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Enzymatic conversions of polar lipids. Principles, problems and solutions

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Abstract

This text provides a brief overview of the principles of enzymatic lipid conversion and some recent advances in the enzymatic conversion of glycerophospholipids and galactolipids. Lipases and phospholipases are used to exchange fatty acids or the polar group in the lipids. The reactions can be carried out either as hydrolysis–esterification sequences or as one-step transferase reactions. The scope and limitations of the different methods are discussed.

Keywords: Enzymatic conversion; Polar lipids; Lipases

1. Introduction

Both glycerophospholipids and galactolipids are abundant in biological materials, mainly as important membrane components. These lipids are potentially useful for many technical applications due to their surface activity, and glycerophospholipids are indeed already widely used as emulsifier in food, cosmetics, etc. Furthermore, the polar lipids can be used to prepare liposomes for drug delivery and other applications.

Fig. 1 shows the general formula of a glycerophospholipid and a common type of galactolipid, digalactosyldiacylglycerol (DGDG). When the polar lipids are extracted from biological materials, they normally contain a wide range of molecular species with different fatty acids and often different polar groups, as well. For some applications, these crude preparations can be used, but in other cases, it is desirable to have a more well-defined product having specified fatty acids in both positions and a specified polar group. The two main ways to obtain well-defined lipids are de novo synthesis and modification of natural lipids. Both enzymatic and chemical methods can be used in both strategies. This text will only deal with enzymatic methods (for a review of chemi-

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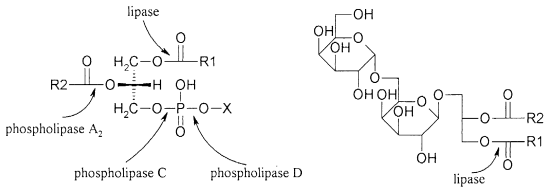

Fig. 1. General formula of glycerophospholipids and DGDG. R1 and R2 are alkyl groups of fatty acids and X depends on the class of glycerophospholipid; in phosphatidylcholine X = $(CH_2)_2N^+(CH_3)_3$. Enzymes that can be used to break or form ester bonds in the different positions of the molecules are indicated.

cal methods, please see Ref. [1]). Most of the examples discussed will deal with modification of natural lipids.

2. Enzymes used

In Fig. 1, the enzymes used to modify the different moieties in the lipid molecules are indicated. In aqueous solution or in emulsions, the predominant reactions are hydrolytic ones, but in organic media with low water activity, high yields in esterification reactions and direct transferase reactions can be achieved.

Many of the lipases that are specific for the 1- and 3-positions in triacylglycerols are specific for the *sn*-1-position of glycerophospholipids, as well, and, recently, it has been shown that this type of enzymes are active in the *sn*-1-position of galactolipids as well [2]. A typical enzyme in this group is the *Rhizopus arrhizus* lipase. Phospholipase A_1 can be used for modifications of the *sn*-1-position of glycerophospholipids, but it is usually more convenient to use a lipase.

For modifications in the *sn*-2-position of glycerophospholipids, phospholipase A_2 is the enzyme of choice. Porcine pancreatic phospholipase A_2 is the most frequently used enzyme in this group.

The most well-studied phospholipase C is the one from *Bacillus cereus*. Concerning phospholipase D, several different enzymes have been used, mainly plant enzymes (for example from cabbage) and microbial ones (for example from *Streptomyces*). Due to the broad substrate specificity of some phospholipase D enzymes, it has been possible to use them for the synthesis of a wide variety of phospholipids [3].

3. Synthetic strategies

The enzymatic exchange reactions can be carried out by using a two-step approach: first, hydrolysis, and then, re-esterification with the moiety to be incorporated (Fig. 2 shows the example of lipase-catalysed exchange of the fatty acid in the *sn*-1-position). Alternatively, the exchange can be achieved in a one-step transferase reaction (Fig. 3 shows the example of lipase-catalysed exchange of the fatty acid in the *sn*-1-position). Typically, hydrolytic reactions are carried out in emulsions, while esterification and transferase reaction normally are carried out in organic solvents with low water content. True transferase reactions are only possible if the mechanism of the enzyme catalysed reaction works via a covalent intermediate (acyl enzyme or phosphatidyl enzyme). However, other enzymes can be used in one-step reactions, as well. In this case, the exchange is achieved because hydrolysis and esterification occur in parallel.

For some exchange reactions described in this chapter, one can make a choice to use either the two-step or a one-step approach. Concerning phospholipase D, the only approach used is the one-step transferase reaction, but concerning the exchange of fatty acids both strategies can be applied. The advantage of the transferase approach is that the reaction is done in only one step. A drawback is that a high excess of the fatty acid to be incorporated is needed since there will be a competition between that fatty

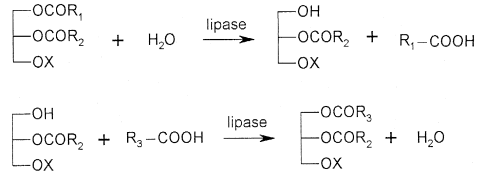

Fig. 2. Exchange the fatty acid in the *sn*-1-position of glycerophospholipids using hydrolysis followed by re-esterification with a new fatty acid. Both steps are catalysed by a lipase. Normally, there is a separation step between the two biocatalytic steps.

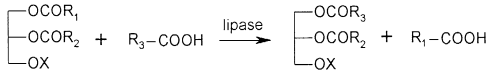

Fig. 3. Exchange the fatty acid in the *sn*-1-position of glycerophospholipids using a one-step transferase reaction (acidolysis). The formula shows the net reaction. The reaction mechanism involves acyl enzymes of both the fatty acid to be removed and the one to be incorporated as well as lyso-phospholipids.

acid and the one(s) already present in the substrate lipid. In the hydrolysis–esterification approach, two reaction steps are needed and in between them a separation step. On the other hand, the fatty acid to be replaced is removed in the separation step which makes it possible to prepare products of very high purity. Also, in this approach, there is often a need for a high concentration of the fatty acid to be incorporated in order to get a good yield in the esterification reaction. In the literature, the one-step approach is the most common one.

4. Important points to consider in enzymatic exchange reactions in lipids

4.1. Equilibrium position

In both the two-step and one-step approach of the enzymatic fatty acid exchange reactions, partially deacylated lipids (lysolipids) constitute reactions intermediates. Therefore, the equilibrium constant in the esterification of the lysolipid will be an important factor determining the yield of fully acylated product. If the reaction is carried out as an esterification, the equilibrium constant will determine the maximal yield (the reaction is under thermodynamic control). The equilibrium constant is quite different for different types of lipids. The esterification of a diacylglycerol to a triacylglycerol is thus considerably more favoured than the corresponding esterification of a lysophospholipid, and the esterification of lysogalactolipids is even more unfavourable. The values of the equilibrium constants give indications of which reaction conditions are needed to obtain acceptable yields in the reactions.

The parameters, which can be changed to obtain a high yield of fully acylated lipid in esterification reactions, are the fatty acid concentration and the water activity (or water concentration). In order to obtain a high yield, the fatty acid concentration should be high and the water activity low. It is usually not a problem to use a high fatty acid concentration, although a slight decrease in reaction rate was observed at very high concentrations [4].

Concerning the possibilities of using low water activity, enzymes differ very much. For most enzymes, the catalytic activity increases with increasing water activity. However, many lipases are active even at low water activity [5], and this makes it possible to obtain high yields in lipid esterification reactions. Pancreatic phospholipase A_2, on the other hand, requires a water activity of about 0.2 to be active, which reduces the obtainable yields compared to the lipase-catalysed reactions. Furthermore, the reaction rate at low water activity is quite low with this enzyme. One way to get a high yield in the esterification in a reasonable time, was to start the reaction at a relatively high water activity to get some product formation in a short time. When the equilibrium position was approached, the water activity was decreased which made possible some further esterification. This procedure was repeated until the water activity became too low for the enzyme. In this way, a yield close to the equilibrium position at the lowest water activity at which the enzyme expresses activity, was reached (60% yield) [4].

In the modification of galactolipids, the equilibrium constants are even less favourable than in the case of glycerophospholipids. This means that very low water activity and high fatty acid concentration must be used to obtain reasonable yields. Under those conditions, acylation of the primary hydroxyl in the digalactosyl residue is favoured as well. If one wants to avoid this, the primary hydroxyl group must be protected (see below).

When the transferase approach is used, high yields can sometimes be obtained although the equilibrium constants are not so favourable. These reactions are under kinetic control. The kinetics for phospholipase D-catalysed transphosphatidylation are often very favourable, which means that high yields can be obtained even at a high water activity. In fact, most of those reactions are carried out in aqueous–organic two-phase systems, which means that the water activity is close to 1.0.

4.2. Acyl migration

One of the most attractive features of enzyme-catalysed reactions is their selectivity. In the reactions involving fatty acid exchange in lipids, the regioselectivity of lipases and phospholipase A_2 is used for the selective exchange of fatty acids in the *sn*-1- and *sn*-2-positions, respectively. Of course, spontaneous migration of the fatty acid residues is a highly unwanted side reaction in those cases. But acyl migration does occur to some extent if the reaction sequence involves lipid species having free hydroxyl groups adjacent to acyl groups, and the reaction conditions are not designed to suppress this side reaction [6] (Fig. 4). Both the hydrolysis–esterification approach and the transferase approach involve lipid species in which acyl migration can occur, and, thus, it is important to choose reaction conditions with care.

Acyl migration is catalysed by acids and bases. Furthermore, support materials used for enzyme immobilisation can catalyse acyl migration. A general strategy to avoid acyl migration is to use highly active enzyme preparations (containing large amounts of enzymes) on a support that does not catalyse acyl migration. Due to the high reaction rate obtained, the residence time in the reactor, and, thus, the time available for acyl migration to occur, is limited. In some cases, acyl migration has been successfully inhibited by borates [7].

Another way to suppress acyl migration is to convert the product to the solid state. This was done in the synthesis of lysophosphatidic acid by acylation of glycerophosphate. When the reaction was carried out at a temperature low enough to make the lysophosphatidic acid precipitate, close to quantitative yields were obtained [8]. On the other hand, if a higher reaction temperature was used, acyl migration was promoted, and this was followed by a second acylation step yielding phosphatidic acid [8]. The same strategy was used to synthesize either lyso-

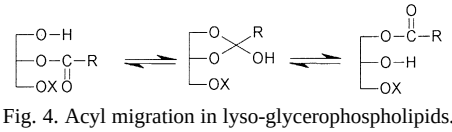

Fig. 4. Acyl migration in lyso-glycerophospholipids.

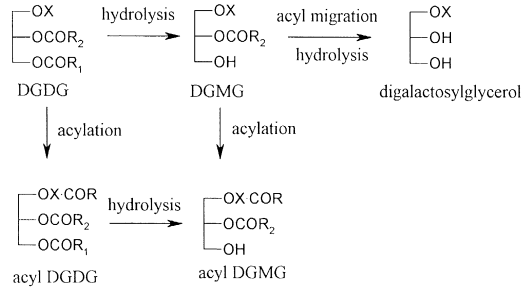

Fig. 5. Reaction scheme for side reactions occurring during lipase-catalysed fatty acid exchange in DGDG. X = digalactosyl residue. The reactions involve acyl migration, hydrolysis and acylation in the carbohydrate part of the molecule.

phosphatidylcholine or phosphatidylcholine by acylation of glycerophosphorylcholine [9]. In the syntheses of the fully acylated lipids, acyl migration was thus used as a step in the sequences leading to the desired products.

In addition to the formation of side products with fatty acids in the wrong positions, acyl migration frequently leads to reductions in the total yield of fully acylated lipid. The reason is that when a regioselective enzyme has removed one fatty acid from the lipid, it cannot remove the other one as long as it remains in its original position. However, if acyl migration occurs, the new lysolipid formed will be a substrate for the enzyme and the second fatty acid

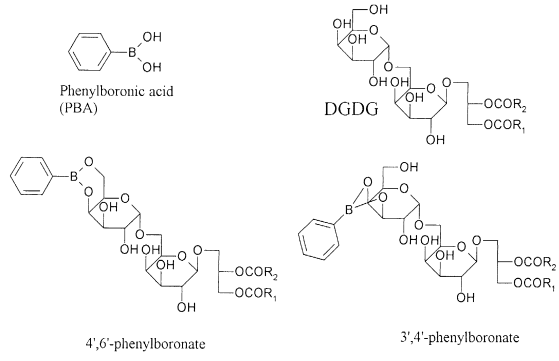

Fig. 6. Possible complex formation between phenylboronic acid and the carbohydrate part of DGDG. The aim of the complexation was to protect the primary hydroxyl group in the 6′ position. The formation of a 4′, 6′-phenylboronate would achieve this. It is also possible that the formation of the 3′,4′-phenylboronate would make the carbohydrate part bulky enough to make it a poor nucleophile and thus reduce the acylation in the 6′ position.

can be removed. The product of this reaction, a completely deacylated lipid, often precipitates under the reaction conditions used, which leads to a decrease in yield of the fully acylated lipid.

4.3. Other side reactions. Extra acylation

An extra point that must be taken into consideration in the enzymatic modification of galactolipids is the risk of acylation in the carbohydrate part of the molecule. DGDG contains a primary hydroxyl group in the outer galactosyl residue and this can be acylated relatively easily by the lipase used for fatty acid exchange in the *sn*-1-position. The product, containing one fatty acid on the carbohydrate part and two fatty acids on the glycerol backbone can of course be an interesting product, but if the aim of the reaction is just to exchange the fatty acid in the *sn*-1-position, this extra acylation should be suppressed. This side reaction makes the reaction scheme for fatty acid exchange in DGDG quite complicated (Fig. 5). Some formation of the digalactosylmonoacylglycerol (DGMG) is necessary due to the mechanism of the reaction. However, its concentration in the final product should be minimised. Acyl migration and further hydrolysis can lead to completely deacylated lipid as with glycerophospholipids.

One way to suppress acylation in the carbohydrate part of DGDG, was to include phenylboronic acid in the reaction mixture [10]. It is known that phenylboronic acid can form complexes with galactosyl residues (Fig. 6). It was not sorted out which complexes actually formed, but it was obvious that the inclusion of an equimolar amount of phenylboronic acid drastically changed the product composition in lipase-catalysed exchange of the fatty acid in the *sn*-1-position of DGDG (Fig. 7). The amounts of products having an acyl group in the carbohydrate part of the molecule constituted about half of the product when the reaction was carried out in the absence of phenylboronic acid, but under 5% was formed with phenylboronic acid addition [10] (Fig. 7).

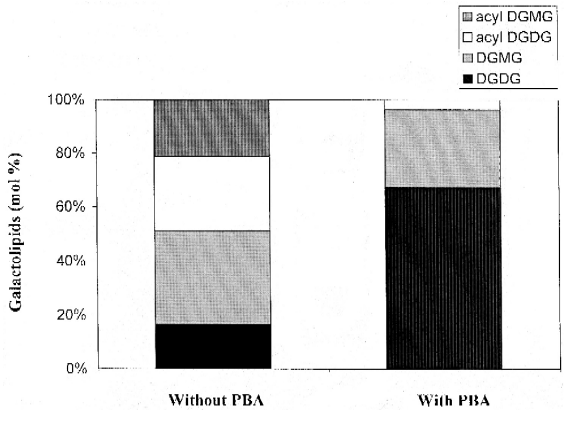

Fig. 7. Product composition in the fatty acid exchange in DGDG in toluene at a water activity of 0.11. The reaction was catalysed by *R. arrhizus* lipase immobilised on polypropylene [10]. For explanation of abbreviations, please see Fig. 5.

5. Conclusions

When designing a process for enzymatic lipid modification, it is beneficial to have information about the equilibrium constants of the reactions. This indicates if the reaction can be carried out under thermodynamic control or if a kinetically controlled approach must be used. Furthermore, the equlibrium constant indicates how other key parameters (water activity and concentrations of other reactants) must be chosen.

In enzymatic lipid conversions, it is important to keep in mind that acyl migration can cause problems. Products with fatty acids in the wrong positions can be formed and acyl migration followed by further hydrolysis can cause a reduction in yield. However, in some cases, acyl migration can be used as a step in the synthetic pathway.

Using the three enzyme lipases, phospholipase A_2 and phospholipase D, it is now possible to convert a mixture of natural glycerophospholipids to a tailor-made product with specified moieties in all positions. Concerning galactolipids, it is possible to exchange the fatty acid in the *sn*-1-position of DGDG using a lipase as catalyst. At the same time, a fatty acid can be introduced on the primary hydroxyl of the digalactosyl residue thus yielding a product containing three fatty acids. If only fatty acid exchange is desired, the primary hydroxyl group can be protected by complexation with phenylboronic acid.

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