, they undergo only weak interactions with the enzyme, leading to minimal inactivation, but unfortunately it also leads to poor binding and therefore the materials often have to be functionalized. Also the mechanical stability of these materials is rather weak which can be increased by cross-linking. Agarose, cellulose derivatives and cross-linked dextrans are commonly used natural supports.

Synthetic organic supports, for instance synthetic polymers, are widely used as immobilization carriers. Ion exchange materials, frequently based on polystyrene
that can be cross-linked with divinylbenzene, have been used for enzyme immobilization. By adding various amounts of divinylbenzene different network densities can be obtained. Furthermore, several copolymers are used in enzyme immobilization. Changing the ratio between the two comonomers, the properties of the copolymer (hydrophilicity/hydrophobicity, amount of functional groups, mechanical properties, porosity, etc.) can be selectively changed. Therefore custom-made carriers for enzyme immobilization for all kinds of different purposes can be produced.

1.4. Methods of immobilization

1.4.1 Physical adsorption

Physical adsorption is a simple and straightforward route for enzyme immobilization, in which the enzyme is bound to a support by hydrophobic, van der Waals or ionic interactions. It is often used because of the ease and low cost of the procedure. A support is added to an enzyme solution and after a few hours of mixing the enzyme-support complex is ready. Secondly, physical adsorption is reversible that enables the reuse of the support. Denatured enzymes can be removed from the support by changes in pH or ionic strength of the reaction medium and it can be replaced with fresh enzyme. However, a drawback is the leaking of enzyme upon use, so not all of the enzyme can be reused and the product might have to be purified.

Several different supports were used for the physical adsorption of lipases. Polymers, carbon nanofibers, silica, and celite have been reported. Due to the interfacial activation, that most lipases show, caused by hydrophobic interactions of the lid (exceptions are lipases form *Pseudomonas glumae, Pseudomonas aeruginosa* and *Candida antartica* lipase B, because of the smaller or missing lid) physical adsorption to hydrophobic surfaces is promising. Octadecyl–sepabeads (Mitsubishi Chemical Corporation) were used to immobilize the lipases from *Candida antarctica* (fraction B), *Mucor miehei* and *Candida rugosa* via interfacial adsorption. The same lipases were immobilized on glyoxyly-agarose via multipoint covalent attachment. Lipases adsorbed on octadecyl–sepabeads, as highly hydrophobic supports, exhibited a clear hyper-activation compared to the soluble enzyme or other types of derivatives. According to the authors, high activity is caused by the hyperactivation of the lipase due to interfacial adsorption to hydrophobic surfaces (Figure 1.7). The closed form of the lipase, considered inactive since the active site is isolated from the reaction medium by a polypeptide chain called the lid, is favoured in solution. But when the lipase comes in contact with a hydrophobic surface, the lid opens and the active
site is fully exposed to the reaction medium because of favourable interactions and the enzyme remains in this open formation (due to the equilibrium state) that is responsible for an increase in enzyme activity.

![Interfacial activation of lipases on hydrophobic supports at low ionic strength](image)

Fig. 1.7. Interfacial activation of lipases on hydrophobic supports at low ionic strength.\[^{[47]}\]

Multipoint covalent attachment of lipases on glyoxylo–agarose promoted a significant improvement in the enzyme stability. However, even these stabilized derivatives have much lower stability than that observed using the octadecyl–sepabeads, both in thermal and organic co-solvent inactivation assays.\[^{[66]}\] Lathouder et al. reported lipase immobilization on cordierite monoliths functionalized with polyethylenimine and with different types of carbon, consisting of carbonized sucrose, carbonized polyfurfuryl alcohol, or carbon nanofibers.\[^{[62,63]}\]

Compared to the free enzyme, the immobilized lipase shows a significantly lower activity. Increased stability, easy catalyst separation and the possibility to reuse the enzyme in immobilized form can compensate for this difference. The commercially immobilized lipase (Novozyme) initially has a significantly higher activity than the monolithic biocatalysts, but deactivates relatively fast. For the monolithic biocatalysts, no deactivation was observed.

The activity of physically adsorbed enzymes is strongly dependent on the pH of the solution and working temperature. Candida rugosa lipase was immobilized on poly(N-methylolacrylamide) by physical adsorption.\[^{[61]}\] The highest productivity and activity were obtained when working at the lowest level of temperature. On the other hand, the immobilization procedure shifted the optimum pH for the Candida rugosa lipase to a more alkaline value. The free lipase reached its maximum activity at pH 7.0, falling to about 75% at pH 8.0, while the poly(N-methylolacrylamide)-immobilized system showed its maximum activity at pH values of 7.5–8.0, retaining about 90% of its maximum activity at pH 8.5. This behaviour has been well described in the literature and can be related to the partial opening of the lipase lid upon immobilization.
1.4.2. Covalent attachment

Covalent attachment is the second most widely used method of enzyme immobilization, in which the enzyme is covalently bound to a support. Covalent binding does not suffer from desorption or leaching of enzyme during catalytic reactions, due to the firm bonding between the enzyme and the support. On the other hand, enhancement of the bond strength between the enzyme and the support can cause changes in the enzyme conformation, often into a less favourable one, which can result in deactivation of the enzyme.\[^{56}\] In immobilization through covalent attachment two different global methods can be outlined: covalent attachment via long spacer arms and covalent attachment via multipoint attachments (Figure 1.8). The spacer arm has the advantage of only moderately restricting the enzymes configuration, while the multipoint attachment is supposed to have a higher stability. The multipoint attachment method is often created by crosslinking with gluteraldehyde.\[^{67}\]

Covalent immobilization was reported by several groups.\[^{56,59,67,68-71}\] Different supports are used for immobilization and most of them are activated carriers with a high content of reactive groups, for example epoxy rings or activated carbonyl groups ready bind mainly to amine groups of the enzyme. Reversibly soluble polymers might be used for lipase immobilization.\[^{68}\] Reversibly soluble polymers have the advantage of immobilization, namely the recovery and reuse of the enzyme, but it also opens up the possibility of performing the reaction in solution, thereby putting aside the problems from solid-liquid mass transfer and pore diffusion.

On the other hand, γ-Fe\(_2\)O\(_3\) magnetic nanoparticles can also be used for the covalent immobilization of \emph{Candida rugosa} lipase.\[^{69}\] The advantage of magnetic particles is the easy separation, simply by applying a magnetic field and furthermore the small size of the particles enables applications in biological systems (Figure 1.9).
Combination of physical adsorption and multipoint covalent linking is a useful approach for lipase immobilization. Physically adsorption of the lipase on a hydrophobic support by interfacial separation can be followed by crosslinking with gluteraldehyde to obtain multipoint covalent attachment. A similar technique to immobilize lipase on a epoxy silica HPLC column has been reported. In both cases, higher enzyme activity was obtained compared with crude enzyme powder. In most cases, the relative activities with covalent attachment are quite low. This can be explained by the fact that covalent bonding between enzyme and support restrict chain mobility within protein molecules and therefore conformational changes that are essential during catalysis are disabled. However, despite this rather low relative activity, immobilized enzymes with an activity approximately the same as the free enzyme have the potential to be used in industry. The costs saved by recovery and reuse of the enzymes can compensate for the loss of activity. An improved stereospecificity can also be the reason for using covalently immobilized enzymes. Lipase from *Pseudomonas fluorescens* showed an increased enantioselectivity upon multipoint covalent immobilization on glyoxyl-agarose.
three-fold improvement in the enantioselectivity was reported. According to the authors this increase is probably due to a distortion of the enzyme structure, since the activity was decreased, or/and due to an increase in rigidity of the enzyme, since the enzyme showed an increased stability.[66]

Another feature that is well illustrated is the influence of the carrier.[68] Three chemically different supports were used for the immobilization of the same enzyme (lipase from *Pseudomonas fluorescens*). Immobilized biocatalysts had the same crude enzyme loading and were used under the same reaction conditions, but showed different relative activity. A 19-fold increase in activity of Eupergit® C250L, a copolymer of methacrylamide, N,N'-methylen-bis(acrylamide) and a monomer carrying oxirane groups, compared to aminopropyl-silica is reported. The enzyme is coupled to the Eupergit® 250L through the free oxirane groups. The increase in activity is probably caused by differences in interactions between support and enzyme, but can also be the result of different particle or pore sizes.

### 1.4.3. Entrapment via inclusion

Another possibility of enzyme immobilization is entrapment via inclusion, meaning that the enzyme is entrapped in a polymer network or a membrane device. Many researchers started with inclusion in polymeric matrices,[72] but more recently inclusion in hydrophobic sol-gel materials[73-76] and entrapment in hollow fibres[77] has been reported. In this immobilization method, the enzyme is retained within a device or material. It does not completely prevent leaking, but it considerably decreases it with regard to physical adsorption. Reetz et al. have established a method that enables the immobilization of lipases via inclusion in sol-gel materials. They studied the effects that several parameters (water content, type and amount of catalyst, variation of the precursor, etc.) have on the stability and enzyme activity. They have examined several lipases and several sol-gel materials with and without additives.[73] The immobilization of enzymes by entrapment in sol-gel-derived materials has turned out to be generally applicable to a wide variety of lipases resulting in significantly increased enzyme activity in an organic medium. One likely reason for the unusually high relative enzyme activity is the high dispersion of the lipase in the sol-gel matrix. An additional factor may be a possible interaction between the lipophilic domains of the lipases and the hydrophobic regions of the organic/inorganic sol-gel matrix. Most literature found on immobilization by inclusion is on sol-gel materials. This technique is based on the production of silica matrices by acid or base catalyzed hydrolysis of silane compounds such as tetraethoxysilane (TEOS). The use of sol-gel matrices, prepared with a combination of alkyl-alkoxysilane precursors of different chain-lengths, for the immobilization of cutinase from *Fusarium solani pisi* has been reported (Figure 1.10).[76] Several additives that
improved the catalytic activity of cutinase as well as of lipase from *Pseudomonas cepacia* were reported.

![SEM micrographs of the sol–gel matrices (1:5 TMOS/n-alkylTMS) with encapsulated cutinase (TMOS, MTMS, PTMS, BTMS and OCTMS stand for tetramethoxysilane, methyltrimetoxysilane, propyltrimetoxysilane, n-butyltrimetoxysilane and n-octyltrimetoxysilane, respectively). The scale bar represents 50 μm.][76]

Enantioselectivity studies with sol-gel encapsulated lipases were also performed.[75] Lipases (*Candida antarctica* type B, *Candida rugosa*, *Mucor miehei*, *Aspergillus niger*, *Burkholderia cepacia*, *Pseudomonas fluorescens*, *Candida rugosa* type VII, *Penicillium roquefortii* and *Thermomyces lanuginosa*) were encapsulated in sol-gel materials produced by the fluoride-catalyzed hydrolysis of mixtures of tetramethyl orthosilicate (Si(OCH₃)₄) and alkylsilanes (RSi(OCH₃)₃). This involves higher enzyme loading, variation of the alkylsilane precursor, and the use of additives such as isopropyl alcohol, 18-crown-6, Tween 80, methyl-β-cyclodextrin and/or KCl. All lipases tested showed significantly improved performance, although in two cases additive effects were not observed. In typical preparative scale reactions only about 250 mg of a lipase-containing gel are needed for 10 g of substrate.

### 1.4.4 Crosslinking of enzyme aggregates

Plenty of carrierless techniques are being developed, such as cross-linked dissolved enzymes (CLEs), cross-linked enzyme crystals (CLECs), cross-linked enzyme aggregates (CLEAs) and cross-linked spray-dried enzyme
CLECs are microcrystals that can be grown from an aqueous solution and can be cross-linked with a bifunctional agent (Figure 1.11). CLEC are macroporous and contain about 50% solvent by volume. The channels allow diffusion of product, substrate and solvent in, through and from the crystals.

**Fig. 1.11.** Optical microscope photograph cross-linked Candida rugosa lipase crystals (CRL-CLEC's) (250x magnification (reproduced at 50% of original size)).

CLEAs can be synthesized more easily than CLEC (Figure 1.12). Therefore CLEAs are possible alternatives to CLEC, since both can be prepared by precipitating the enzymes and subsequent cross-linking with for instance gluteraldehyde. CLEAs of penicillin acylase were reported by Cao *et al.* to give comparable activities as CLEC and showed furthermore a greater tolerance for organic solvents.

Enantioselectivity of a crude, commercial available lipase with a highly purified enzyme crystal was compared. The increased enantioselectivity of the CLEC is attributed to the purity of the enzyme crystals in comparison to the crude enzyme preparation.

Overbeeke *et al.* used the lipase from *Candida rugosa* for their experiments to see the influence of CLEC purity on the open and closed conformation of the lid on the enantioselectivity. According to the authors, changes in the active site are coupled to changes in the opening and closing of the lid. There is a twofold effect of the lid opening. Firstly, the activation barrier leading to Michaelis–Menten complex can be affected. Secondly, lid opening induces small changes in the active site structure influencing the enantioselectivity. This idea is strengthened by observations made by Peters *et al.* who found that substitution of the active site serine in *Humicola lanuginosa* lipase by alanine influenced the dynamic properties of the lid covering the active site. Furthermore, comparison of the open and closed
structures of *Candida rugosa* lipase reveals differences in the orientations of the side chains in the active site. Changes in the active site are thus coupled to changes in the opening and closing of the lid. Whether the E-values subsequently increase or decrease depends among others on the substrate used.

Fig. 1.12. *Comparison of the formation of CLEAs versus CLECs.*[90]

The catalytic activity of CLEAs of different lipases (*Candida antarctica* lipase A and B, *Thermomyces lanuginosus* lipase, *Rhizomucor miehei* lipase and *Aspergillus niger* lipase) was examined.[84] The authors reported the effect that additives such as sodium dodecyl sulfate, Triton X-100 or dibenzo-18-crown-6, have on the catalytic activity of the CLEA. Higher activity of the CLEA than the free enzyme was observed.

Lalonde *et al.* have also examined the thermostability in water-miscible organic solvents of crude and pure *Candida rugosa* lipase (CRL) and CRL-CLECs at 40 °C.[81] The catalytic activity of the CLEC shows an enormous increase in thermostability in comparison with the crude and free enzyme preparations. The stability of CLECs in 50% organic solvent solutions displays an amazing increase with regard to the crude enzyme preparation. The authors attribute these effects to a combination of crystallinity in the material and covalent cross-linking between the molecules. Due to the crystallinity the enzymes are closely packed, resulting in many protein-protein interactions, which can stabilize the enzyme crystals against unfolding, aggregation or dissociation.

1.5. Aim and outline of this thesis

The goal of this research is to extend the knowledge of enzyme immobilization as a process and to move beyond general correlations to a better understanding on a molecular level of how immobilization on surfaces can stabilize and activate
protein catalysts. This thesis also deals with attempts to improve enzyme activity and stability, trying different materials as carriers for enzyme immobilization, varying immobilization conditions, altering the porosity properties of the carrier, etc., but always with a strong background knowledge of the material properties.

In Chapter 2, synthesis of highly porous copolymers, as carrier for Candida antarctica lipase B immobilization, was performed. Chemically identical copolymers were obtained, differing in their porosity properties. The influence of the resin properties on the loading of Cal-B during immobilization and on the enzyme activity of the immobilized Cal-B was studied.

Since the synthesized copolymers contain an epoxy ring, Chapter 3 deals with the carrier modification. The epoxy group can be easily replaced with other functional groups that interact differently with the enzyme (either creating physical or chemical connections). The influence of the amount of the modifier on enzyme loading and activity was also discussed.

A strategy for chemical modification and cross-linking of Cal-B is presented in Chapter 4. Different epoxides and diepoxides are used for this purpose. The influence of the length of the epoxide arm in the modification process, as well as the length of the spacer arm in the cross-linking process, on the final enzyme preparation properties was investigated.

Chapter 5 focuses on surface characterization of the Cal-B immobilized on silicon wafer, using an aminopropyltriethoxysilane-glutaraldehyde modification approach. It was revealed that every single step in the immobilization procedure has a significant influence on the final enzyme features. Therefore, a systematic study of the various steps of immobilization was conducted. Furthermore, the reactivity of such immobilized enzyme is also discussed.

Chapter 6 deals with the properties of the immobilized Cal-B on hydrophobic polystyrene nanoparticles prepared by a nanoprecipitation process. The influence of the pH of the buffer solution used during the immobilization process on the loading and activity of Cal-B was studied.

Finally, Appendix A gives an overview of the porosity properties of the synthesized poly(GLA-co-EGDMA). Altering the porosity parameters of the carrier, as result of enzyme immobilization was also assessed.

1.6. References

References

References