Lipase – characterization, applications and methods of immobilization

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Introduction

As of today the possibilities of using enzymes are very widespread, starting from scientific studies (in order to better understand the biochemical aspect of the enzymes' functioning mechanisms), cosmetic studies (enzymes in skincare products), medical diagnostics and chemical analysis, therapeutic uses (anticoagulants, anti-inflammatory agents), up to an industrial catalysis of such syntheses as e.g. the production of wine and beer, dairy products, in the sugar or pulp and paper industry, sewage treatment and many others. All this contributes to the constant increase in demand for these valuable products.

Approximately 4,000 enzymes are known, from which 200 have practical applications. These are especially enzymes of bacterial origin. At least 75% of enzymes with industrial significance (including lipases) have hydrolytic properties [1].

General characteristics of lipase

Lipase (triacylglycerol acylhydrolase) is an omnipresent enzyme with important physiological significance and industrial potential. Lipases catalyze the hydrolysis of triglycerides to glycerol and free fatty acids. They are soluble in water and hydrolyze insoluble substrates to more polar lipolytic products. The first lipase was identified by Claude Bernard in 1856 and from then on they have been identified in microorganisms, plants and animals [1,2].
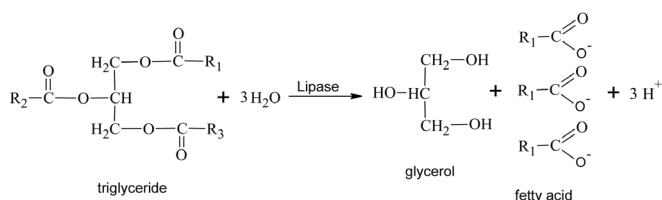

Fig. 1. **Hydrolysis of triglycerides with the participation of lipases**

Lipases are serine hydrolases containing $G-X_1-S-X_2-G$ sequences as the catalytic part of the particle, where G = glycine, S = serine, X_1 = histidine, X_2 = glutaminic or aspartic acid. Such structure is characteristic also for serine proteases. The knowledge of their 3-dimensional structure plays a significant role in designing and structuring lipases for specified purposes [3]. It has been shown that many lipases are phylogenetically connected.

Lipases are unique in terms of catalyzing fat hydrolysis to fatty acids and glycerol, which takes place on the water-lipid border, and in terms of a reverse reaction in a non-water environment. After contact with an insoluble substrate in water, a change in the enzyme's conformation occurs. This phenomenon of interfacial activity is nowadays an area of intensive studies performed by crystallographers, biochemists, molecular biologists, chemists and biochemical engineers [3].

The properties of lipases, just like lipases themselves, vary. A definitely desirable feature is the thermal stability of this enzyme. The source of thermally resistant lipases are first and foremost bacteria, and the thermal durability is linked to their structure. The thermal stability is influenced by such factors as pH and the presence of heavy metals. All activities at the level of protein engineering are meant to improve their stability [4]. A considerable improvement of the resistance of many lipases may be obtained by their immobilization on substrates and in many types of carriers.

Occurrence of lipase

Lipase is a physiologically necessary enzyme. It occurs in many plant and animal organisms, as well as in microorganisms. However, its richest source are bacteria, fungi and yeast [5].

Lipolytic enzymes are widespread in the plant world; however, the knowledge and experience pertaining to plant-originating lipases is still limited in comparison to the information on lipases from mammals and microorganisms, and this is especially connected to the difficulties with their isolation.

Among mammals, three groups of lipolytic enzymes are distinguished: lipases isolated to the digestive tract, tissue lipases and milk lipases. Mammal tissues and organs which contain lipases include the heart, brain, muscles, veins, kidneys, spleen, lungs, liver, fatty tissue and plasma [6].

Microbiological lipases, especially those originating from bacteria, are more stable than those from plants or animals. They possess unique qualities and, because of them, are used more often for industrial purposes. The knowledge about lipolytic bacterial enzymes is developing with surprising rate. The classification of bacterial lipases has been based mainly on the sequence of amino acids and some basic biological properties. Bacterial lipases from the *Pseudomonas* kind constitute the main group from which two types are distinguished – one is connected with the auxiliary protein in order to show proper activity, and the other one is not [5,6].

Isolation and purification

Lipases available on the market are usually isolated from higher eukaryotic organisms and microorganisms which include bacteria, fungi, yeast and actinomycetes. Lipase-producing microorganisms occur in various environments such as industrial waste-collection plants, plants producing vegetable oils, dairies, oil-polluted soils, decomposing food, composting plants, coal heaps or hot springs.

A reliable method discovering the activity of lipase in microorganisms is the one used in an unchanged environment with the Tween 80 surfactant for the identification of the lipolytic activity. The occurrence of non-transparent areas around the inspected colony means that the given organism produces lipase. This method may be modified by using various Tween surfactants in connection with Nile blue (oxazine dye). Often, also an inoculation is used of microorganisms producing lipase on agar plates, with the use of tributyrin as the substrate. The transparent areas around the colony indicate the production of lipase [7].

Microbiological lipases are obtained mainly by a submerged fermentation. Its production is influenced by: the concentration of coal and nitrogen source, environmental pH, temperature and concentration of dissolved oxygen [8]. The majority of microbiological lipases are active in a basic habitat (pH 7.0-9.0). For this reason, the activity of these enzymes is so strongly dependent on pH changes in the reaction mixture which influence the catalytic potential of lipases. Heavy metal ions are also effective lipase antagonists and modulators. On the other hand, chelating agents, e.g. EDTA, inhibit only the activity of metal lipases [9].

Because of the possibility of intensive production, good stability and numerous stereospecific properties, microbiological lipases still draw scientists' attention. Lipases have been isolated and studied from

such microorganisms as *Pseudomonas* [10], *Geotrichum* [11], *Candida rugosa* [12], *Aspergillus* [13], *Rhizophus* [14], *Mucor hirmalis f. hiemalis* [15], *Streptomyces rimosus* [16], *Penicillinum* [17]. These lipases not only differ from each other molecularly, but they also possess different catalytic properties [18].

Apart from the catalyst function, microbiological lipases used in waterless solvents offer also new possibilities, e.g. the improvement of the enzyme's thermal stability or the possibility of using hydrophobic substrates [19].

The majority of industrial products do not require homogenous lipase agents; however, the specified degree of purification allows for an effective and successful application. Moreover, the purification of enzymes enables a proper determination of the sequence of their main amino acids in the 3-dimensional structure. X-ray examinations of pure lipase allowed to determine the relation between the structure and the function, and they have contributed to a better understanding of the kinetic mechanism of lipase's functioning in hydrolysis or synthesis reactions. The purity of lipase products is very important in an industry using enzymes for the biocatalytic production of pharmaceuticals or cosmetics [3, 20].

Many lipases have been carefully purified and characterized; it was shown that their activity and stability depend on the pH, temperature, influence of metal ions and chelating factors. The purification methods used up to date involved mainly the use of such techniques as precipitation, chromatography, gel filtration. In some cases, an affinity chromatography has been used in order to reduce the number of purification levels [1].

The main limitations in traditional methods include low efficiency and time consumption. Alternative new technologies, such as membrane processes, biphasic water systems and immuno-cleansing gradually stand against the outdated methods of lipase purification. Currently, the industry is searching for new methods which are relatively cheap, fast and highly efficient [3].

Many lipases are active in organic solvents during the catalysis of a number of chemical reactions including esterification, transesterification, regioselective acylation of glycols and methanol, synthesis of peptides and other substances. It is expected that lipase will become an equally important industrial enzyme as protease or carbohydrase.

Application of lipase

Because of their significance, for a long time lipases have been the subject of numerous studies which concentrate mainly on the structure of this enzyme, the mechanism of functioning, kinetics, sequencing and cloning genes, as well as on the general characteristics of their occurring.

Lipases are commonly used in processing fats and oils, as additions to detergents and degreasing agents, in food processing, chemical and pharmaceutical syntheses, in paper production and the cosmetic industry. They may also be used in increasing the speed of fatty and polyurethane waste decomposition [1].

Lipases used as additions to detergents must fulfill the following criteria: low substrate selectivity, resistance to fairly raw washing conditions (pH 10-11, 30-60°C), resistance to destructive surfactants and enzymes (e.g. linear AAS and proteases) which are very important components of detergents.

Fats constitute an important component of food. The nutrition and taste values, as well as the physical properties of triglycerides are largely dependent on such factors as the position of the fatty acid in the glycerol chain, the length of the fatty acid chain and the degree of its saturation. Lipases enable the modification of lipid properties by a change of the fatty acid chains' position in glycerides and the substitution of one or more fatty acids with others. This way is relatively cheap and less desired lipids may be modified in order to obtain more valuable fats [21].

Hydrophobic components of wood (especially waxes and triglycerides) are the cause of major problems in the pulp and paper industry. A perfect solution turned out to be the use of lipases which remove undesirable, hydrophobic components [22].

The use of lipases in chemical organic syntheses is gaining more and more recognition. They are used in the catalysis of various kinds of chemo-, regio- and stereoselective changes.

Enzymes catalyze the hydrolysis of triglycerides which do not mix with water at the interphase. In specified conditions, the amount of water in the mixture determines the direction of the reaction, catalyzed by the lipase. With a small amount of water or its lack, rather the esterification and transesterification reactions occur. An excess of water favors hydrolysis [23]. Selected reactions occurring with the use of lipase have been presented in table 1.

Table 1. Selected reactions occurring with the use of lipase [6]

HYDROLYSIS	
$R_1-\overset{O}{\overset{\|}{C}}-O-R_2 \ + \ H_2O$	$R_1-\overset{O}{\overset{\|}{C}}-OH \ + \ R_2-OH$
SYNTHESYSIS OF ESTERS	
$R_1-\overset{O}{\overset{\|}{C}}-OH \ + \ R_2-OH$	$R_1-\overset{O}{\overset{\|}{C}}-O-R_2 \ + \ H_2O$
TRANSESTRIFICATION Alkoholysis	
$R_1-\overset{O}{\overset{\|}{C}}-O-R_2 \ + \ R_3-CH_2-OH$	$R_1-\overset{O}{\overset{\|}{C}}-O-R_3 \ + \ R_2-CH_2-OH$
Interestrification	
$R_1-\overset{O}{\overset{\|}{C}}-O-R_2 \ + \ R_3-\overset{O}{\overset{\|}{C}}-O-R_4$	$R_1-\overset{O}{\overset{\|}{C}}-O-R_4 \ + \ R_3-\overset{O}{\overset{\|}{C}}-O-R_2$
Acidolysis	
$R_1-\overset{O}{\overset{\|}{C}}-O-R_2 \ + \ R_3-\overset{O}{\overset{\|}{C}}-OH$	$R_3-\overset{O}{\overset{\|}{C}}-O-R_2 \ + \ R_1-\overset{O}{\overset{\|}{C}}-OH$

Lipase immobilization

Lipases are readily and often used in biotechnological processes. Their activity may be improved by immobilization. Because of this, new studies are conducted which pertain to the activity and application of immobilized lipase enzymes [24].
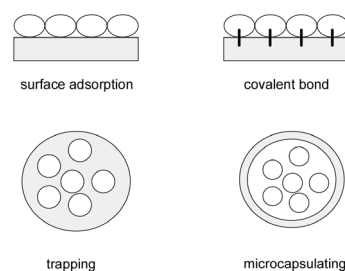

surface adsorption   covalent bond

trapping   microcapsulating

Fig. 2. Ways of immobilizing the lipase on the surface and inside of the carrier

There exist many methods of immobilizing lipase, starting from adsorption or precipitation on hydrophobic materials, covalent bonding to functional groups, trapping in polymer gels, adsorption on macroporous, anionic ion-exchange resins, or microencapsulation in lipid membranes. Among the mentioned methods, covalent bonding of lipase is dominating.

On non-organic substrates (aluminium oxide, silica and porous glass) Moreno et al. [25] covalently immobilized the *Candida cylindracea* lipase, previously activating the carriers with cyanuric acid. Derivative immobilizing on silica and aluminum oxide were characterized by a higher activity and thermal resistance than pure enzymes or even those immobilized on porous glass.

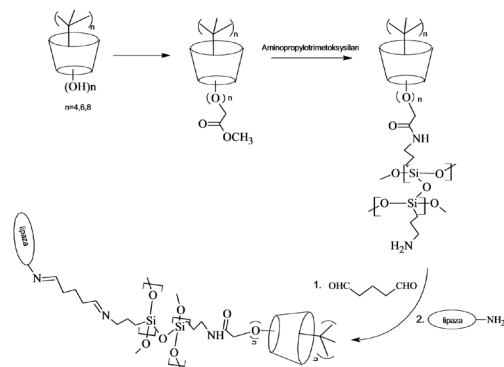Lipase (*Candida rugosa*) has been covalently immobilized also by

Fig. 3. Synthesis of calixarene polymers and covalent immobilization of *Candida rugosa* lipase [26]

Erdemir et al [26] using aminofunctional calixarenes on substrates of silica polymer (Figure 1). This way, a stable biocatalyst has been obtained with the possibility of repeated use and a lot better hydrolytic activity compared to the free enzyme.

Lipase has also been covalently immobilized on silica gel by Ozyilmaza [27] who used two bonding factors – glutarate aldehyde and 1,6-diaminohexane. The *Candida lipolitica* lipase has been catalyzed on modified palygorskite substrates by Huang et al [28].

Ozmen et al [29] covalently immobilized the *Candida rugosa* lipase on β-cyclodextrin polymers cross-linked with glutarate aldehyde and hexamethylenediizocyjanian. Aldehyde groups of the substrate and amino groups of the enzyme provide multipoint connections in the compound with which a stable enzyme derivative is obtained.

Lipase has been covalently bound on organic carriers also by Mieletić et al, [30], Kartal et al [31], Huang et al [32], using various copolymers and resins.

Often, lipases are immobilized also by means of adsorption. Lee et al [33] used the adsorption method to immobilize pig pancreatic lipase on magnetite nanoparticles. Based on the discovery that lipase is strongly adsorbed on hydrophobic substrates, magnetite nanoparticles have been modified with sodium dodecyl sulfate. This method has proved to be interesting because the bound enzyme may be recovered by magnetic separation. On magnetic nanoparticles protected by ionic fluid, with a different length of the cationic chain (C1, C4, C8) and various anions (Cl$^-$, BF$_4^-$, PF$_6^-$), also the *Candida rugosa* lipase was immobilized with the use of the ionic adsorption method [34].

Salis et al [35] conducted studies on the influence of the substrate on the enzyme load and activity of the immobilized lipase (*Pseudomonas fluorescens*). Various functional groups occurring on the porous substrates used by them were responsible for the physical (polypropylene, organosilicate, SBA-15) or chemical adsorption (polymethacrylate and modified SBA-15).

With regard to lipase trapping, alginate substrates are used relatively often for this purpose [36, 37] because of their delicately gelating properties and nontoxicity. Cheirsilp et al [38] used *Pseudomonas* lipase immobilized with calcium alginate to obtain monoacylglycerols from palm oil.

For trapping enzymes the sol-gel technique is used which is proven in case of bioparticles susceptible to high temperatures. Up till now the immobilization of lipases via this technique has been carried out with the use of silica materials [39, 40, 41]. Pirozzi et al [42] have trapped lipase in their studies in zirconium oxide obtained with the sol-gel method. This technique has allowed to create a substrate and to trap the enzyme in it in one stage.

Trapping and encapsulation of enzymes in natural polymers is becoming increasingly popular because of the low cost, repeatability of the process and nontoxicity. Jegannathan et al [43] have presented a fairly innovative way of encapsulating the *Burkholderia cepacia* lipase in the naturally occurring κ – Carrageenin polysaccharide with the use of coextrusion to create a capsule with a liquid nucleus.

Conclusions

Lipases are thoroughly and commonly occurring enzymes with numerous industrial applications. In the enzyme catalysis lipases represent the class of most often used biocatalysts. Immobilizing lipase is used with success in organic syntheses, chemo-, regio- and stereoselective reactions. Because of such great significance, lipase still remains the subject of constant studies by biotechnologists, bioengineers and chemists.

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