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Enzyme-catalysed Lipid Modification

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Lipids are of great importance, for example in the food, chemical and pharmaceutical industries. For many applications, lipids isolated from biological material can be used directly or after more or less sophisticated purification procedures. However, for certain applications, synthetic lipids are more suitable, and enzymes have proven to be useful catalysts for their preparation. The synthesis can be carried out starting from building blocks like fatty acids and glycerol, but often a natural lipid material is used as starting material and enzymatic conversions are employed to modify its composition and thereby its properties. From the application point of view, key characteristics of the lipid can be its melting behaviour (Macrae, 1985), its ability to interact with other biomolecules (Millqvist *et al.*, 1994) or other physical properties. Furthermore, for lipids used in foods, the health aspects of the fatty acid content are of vital importance (Sridhar and Lakshminarayana, 1992). In this chapter the use of enzymes in the preparation of acylglycerols (tri-, di- and monoglycerides), phospholipids and fatty acid esters will be covered. A complicating factor is that these substances are poorly soluble in water, which is the solvent normally used for enzymatic conversions. Accordingly, emulsions have commonly been used as reaction media, but recently the introduction of biocatalysis in organic media has greatly widened the scope of enzymatic lipid modification (Tramper *et al.*, 1992). The enzymes used for lipid conversions are mainly lipases and phospholipases.

Lipase structure and mechanism

The lipases constitute a group of enzymes which has attracted increasing interest in recent years. One reason for this is the variety of substances that can be synthesized in lipase-catalysed reactions. Reviews concerning the use of lipases for the modification of fats and other lipids (Mukherjee, 1990) and concerning the use of lipases in organic synthesis have been published (Haraldsson, 1992). Furthermore, lipases are of fundamental interest in enzymology because of their special properties, enabling them to be active at interfaces and thereby convert water-insoluble substrates (Brockman, 1984).

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

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The first three-dimensional structures of lipases were determined only recently (Brady *et al.*, 1990; Winkler, D'Arcy and Hunziker, 1990). It was shown that both the *Rhizomucor miehei* lipase and human pancreatic lipase have active sites with a trypsin-like catalytic triad involving the amino acid residues serine, histidine and aspartic acid. Somewhat later, a *Geotrichum candidum* lipase was shown to have a slightly different catalytic triad containing glutamic acid instead of aspartic acid (Schrag *et al.*, 1991). The information concerning the three-dimensional structures, in combination with studies of the catalytic activity of lipases have shown that these enzymes work according to the same mechanism as the well-known serine proteases. The serine hydroxyl group of the lipase makes a nucleophilic attack on the carbonyl carbon of the acyl donor, and an acyl enzyme is formed as an intermediate (*Figure 1*). In aqueous solution the acyl enzyme is hydrolysed by water.

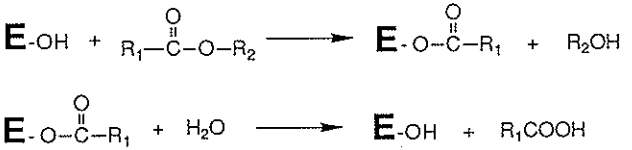


Figure 1. Mechanism of lipase-catalysed hydrolysis of an ester. E-OH: lipase molecule with the serine hydroxyl group of the active site.

Lipase-catalysed reactions

The physiological role of lipases is to hydrolyse triglycerides. Most of these substrates are not soluble in water to any appreciable extent. Therefore the reactions take place in emulsions. Lipases are adapted to this and are activated by the presence of an interface between an aqueous and an organic phase (Brockman, 1984). Consequently, with substrates of moderate solubility in water, the reaction rate of the lipase-catalysed reaction is normally very low as long as the substrate concentration is below the solubility limit of the substrate. Once this limit is exceeded and a separate substrate phase is formed, high reaction rates are obtained. The products of the hydrolysis of triglycerides are diglycerides and free fatty acids (*Figure 2*). The diglycerides can be further hydrolysed to monoglycerides and eventually to glycerol, with the liberation of the other esterified fatty acids. The substrate specificity of most lipases is quite broad, so apart from triglycerides many other esters can be hydrolysed with high reaction rates.

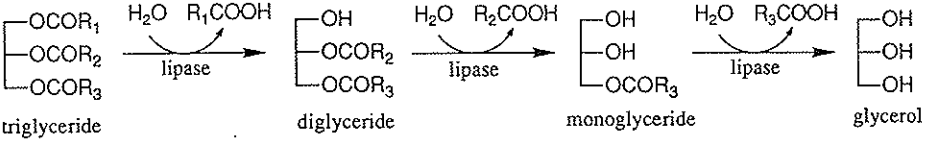


Figure 2. Lipase-catalysed hydrolysis of a triglyceride.

From the synthetic point of view, hydrolysis reactions are of limited interest. Thus the interest for lipases for synthetic purposes was modest until recently, when enzymology in organic media became an active research area (Tramper *et al.*, 1992). By using organic media with low water content, hydrolytic enzymes in general, and lipases in particular, can be used for many synthetic reactions. In organic media, other

nucleophiles can compete successfully with water in the deacylation of the acyl enzyme. This is the basis of most of the recent applications of lipases in organic media. Furthermore, the equilibria of the reactions in organic media are frequently altered so that large amounts of the condensation product are present at equilibrium (Valivety *et al.*, 1991).

Lipases can thus catalyse esterification, which is the reversal of the ester hydrolysis. A carboxylic acid acts as acyl donor and an alcohol carries out the deacylation of the acyl enzyme (*Figure 3*). Another way to prepare esters is to carry out an alcoholysis reaction. Alcoholysis is the reaction analogous to hydrolysis with an alcohol acting as a nucleophile instead of water (*Figure 3*). Organic media should be used to suppress the hydrolysis which occurs at high water content. Alcoholysis reactions can be used to prepare esters of fatty acids directly from triglycerides. Since triglycerides constitute an abundant raw material, alcoholysis is an attractive one-step process for the production of fatty acid esters (Shaw, Wang and Wang, 1991; Kanasawud *et al.*, 1992).

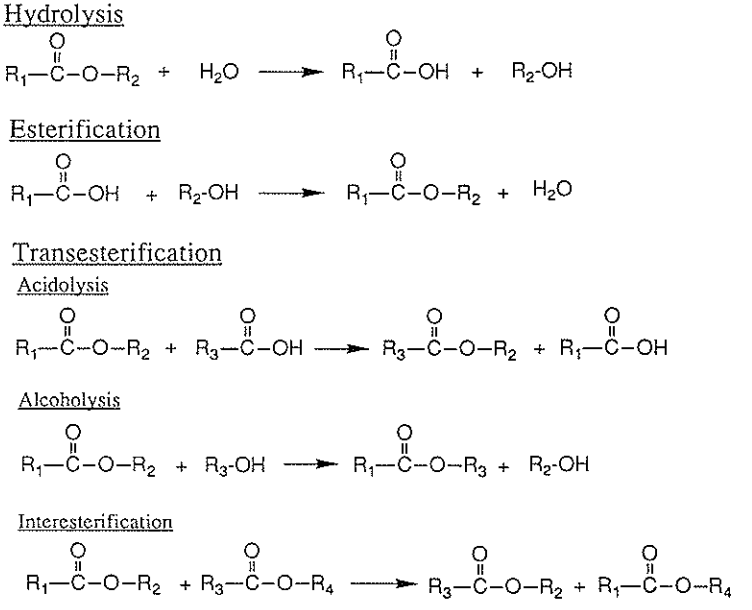


Figure 3. Lipase-catalysed reactions.

Other kinds of lipase-catalysed reactions are slightly more complex, since they involve more than one acyl-enzyme intermediate. In the modification of triglycerides, acidolysis reactions are often used (*Figures 3* and *4*) (Macrae, 1985). First, the triglyceride acylates the enzyme, leaving a diglyceride as reaction product. The acyl enzyme can then be deacylated by water or another nucleophile. The free fatty acid to be incorporated can acylate the enzyme as well, and thereby form a second acyl enzyme. If this is deacylated by a diglyceride, a triglyceride containing the new fatty acid is formed. It is clear that many different reactions occur, involving all triglycerides, partial glycerides, free fatty acids and acyl enzymes containing all the fatty acids present. A detailed kinetic analysis of these reactions is therefore quite complex (Kyotani *et al.*, 1988; Reyes and Hill, 1994). If the reaction is continued long enough,

an equilibrium mixture will eventually be formed. It is not necessary that the fatty acid to be incorporated into the triglyceride is present in the form of a free fatty acid. Fatty acid esters and triglycerides are other possible acyl donors, which are used in practical applications. These reactions are called interesterification reactions (*Figure 3*).

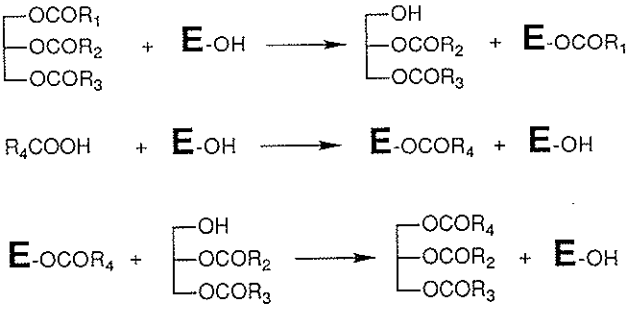


Figure 4. Mechanism of lipase-catalysed acidolysis. The lipase can form acyl enzymes, both in reactions with triglycerides (the first reaction) and with free fatty acids (the second reaction). Several nucleophiles can deacylate the acyl enzymes, for example diglycerides (the third reaction).

Lipase specificity

One of the most important features of enzyme-catalysed reactions is their specificity. This is often a main reason why an enzymatic conversion is chosen instead of a traditional organic-chemical route. In lipase-catalysed conversions of triglycerides, different kinds of specificity occur (Jensen, Dejong and Clark, 1983; Jensen, Rubano Galluzzo and Bush, 1990) (*Table 1*). Lipases can show different specificity towards different classes of lipids. The natural lipase substrates are the triglycerides. However, some enzymes show the typical characteristics of lipases although they convert triglycerides more slowly than other related substrates. One typical example is the mono- and di-glyceride lipase from *Penicillium camembertii* (Isobe *et al.*, 1992).

Table 1. Different types of lipase selectivity

Lipid-class selectivity
Regioselectivity
Fatty-acid selectivity
Nucleophile selectivity
Stereoselectivity

The most important type of lipase specificity utilized for lipid conversions is regiospecificity. A relatively large group of lipases, including pancreatic lipase and many fungal lipases, show specificity for the 1- and 3-positions of triglycerides. This has been widely used in triglyceride modification when modifications in the 1- and 3-positions are desired but the correct fatty acid is present already in the 2-position (see below). A lipase with strong selectivity for the 2-position has not yet been discovered, but a lipase from *Geotrichum candidum* showed moderate preference for this position (Sugihara, Shimada and Tominaga, 1991).

Lipases can show selectivity for certain fatty acids. The clearest example is a lipase from *Geotrichum candidum* which is selective for fatty acids having a *cis*-9 double

bond (Jensen, 1974). Only some strains of *Geotrichum candidum* have a lipase with high selectivity, while others have less selective lipases or may be mixtures of lipases with different selectivity (Sonnet, Foglia and Baillargeon, 1993). A few lipases show selectivity for short fatty acids in triglycerides. Both *Syncephalastrum racemosum* lipase and *Penicillium caseicolum* lipase showed the highest rates for hydrolysis of tributyrin (Chopra, Chander and Singh, 1982; Alhir, Markakis and Chandan, 1990). The latter lipase is of importance for flavour development during the ripening of certain types of cheese. Lipases from plant seeds often show selectivity for the triglycerides present in the seeds. Examples are the selectivity of corn lipase for trilinolein and triolein, castor bean lipase for triricinolein, rapeseed lipase for trierucin and elm seed lipase for tricaprin (Lin, Yu and Huang, 1986). Furthermore, the seed lipase of *Vernonia galamensis* has recently been shown to be selective for vernolic acid (12,13-epoxy-oleic acid) (Ncube *et al.*, submitted). Most lipases except those mentioned show fairly equal activity on a wide range of fatty acids (Berger and Schneider, 1991).

The fatty-acid selectivity of lipases can be used to enrich certain fatty acids. Several lipases discriminate against fatty acids with a *cis*-5 double bond. The strong selectivity expressed in esterification reactions was used to convert the other fatty acids to esters while the Δ5 unsaturated acids were enriched in the free fatty acid fraction (Hayes and Kleiman, 1993). The same principle was used to enrich erucic acid from a rape-seed-oil hydrolysate. In this case *Geotrichum candidum* lipase catalysed the esterification, leaving erucic acid in the free fatty acid fraction (Sonnet, Foglia and Feairheller, 1993). In both cases the fatty-acid selectivity was less pronounced in hydrolysis reactions than in esterification.

It should be pointed out that the fatty-acid specificity seen in a lipase-catalysed reaction is not solely due to the specificity of the lipase. The reaction conditions, especially the temperature, may influence the specificity. Normally, fatty acids react more effectively when the temperature is above the melting temperature of that particular fatty-acid chain. By lowering the temperature it was possible to induce selectivity for linolenic acid, since at 10°C this chain was still liquid while fatty-acid chains with less unsaturation had higher melting points (Kaimal and Saroja, 1988).

In the normal hydrolytic reaction catalysed by lipases, water acts as nucleophile in the deacylation of the acyl enzyme. However, in organic media other nucleophiles can successfully compete. In fact, most lipases have quite broad specificity for the nucleophile, and many of them accept even quite bulky nucleophiles as diglycerides, as well as many alcohols of interest for synthetic purposes in organic chemistry. In most cases, primary alcohols are the best nucleophiles, while secondary alcohols are less reactive and tertiary alcohols seldom react at all (Rangheard *et al.*, 1992). One of the best-investigated lipases in this respect is the *Rhizomucor miehei* lipase, which accepts a very wide range of alcohols containing a variety of other functional groups as well (Miller *et al.*, 1988).

Still another kind of specificity is stereospecificity. Methods to study this specificity with triglyceride substrates have been developed only recently (Rogalska, Ransac and Verger, 1990). The degree of steroselectivity is normally moderate and varies for different substrates. It has even been observed that a switch in stereoselectivity occurs, so that a few lipases show preference for the sn-3 position of trioctanoin and for the sn-1 position in triolein (Rogalska *et al.*, 1993). It should be pointed out that

the stereoselectivity of lipases has been widely used in the resolution of racemic mixtures of esters other than acylglycerols (Haraldsson, 1992). This is the most widespread use of lipases in organic synthesis.

Acyl migration

As mentioned above the 1,3-specific lipases have been used extensively in triglyceride conversions. A side-reaction that can occur in these transformations is the spontaneous acyl migration in the di- and mono-glycerides present in the reaction mixture (Bloomer, Adlercreutz and Mattiasson, 1991). As a result, the fatty acid composition in the 2-position might be changed, even though the enzyme itself is completely 1,3-specific. Similarly, in the preparation of pure 2-monoglycerides, 1,2-diglycerides, etc (see below), it is important to suppress acyl migration or else an isomer mixture will be formed. The rate of acyl migration is solvent dependent; it decreases with increasing solvent polarity (Sjursnes and Anthonsen, 1994).

Modes of using enzymes in organic media

Research concerning enzymatic reactions in organic media has been quite active during the past decade. In most applications an organic solvent is added to the reaction mixture. However, sometimes the substrates can constitute both substrates and solvent for the reaction. This is an attractive approach for practical applications, for example in the fat area. From the theoretical point of view, the solvent-free systems can be treated in the same way as those containing organic solvents.

There are several modes of using enzymes in organic media (Adlercreutz and Mattiasson, 1987; Tramper *et al.*, 1992) (*Figure 5*). The most straightforward way is simply to add an organic solvent to the aqueous solution of the enzyme. If the solvent is water-miscible, a homogeneous system is formed, otherwise a two-phase system. In both these cases enzyme inactivation is a key issue. In the homogeneous system, the enzyme is often active up to a certain concentration of solvent (Khmelnitsky *et al.*, 1991). In the two-phase system, partial inactivation of many enzymes occurs at the interface. However, as mentioned above, lipases normally work at interfaces and are activated by these (Brockman, 1984) and they are therefore suited for systems containing water-immiscible solvents.

In order to take full advantage of the organic reaction-medium, the water content in the reaction mixture should be kept quite low. Lyophilized enzyme powders can be directly suspended in organic media, and for some small-scale applications this is a practical way to use enzymes (*Figure 5c*). However, sometimes problems occur due to the aggregation of the enzyme powder. To decrease these problems and to facilitate the handling of the enzyme in general, it is common practice to immobilize the enzyme on a support material before using it in the organic medium (Reslow, Adlercreutz and Mattiasson, 1987) (*Figure 5d*). Because the enzyme is not soluble in the organic environment, there is no need for covalent bonds between the support and the enzyme. Therefore simple immobilization methods such as adsorption or deposition can be used. This kind of enzyme preparation is used at present in potential industrial processes employing enzymes in organic media (Macrae, 1985).

Still another mode of using enzymes in organic media is to solubilize the enzymes

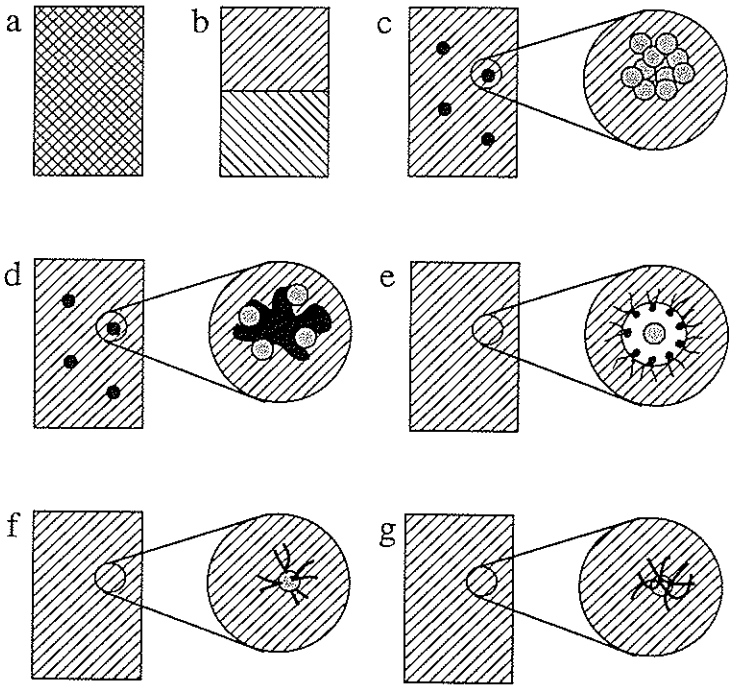


Figure 5. A schematic presentation of different ways to use enzymes in combination with organic solvents: (a) homogeneous mixture of water and water-miscible solvent; (b) two-phase system containing water and a water-immiscible solvent; (c) enzyme powder suspended in solvent, the small circles in the enlarged figure represent enzyme molecules; (d) enzyme on a support suspended in solvent; (e) enzyme solubilized in a microemulsion containing water, organic solvent and surfactant; (f) covalently modified enzyme dissolved in organic solvent; (g) non-covalent enzyme–polymer complex dissolved in organic solvent.

in microemulsions (Martinek *et al.*, 1989) (*Figure 5e*). Provided the surfactant does not inactivate the enzyme, good enzyme stability can often be achieved in microemulsions. However, product isolation is complicated by the presence of the surfactant. Another way to solubilize enzymes in organic media is to couple them covalently to polyethylene glycol (Inada *et al.*, 1986) (*Figure 5f*). The resulting modified enzyme can be dissolved in solvents like aromatic hydrocarbons and chlorinated hydrocarbons. Mass transfer is quite rapid in these systems, but the covalent modification often results in partial inactivation of the enzyme. Recently it has been shown that non-covalent surfactant–enzyme complexes and polymer–enzyme complexes can be dissolved in organic solvents (Okahata and Ijiro, 1992; Otamiri, Adlercreutz and Mattiasson, 1992; Blinkovsky, Khmelnitsky and Dordick, 1994) (*Figure 5g*). The main drawback is the presence of the surfactant or polymer during product isolation. Otherwise, this is quite an attractive method.

In all these examples small amounts of water are needed to make the enzyme catalytically active. Water present in the reaction mixture partitions between the solvent, the enzyme and the support or other components present. The amount of water bound to the enzyme governs its catalytic activity. Because of the widely different abilities of solvents to dissolve water, and of supports to bind water, the total water concentration in the reaction mixture gives little information about the hydra-

tion of the enzyme. When studying the effects of solvents and supports on enzyme catalysis, it is misleading to compare results obtained at a fixed water concentration (Valivety, Halling and Macrae, 1992a). For proper comparisons it is necessary to carry out the experiments at fixed thermodynamic water activity (Halling, 1984; Adlercreutz, 1991; Valivety, Halling and Macrae, 1992a).

Preparation of esters

Enzymatic esterification can be carried out under mild conditions and therefore it results in products of high purity. There are industrial processes for the lipase-catalysed production of isopropylmyristate and a few other esters (Staal, 1990). These are mainly used as ingredients in skin-care products and cosmetics. Furthermore, it has been shown that enzymatic esterification of polyunsaturated fatty acids can be carried out without any oxidation or other side-reactions (Bloomer, Adlercreutz and Mattiasson, 1992). An important issue concerning the esterification reactions is to remove the water formed in the reaction so that the equilibrium is shifted towards the formation of ester. If all reactants and products except water are essentially non-volatile, water can simply be evaporated at reduced pressure. Wax esters can be produced in this way (Eigtved, Hansen and Miller, 1988; Trani, Ergan and André, 1991), as well as triglycerides (Ergan, Trani and André, 1990). If volatile alcohols are used as substrates, somewhat more sophisticated distillation processes can be applied to remove water (Hills, Macrae and Poulina, 1989) or, alternatively, water can be absorbed by molecular sieves or other absorbents (Knox and Cliffe, 1984; Omar, Nishio and Nagai, 1988). Often ester yields in excess of 99% can be achieved (Bloomer, Adlercreutz and Mattiasson, 1992). Sometimes fairly good yields can be obtained just by carrying out the reaction in a two-phase system in which the ester is extracted into the organic phase (Monot *et al.*, 1991; Borzeix, Monot and Vandecasteele, 1992). Several flavour esters have been prepared using this technique (Langrand *et al.*, 1990).

Fatty acid esters can be prepared directly from triglycerides using an alcoholysis reaction. With some lipases the reaction can be carried out without extra solvent (Shaw, Wang and Wang, 1991; Kanasawud *et al.*, 1992). The yield can sometimes be improved by adsorption of the other product, glycerol, on an adsorbent such as silica gel (Stevenson, Stanley and Furneaux, 1994).

Triglyceride modification

Fats and oils mainly consisting of triglycerides are very important products, especially in the food area. The physical properties of a fat depend on its fatty acid composition. In order to obtain products with the desired properties, fractionation is used to a large extent. However, sometimes there is a need to exchange fatty acids present in the triglycerides with other fatty acids.

It was observed long ago that lipases can be used to incorporate new fatty acids into triglycerides (Borgström, 1954). Research has been intense during the past decades with the aim to prepare fats with desirable properties. An important example is the synthesis of cocoa-butter substitutes. Cocoa butter is widely used in chocolate and related products, because of its unusual melting characteristics. It has a rather narrow

melting range around 30°C due to its high content of the triglycerides oleyl-distearoylglycerol and oleyl-palmitoyl-stearoylglycerol. Because of the high price of natural cocoa butter, large efforts have been spent on the preparation of substitutes with similar properties from cheaper raw materials. In the production of cocoa-butter substitutes, lipases have proven to be quite useful (Macrae, 1985). Using 1,3-specific lipases it is possible to manipulate the fatty acid composition in these positions without changing the fatty acids in the 2-position. Since vegetable oils often contain oleic acid in the 2-position, the task for the bioconversion is to introduce the proper saturated fatty acids in the 1- and 3-positions. Processes have been developed starting from, for example, a fraction of palm oil containing 2-oleyl-1,3-dipalmitoylglycerol as the main component (*Figure 6*). Transesterification with stearic acid or an ester thereof yields a cocoa-butter substitute (Macrae, 1985). Alternatively, oils containing large amounts of oleic acid-rich triglycerides, such as olive oil, can be transesterified with, for example, hydrogenated cotton seed oil which contains mainly triglycerides of palmitic and stearic acids (Chang, Abraham and John, 1990). Other suitable starting materials are oils from sunflower seeds or rape seeds. The oleic acid present in the 2-position in the substrate should remain there. However, due to acyl migration in di- or monoglycerides, the fatty acid incorporated in the 1- and 3-positions might end up in the 2- position (see above). Consequently, triglycerides with fatty acids in all three positions are formed; these are not accepted in cocoa-butter substitutes because their melting points are too high. Because of the mechanism of the reaction (*Figure 4*) diglycerides are necessary intermediates in the reactions. In order to minimize the spontaneous acyl migration, it is important to have a high catalytic activity in the reactor so that the enzymatic reaction is fast (Bloomer, Adlercreutz and Mattiasson, 1991). Then the residence time in the reactor is short and the extent of spontaneous reactions is therefore small. It is thus possible to suppress this side-reaction so that it is negligible. Several patents concerning lipase-catalysed production of cocoa-butter substitutes have been filed (Matsuo *et al.*, 1980; Macrae and How, 1983; Nakamura *et al.*, 1987) and industrial production started a couple of years ago.

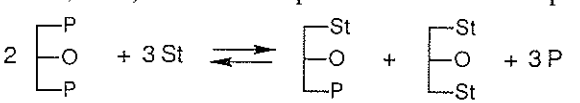


Figure 6. Schematic presentation of the conversion of 2-oleyl-1,3-dipalmitoylglycerol to 2-oleyl-3-palmitoyl-1-stearoylglycerol and 2-oleyl-1,3-distearoylglycerol in a lipase-catalysed acidolysis reaction. The reaction products are the main components of cocoa butter.

Other structured lipids containing suitable fatty acids have been evaluated for different applications. Triglycerides with medium-chain fatty acids in the 1- and 3-positions have been suggested for use in intravenous nutrition. The fatty acids in these positions are cleaved off by lipoprotein lipase in the bloodstream more effectively than longer fatty acids (Björkling, Godtfredsen and Kirk, 1991).

Since polyunsaturated fatty acids like eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) have a number of health-promoting effects, large efforts have been spent on the incorporation of these fatty acids into different kinds of fats and oils. Either moderate amounts have been incorporated in fats which normally contain no polyunsaturated fatty acids or very small amounts, like groundnut oil (Sridhar and Lakshminarayana, 1992), or products highly enriched in polyunsaturated fatty acids are prepared (Li and Ward, 1992). Fish oils contain large amounts of

polyunsaturated fatty acids and they are therefore suitable starting materials for further enrichment (Haraldsson *et al.*, 1989; Haraldsson and Almarsson, 1991). Phospholipids have been suggested as another type of lipid for the administration of polyunsaturated fatty acids to humans. Consequently, incorporation of these fatty acids into phospholipids has been investigated (see below; Hosokawa *et al.*, 1991, 1993).

Because of the surplus of butter fat available in some countries, research has been conducted with the aim of preparing new products from this source. Possibilities investigated are transesterification to obtain fats with modified fatty acid composition (Kalo, Huotari and Antila, 1990) and monoglyceride production (Yang and Parkin, 1994).

Hydrolysis of triglycerides to obtain free fatty acids and glycerol is not included in this chapter. This topic was covered by Mukherjee (1990).

Preparation of mono- and diglycerides

Regio-isomerically pure 1(3)-monoglycerides have been prepared from glycerol and fatty acids or fatty acid esters using 1,3-specific lipases as catalysts in organic solvents (Hayes and Gulari, 1991; Berger and Schneider, 1992) (*Figure 7*). In order to increase the interface between the polar glycerol and the apolar solvent containing the fatty acid, the glycerol was adsorbed on to a solid support. The monoglyceride yields were above 75% with diglycerides as the main by-product (Berger and Schneider, 1992). One way to increase the monoglyceride yield is to adsorb the monoglyceride continuously during the reaction (van der Padt *et al.*, 1992). 1,3-Specific lipases have been used to prepare 1,3-diglycerides as well (Berger, Laumen and Schneider, 1992).

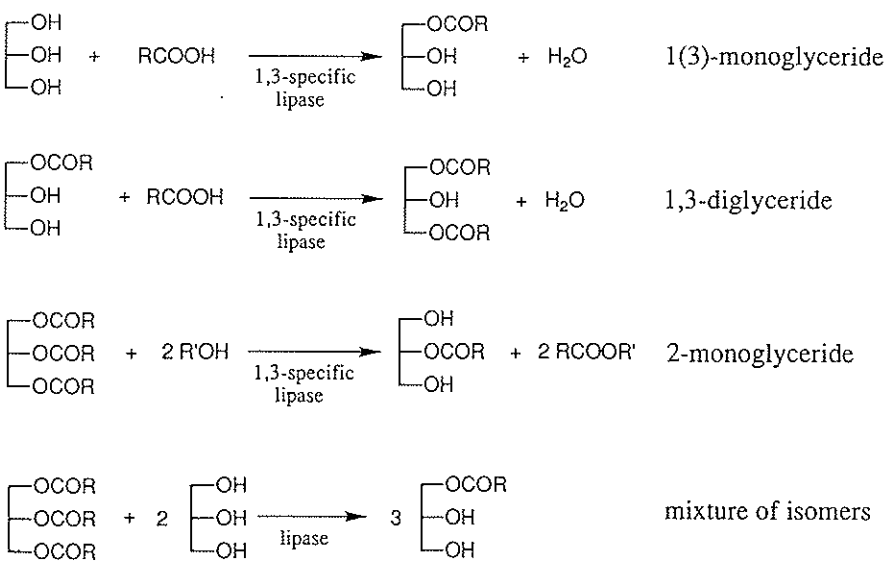


Figure 7. Lipase-catalysed preparation of mono- and diglycerides. Details concerning the different methods are given in the text.

Alternatively, monoglycerides can be prepared via lipase-catalysed glycerolysis of triglycerides (*Figure 7*). This has been achieved in a solvent-free system (McNeill, Shimizu and Yamane, 1991). High yields (67–90%) were obtained when the reactions were carried out at temperatures low enough for the precipitation of the monoglyceride. This led to solidification of the entire reaction mixture.

2-Monoglycerides have been prepared using 1,3-specific lipases for the hydrolysis (Holmberg and Osterberg, 1988) or alcoholysis (Millqvist, Adlercreutz and Mattiasson, 1994) of triglycerides (*Figure 7*). In the hydrolysis reaction carried out in a microemulsion, acyl migration was a problem, but in the alcoholysis reaction acyl migration was slow provided a suitable solvent, such as methyl-tert-butyl ether, was used.

Phospholipid modification

In nature, phospholipids play important roles as components of cell membranes, etc. Often different classes of phospholipids (having different polar groups) occur together and they constitute complex mixtures of molecular species having different fatty acid groups. Phospholipids differing in the polar part can be separated by chromatographic methods. However, separation with respect to the fatty acids often becomes too complicated due to the large number of possible combinations. For some applications these mixtures can be used as such, but for more specific applications one needs preparations which are uniform both concerning the polar part and the fatty acids. Pure phospholipids can be synthesized chemically, enzymatically or by a combination of these methods. Natural phospholipid mixtures are sometimes used as starting materials. A convenient method to prepare a new, well-defined phospholipid is to use a starting material containing the same fatty acid in both positions; these can be synthesized chemically. If a phospholipid containing two different fatty acids in the sn-1 and sn-2 positions is desired, one of the fatty acids can be exchanged using enzymatic methods (Svensson *et al.*, 1993) (*Figure 8*). The enzymatic exchange can be done either in two steps – hydrolysis and esterification – or in a one-step transesterification reaction. With the two-step procedure one can obtain a pure product provided the lysophospholipid is purified after the hydrolytic step. In the transesterification there is a need for a large excess of the new fatty acid because the fatty acid composition in the reactive positions of the product will reflect the equilibrium mixture of the fatty acids available for reaction. On the other hand, the transeterification reaction is less laborious and gives a higher yield than the hydrolysis–esterification route.

Below, enzymatic modification of the different parts of the phospholipid molecules are treated separately. Most work concerning exchange of fatty acids has been carried out with phosphatidylcholine, but there are indications that the same methods can be used for other classes of phospholipids as well (Svensson, Adlercreutz and Mattiasson, 1992).

Both phospholipases and lipases have been used to modify phospholipids. Several types of phospholipases exist. They are specific for the cleavage of different bonds in the phosolipid molecules. Phospholipases A and B cleave off the fatty acids, and phospholipase C and D hydrolyse the ester bonds on either side of the phosphate group (*Figure 9*). The use of the new techniques employing enzymes in organic

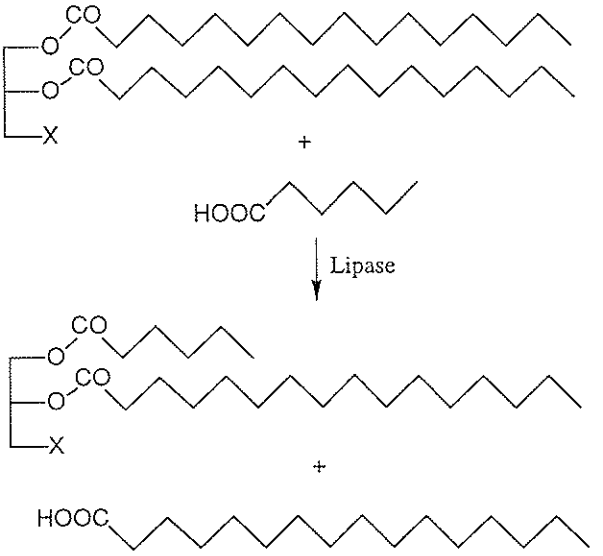


Figure 8. Conversion of dipalmitoyl-phosphatidylcholine to 1-hexyl-2-palmitoyl-phosphatidylcholine in a lipase-catalysed acidolysis reaction (Svensson *et al.*, 1993).

media, makes it possible to reverse these reactions and synthesize phospholipids with phospholipases as catalysts.

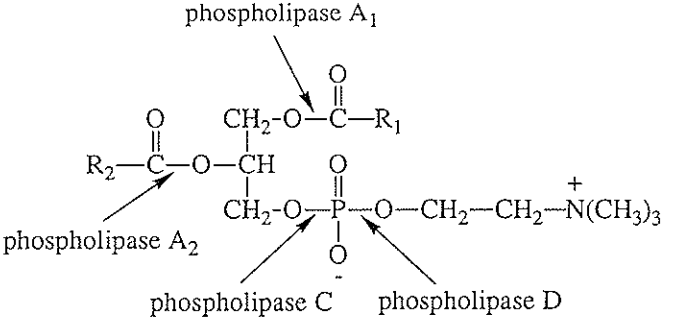


Figure 9. Phosphatidylcholine molecule showing the cleavage sites of the phospholipases.

Exchange of the fatty acid in the sn-1 position of phospholipids

Phospholipase A_1 has not been used to any large extent for lipid modification. It was long ago observed that lipases can be used to incorporate new fatty acids into phospholipids (Brockerhoff *et al.*, 1976), and since lipases are quite convenient to use they have been widely employed for the exchange of fatty acids in the sn-1 position of phospholipids. The lipases specific for the 1- and 3-positions in triglycerides have been shown to be specific for the sn-1 position of phospholipids. The reactions are often carried out as acidolysis reactions. In the first attempts, the fatty acid and the phospholipid were dispersed as micelles in an aqueous buffer, but the yields were moderate (Brockerhoff *et al.*, 1976) (*Table 2*). A main point in the synthesis is the minimization of hydrolysis. Organic media with low water contents have therefore

been tried for the reactions. Compared to the acidolysis of triglycerides, the phospholipid reactions give considerably more hydrolysis even in the organic media. The transesterification results described in the literature are summarized in *Table 2*. The reports include several different ways of carrying out the reactions. Covalent modification of the lipase with polyethylene glycol made it possible to dissolve the enzyme in benzene (Yoshimoto *et al.*, 1986). A two-phase system consisting of water and hexane was used by Yagi *et al.* (1990), and resulted in slightly higher yield. Water/hexane was used by Totani and Hara (1991) as well. In order to reduce hydrolysis, water was replaced by glycerol. A microemulsion with iso-octane as organic solvent and Aerosol-OT as surfactant was used to incorporate polyunsaturated fatty acids into phosphatidylcholine (Holmberg and Eriksson, 1992). Immobilization of the lipase on a porous support and suspending this enzyme preparation in the reaction mixture have been used successfully in a number of recent reports (Svensson, Adlercreutz and Mattiasson, 1990, 1992; Pedersen, 1991; Mutua and Akoh, 1993). Toluene was a good solvent for the incorporation of saturated fatty acids into phosphatidylcholine (Svensson, Adlercreutz and Mattiasson, 1990) while hexane was the best for the incorporation of eicosapentaenoic acid (Mutua and Akoh, 1993).

Table 2. Lipase-catalysed transesterification of phospholipids (from Svensson, 1994)

Yield of modified phospholipid (%)	Incorporation of fatty acid (%)	Reference
14–23	18	Brockerhoff *et al.* (1976)
20	8.5	Yoshimoto *et al.* (1986)
25	25	Yagi *et al.* (1990)
40	25	Svensson, Adlercreutz and Mattiasson (1990)
<25	67	Pedersen (1991)
47	32	Totani and Hara (1991)
60	50	Svensson, Adlercreutz and Mattiasson (1992)
75	25	Holmberg and Eriksson (1992)
?	17.7	Mutua and Akoh (1993)

The yield of modified phospholipid and the incorporation of the new fatty acid(s) into the phospholipid are noted. Complete exchange of the fatty acid in the sn-1 position would give an incorporation of 50%.

Apart from the yield, the extent of incorporation of the new fatty acid is of importance. Since the reaction should be specific for the sn-1 position of the phospholipid, 50% incorporation is what is desired. Higher values of incorporation show that some of the new fatty acid has been incorporated in the sn-2 position, indicating poor regioselectivity in the reaction or acyl migration (Pedersen, 1991). To evaluate the importance of different reaction parameters on the reaction, it was helpful to plot the yield versus the incorporation of the new fatty acid (*Figure 10*). In order to obtain high yields of modified phospholipid, it was necessary to keep the water content low. In the system with immobilized lipase, this was achieved by adjusting the thermodynamic water activity of the reaction mixture (Svensson, Adlercreutz and Mattiasson, 1992). A low water activity resulted in a low reaction rate but the yields were considerably higher than at high water activity. The best results were obtained with *Rhizopus arrhizus* lipase immobilized on a polypropylene support (Svensson, Adlercreutz and Mattiasson, 1992). Fatty acid esters have been tried as

acyl donors instead of free acids but the yields were lower (Svensson, Adlercreutz and Mattiasson, 1990).

The regioselectivity in the lipase-catalysed transesterification of phospholipids is quite high. In the sn-2 position less than 1% incorporation was found when almost complete exchange had occurred in the sn-1 position (Svensson, Adlercreutz and Mattiasson, 1992).

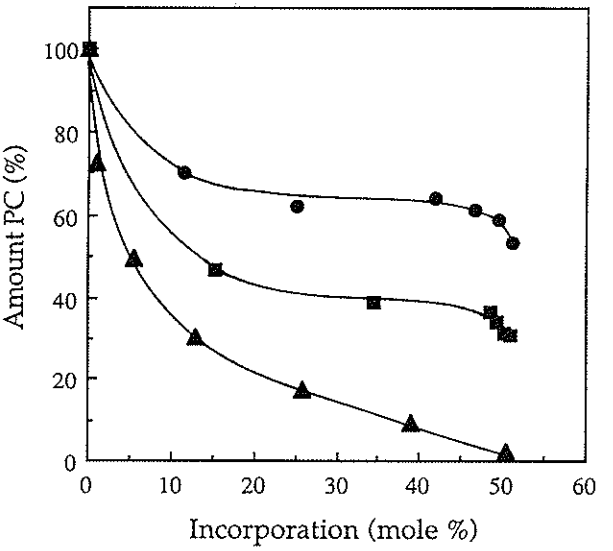


Figure 10. Lipase-catalysed acidolysis of phosphatidylcholine. The time course of the reaction is illustrated as the amount of remaining phosphatidylcholine as a function of the incorporation of the new fatty acid, heptadecanoic acid. The reactions were carried out in toluene using Lipozyme IM 20 at a water activity of 0.43 (triangles) or *Rhizopus arrhizus* lipase adsorbed on polypropylene at a water activity of 0.43 (squares) or 0.11 (circles) (Svensson, Adlercreutz and Mattiasson, 1992).

Exchange of the fatty acid in the sn-2 position of phospholipids

Only phospholipase A_2 has been successfully used to modify the fatty acid composition in the sn-2 position of phospholipids. One could hope that non-specific lipases would be useful for the exchange of fatty acids in both positions, but this has not yet been realized.

The fatty acid in the sn-2 position can easily be hydrolysed off using phospholipase A_2. The reversed reaction proceeds with rather low yields, even in organic media with low water content. In a screening of different enzymes, phospholipase A_2 from *Naja naja* provided the best yield (6.5%) (Pernas *et al.*, 1990) (*Table 3*) and toluene was used as solvent. There is a large interest in incorporating polyunsaturated fatty acids into phospholipids for use in food. The condensation of lysophospholipid and polyunsaturated fatty acids in a microemulsion provided a yield of 6% using phospholipase A_2 from porcine pancreas (Na *et al.*, 1990). By using a solvent-free system, polyunsaturated fatty acids from fish oil were incorporated into phospholipids using porcine pancreatic phospholipase A_2 (Hosokawa *et al.*, 1991). The yield reported was 15%, which was improved to around 20% (Hosokawa *et al.*, 1993). Even better yields

Table 3. Phospholipase A_2-catalysed esterification of lysophospholipid and fatty acid

Yield of phospholipid (%)	Reference
6.5	Pernas *et al.* (1990)
6	Na *et al.* (1990)
15	Hosokawa *et al.* (1991)
>20	Lilja-Hallberg and Härröd (1992)
20	Hosokawa *et al.* (1993)
14	Svensson (1994)

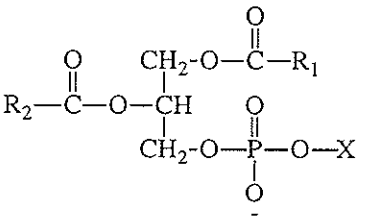
(>20%) were obtained in a similar study using porcine pancreatic phospholipase A_2 immobilized on a polymer carrier and with iso-octane as solvent (Lilja-Hallberg and Härröd, 1992).

In the esterification reaction, the theoretically maximal yield is determined by the equilibrium position. This in turn depends on the concentrations or activities of the reactants. If a high yield with respect to the lysophospholipid is desired, it is beneficial to use a large excess of the free fatty acid. Furthermore, the water activity in the reaction mixture should be kept low, since water is a reaction product. A systematic investigation of the influence of the water activity on the yield in the preparation of phosphatidylcholine from lysophosphatidylcholine and oleic acid has been carried out. The best yield in this study (14%) was achieved at a water activity of 0.33 (Svensson, 1994). At lower water activity the porcine pancreatic phospholipase A_2 immobilized on XAD-8 was not active. The lack of catalytic activity at low water activity is a serious drawback in this type of reaction. Lipases often show good catalytic activity even at very low water activity (Valivety, Halling and Macrae, 1992b), while phospholipases and most other enzymes require higher water activity.

It is noteworthy that transesterification reactions do not seem to work well with phospholipase A_2 (Na *et al.*, 1990; Pernas *et al.*, 1990). The reason might be due to differences in the catalytic mechanism of lipases and phospholipase A_2. The catalytic mechanism of phospholipase A_2 from bovine pancreas has been studied in detail. It shows large similarities with the mechanism of the lipases. However, phospholipase A_2 lacks the serine residue which acts as a nucleophile in the case of lipases. Instead, a water molecule is present in this position in the active site (Dijkstra *et al.*, 1981). As a result, no acyl enzyme is formed. The 'transesterification' reaction in this case would be a hydrolysis reaction followed by esterification. Since both these reactions can be carried out separately, the problem is probably that it is difficult to find conditions under which both reactions occur to an appreciable extent. There are a couple of reports that the transesterification reaction can, indeed, be carried out (Mukherjee, 1990; Mutua and Akoh, 1993).

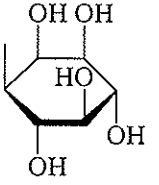
Preparation of lysophospholipids

Lysophospholipids have been prepared from phospholipids using lipases as catalysts. Since the lipase-catalysed reactions are selective for the sn-1 position, the reaction product has the remaining fatty acid in the sn-2 position. Both hydrolysis reactions (Haas *et al.*, 1993; Morimoto *et al.*, 1993) and alcoholysis reactions (Sarney, Fregapane and Vulfson, 1994) have been used. If acyl migration occurs, the isomeric



R_1,R_2: Fatty acid alkyl chains

X: $-CH_2CH_2N^+(CH_3)_3$	Phosphatidyl choline
$-CH_2CH_2NH_3^+$	Phosphatidyl ethanolamine
$-CH_2CH(OH)CH_2(OH)$	Phosphatidyl glycerol
$-CH_2CH(NH_3^+)COOH$	Phosphatidyl serine



Phosphatidyl inositol

-H Phosphatidic acid

Figure 11. Different classes of glycerophospholipids.

purity of the product might decrease. Furthermore, the yield will decrease due to further hydrolysis of the second fatty acid. Quite pure products have been obtained in boric acid–borax buffers, while other buffers provided products of lower purity (Morimoto *et al.*, 1993).

Conversions from one phospholipid class to another

Phospholipase C or D can be used to convert one phospholipid class to another (*Figure 11*). The most common starting material is phosphatidylcholine. Phospholipase D from cabbage was used to convert phosphatidylcholine to phosphatidylglycerol in a membrane reactor (Lee *et al.*, 1985). Ether was added to dissolve the phospholipid and stabilize the enzyme. Later, the same research group compared a micelle and an emulsion system as reaction media for this reaction (Juneja *et al.*, 1987a). In the micelle system, the initial reaction rate was high but the competing hydrolysis reaction resulted in large amounts of phosphatidic acid. In the emulsion of buffer/glycerol in ether containing the phosphatidylcholine, this side-reaction was effectively suppressed so almost quantitative conversion to phosphatidylglycerol was achieved. In these studies, dissolved enzyme was used. However, it was found advantageous to immobilize the enzyme by adsorption on octyl-Sepharose CL-4B (Juneja *et al.*, 1987b). With the immobilized phospholipase D, repeated batch and continuous reactions were carried out. A similar enzyme preparation, but with phospholipase D from a *Streptomyces* species was used to convert phosphatidylcholine

to phosphatidylserine (Juneja *et al.*, 1992). The yield in the reaction was improved by the addition of choline oxidase which oxidizes the reaction product choline and therefore favours further formation of phosphatidylserine. In this reaction hydrogen peroxide was formed, and this substance inactivated the enzymes to some extent. The addition of catalase to decompose hydrogen peroxide further improved the process (Juneja *et al.*, 1992).

At least some phospholipase D enzymes have broad nucleophile specificity. This fact has been used to synthesize unnatural phospholipids. A phospholipase D from *Streptomyces prunicolor* accepted as nucleophiles pentoses, hexoses, glycosides, amino sugars, alcoholic sugars, di- and oligosaccharides, amino acids, nucleic acids, dinitrophenol and primary and secondary alcohols (Kudo, 1988). Other interesting examples are the transphosphatidylation of phosphatidylcholine with azasugars, nucleosides, peptides and inhibitors (Wang *et al.*, 1993). For these reactions an enzyme from *Streptomyces* was active but the cabbage enzyme and other enzymes were not. The unnatural phospholipids were intended for incorporation into liposomes for use in drug delivery and targeting. Another interesting example is the preparation of sialic acid derivatives. Here, the sialic acid was coupled to the rest of the molecule via a spacer (Ri *et al.*, 1993) (*Figure 12*).

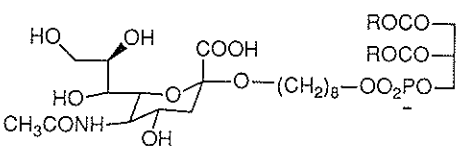


Figure 12. Sialic acid derivative prepared by phospholipase D catalysed transphosphatidylation of phosphatidylcholine (Ri *et al.*, 1993).

The natural reaction of phospholipase C is to break the bond between the glycerol backbone and the phosphorus in glycerophospholipids (*Figure 9*). However, it can hydrolyse the corresponding bond in sphingophospholipids as well, to form phosphorylcholine and a ceramide (Kanfer and Spielvogel, 1975) (*Figure 13*). Furthermore, it has been shown that phospholipase C possesses transphosphatidylation activity. The enzyme from different *Clostridia* was used to transfer the phosphorylcholine part of phosphatidylcholine to ceramide so that sphingomyelin was formed (Kanfer and Spielvogel, 1975).

A somewhat unusual reaction has been described for phospholipase D. It catalyses a transphosphatidylation reaction with phosphatidylglycerol both as donor and acceptor of the phosphatidyl group under the formation of cardiolipin (Stanacev, Stuhne-Sekalec and Domazet, 1973) (*Figure 14*). In fact, this is the route for cardiolipin biosynthesis in various species of bacteria.

Concluding remarks

Research concerning enzymatic lipid modification has developed rapidly during the past decade due to the increased knowledge about biocatalysis in organic media. In organic media, lipids can be handled effectively and the equilibrium positions are favourable for the synthesis of many interesting lipids. Compared to chemical processing, enzymatic conversions offer advantages concerning mild reaction condi-

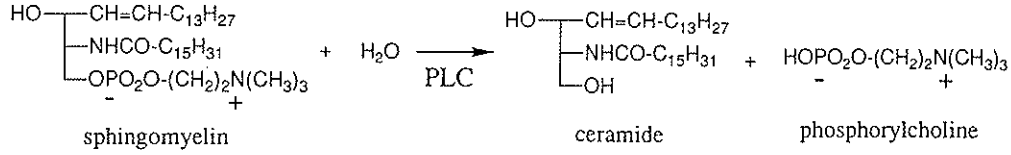


Figure 13. Preparation of ceramide by phospholipase C- catalysed hydrolysis of sphingomyelin (Kanfer and Spielvogel, 1975).

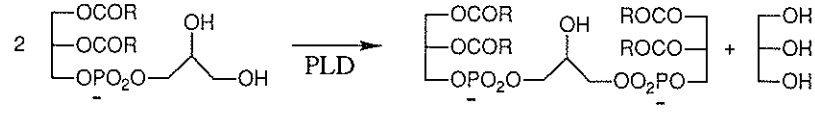


Figure 14. Phospholipase D-catalysed conversion of phosphatidylglycerol to cardiolipin (Stanacev, Stuhne-Sekalec and Domazet, 1973).

tions, which has proven to be important, for example in ester synthesis and concerning selectivity in the reactions. The selectivity of lipases has made it possible to synthesize tailor-made triglycerides, partial glycerides and phospholipids. It is likely that in the future a wide range of conversions of complex lipids will be developed. At present, our knowledge about the lipases is rapidly increasing, while much less is known about other lipid-converting enzymes. Further synthetic possibilities will certainly appear with increasing knowledge about these enzymes, and new enzymes will be discovered in screening programmes.

Acknowledgement

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