Chapter 4

ENZYMATIC MODIFICATION OF OILS AND FATS

As shown in CHAPTER 3, the current chemical modification processes of oils and fats usually require high temperature and pressure which degrade the oils and fats and introduce impurities. In contrast, lipases can work under milder conditions. In addition, lipase's specificity allow modification of oils and fats in more sophisticated way. This chapter outlines basics and application of lipases for modification of acylglycerols.

4-1 Catalytic properties of lipases

4-1-1 What is lipase ?

Lipase, or triacylglycerol (TAG) acylhydrolases (E.C. 3.1.1.3) is a kind of carboxy esterase. Under physiological conditions, this enzyme catalyzes hydrolysis of oils and fats, so the biological role of lipase is metabolism of lipids. Besides, lipases catalyze various reactions (shown below) other than hydrolysis, under controlled conditions. The capability of catalyzing various reactions makes lipases very useful biocatalysts for modification oils & fats, and other synthetic chemistry.

4-1-2 Reactions catalyzed by lipases

Lipases (and esterases) catalyze three types of reactions (Figure 4-1-1). The catalytic action of lipases is reversible. They catalyze hydrolysis in an aqueous system, but also esterification (reverse reaction of hydrolysis) in a microaqueous system, where water content is very low.

Transesterification is categorized into four subclasses according to the chemical species which react with the ester. **Alcoholysis** is the reaction with an ester and an alcohol, while **acidolysis** is the one with an ester and an acid. **Interesterification** is a reaction between two different esters, where alcohol and acid moiety is swapped. In **aminolysis** , an ester is reacted with an amine, generating an amide and an alcohol.

Some of the reactions in Figure 4-1-1 are, in principle, the same as the ones for chemical modifications shown in CHAPTER 3. However, there are big differences between the chemical and lipase-catalyzed reactions. First, the lipase-catalyzed reactions can proceed under milder conditions than the chemical reactions. In one aspect, this is advantageous, because undesired side reactions such as heat degradation of the substrates can be avoided. But in another aspect, it may be a drawback because lipases can work only under mild conditions, i.e. lipase is much weaker than the chemical catalysts.

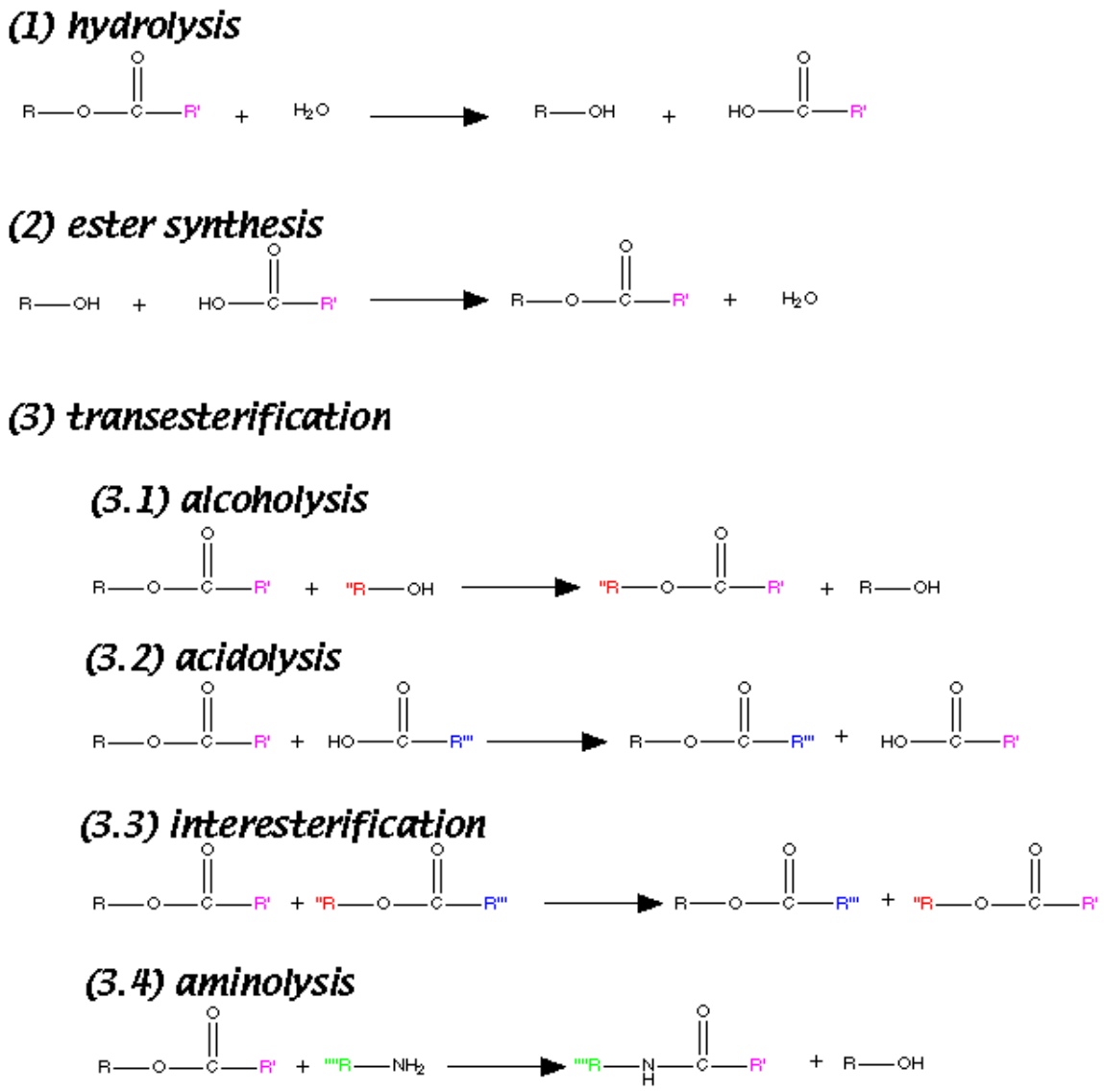

Figure 4-1-1: Types of reactions catalyzed by lipases.

Second, (and more importantly), the lipase-catalyzed reactions are specific (or selective) for particular acyl groups, particular positions of the substrates, or particular stereoisomers. This specificity enables the modification of oils and fats in more sophisticated way.

4-1-3 Specificity of lipases

1) Positional specificity

It is often stated that lipases can be placed according to their **positional specificity** into two groups: 1,3-positional-specific and non-positional-specific. Usually, pancreatic and fungal lipases are 1,3-positional-specific, while yeast and bacterial ones are non-positional-specific or weakly 1,3-positional-specific. However, it should be noted that the positional specificity of lipases is not strictly divided into the two categories, but it varies widely in the range of very distinctly 1,3-positional-specific to very weakly specific or completely non-positional-specific.

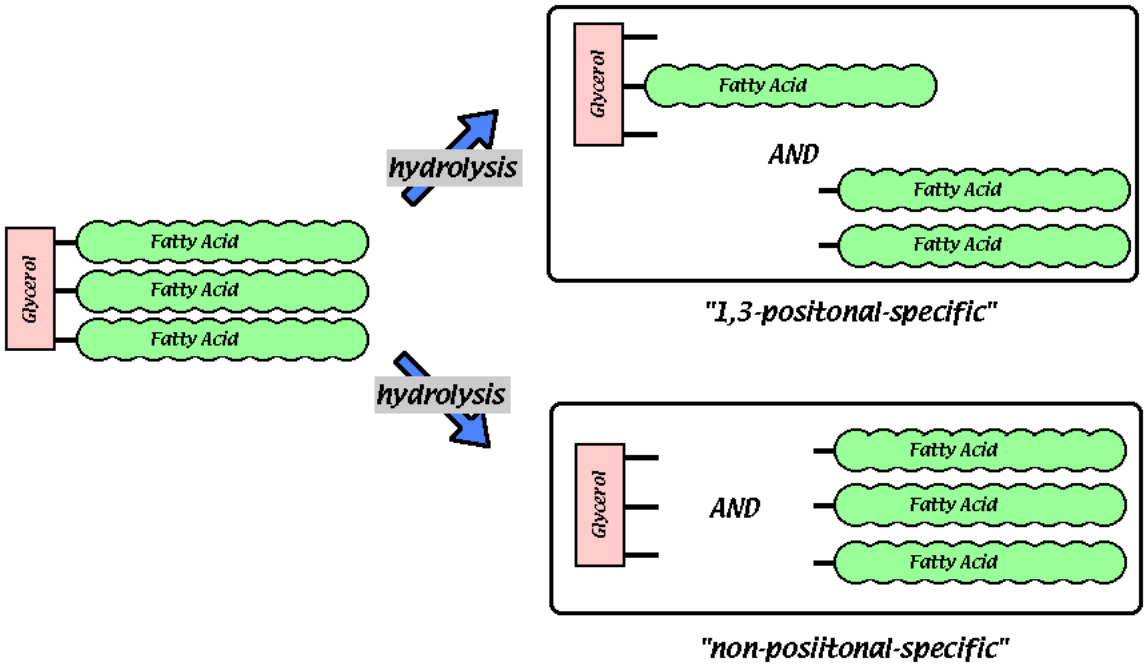

Figure 4-1-2: Positional specificity of lipases on TAGs.

Non-enzymatic **acyl migration** (Figure 4-1-3) from 2- to 1,3-position in mono- and diacylglycerols makes this categorization more complicated to establish (Figure 4-1-4). The migration is spontaneous and promoted by acid, alkaline and heat.

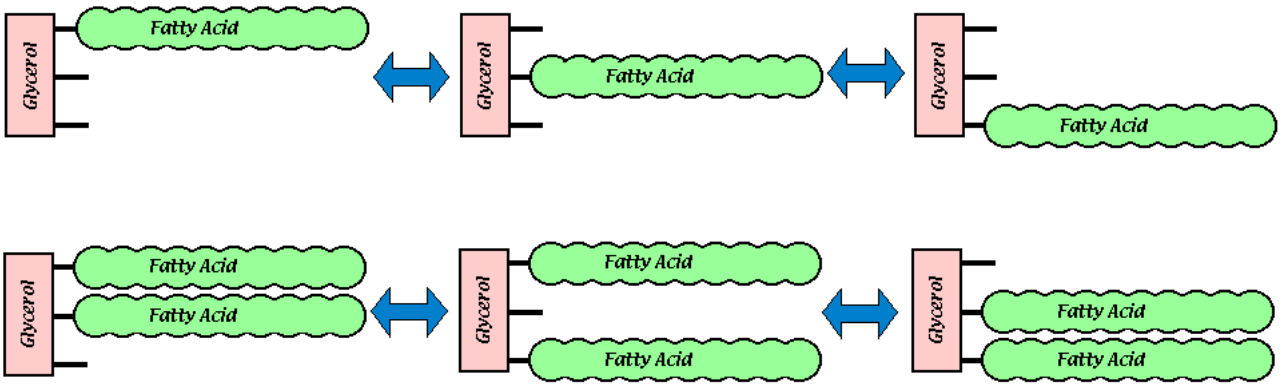

Figure 4-1-3: Non-enzymatic acyl migration of partial glycerides. In mono and diacylglycerol molecule, acyl groups move to the adjacent free -OH group within the same molecule. This is spontaneous and promoted by acid, alkali, and heat.

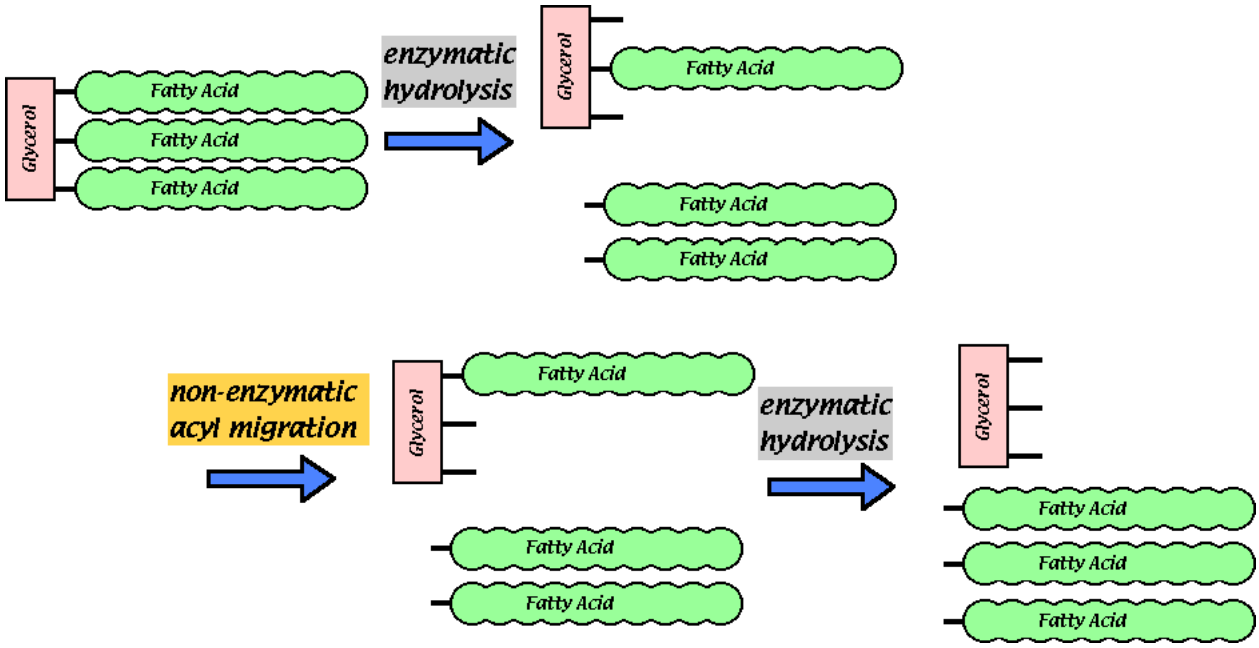

Figure 4-1-4: Is this lipase non-positional- specific or 1,3-positional-specific? The non-enzymatic acyl migration confuses the situation.

2) Fatty acid specificity

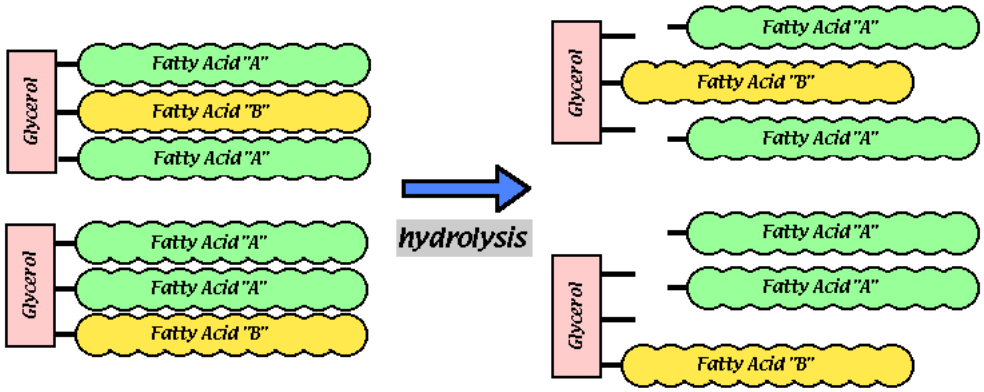

Figure 4-1-5: Fatty acid specificity of lipases. Suppose that "Fatty acid A" is well-hydrolyzed acyl group, while "Fatty acid B" is resistant against the lipase's action. Incubation of the substrate with the lipase will release "Fatty acid A" but "Fatty acid B" residues remain unreacted.

Fatty acid specificity of lipases arises from the difference of reaction rates on the ester linkages with different fatty acids. It is usually determined by comparing the relative hydrolysis rate of various fatty acid esters with different acylgroups. In fact, the specificity spectrum diverges significantly, which indicates that lipases seem not to have strict fatty acid specificity.

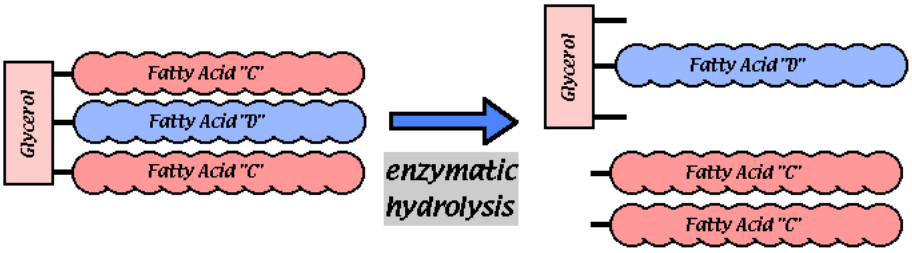

Figure 4-1-6: Is this lipase prefers "fatty acid C" over "fatty acid D", or just 1,3-positional-specific? If the lipase is 1,3-positional-specific, it will be difficult to make conclusion.

Two facts make the determination of fatty acid specificity more ambiguous: 1) it is superimposed by the positional specificity when natural oils and fats are used for tests (Figure4-1-6), and 2) the results are affected by the physical properties of the substrate (solid or liquid, droplets' size for emulsions, solubility in water for low carbon-number TAGs, etc.). To avoid these problems, a method was proposed to test the relative fatty acid specificity of a lipase using a chemically interesterified oil, of which fatty acid positional distribution is completely random. According to the results, polyunsaturated fatty acids are the most resistant to lipase-catalyzed hydrolysis.

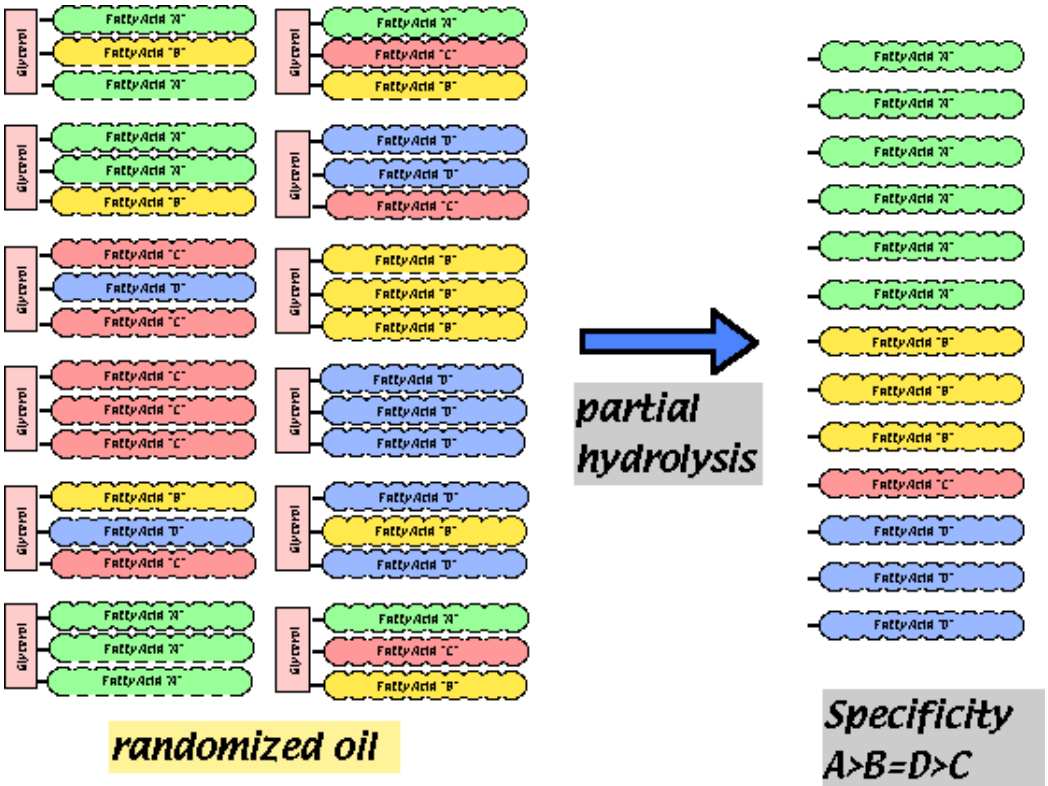

Figure 4-1-7: Determination of relative fatty acid specificity of lipase. Chemically interesterified oil with completely random fatty acid distribution is partially hydrolyzed with the lipase that is tested, followed by analysis of the composition of the released fatty acids. This method can cancel the influence of the positional specificity of the lipase tested.

4-2 Enzymatic reactions in organic media

4-2-1 Do enzymes work in organic media?

Generally, enzymes work under physiological conditions, i.e. in water. But some of the enzymes are still active in media other than water.

The notion that enzymes are catalytically active in organic solvents was known as early as the turn of the 20th century. However, it was not until the mid-1980 that Prof. Klibanov in USA and Prof. Fukui in Japan advocated the full exploitation of the potential use of enzymes in organic media. The ability of enzymes to catalyze useful synthetic transformations in organic media is now beyond doubt. There are some advantages in using enzymes in organic media as opposed to aqueous media, including:

1) shifting thermodynamic equilibrium to favor synthesis over hydrolysis,
2) reduction of water-dependent side reactions,
3) elimination of microbial contamination, and
4) suitable for reactions with substrates insoluble and/or unstable in water.

4-2-2 Enzymatic reactions in solvent-free system

Enzymatic reactions in organic media are actually divided into two systems: reactions performed in **organic solvent systems** and in **solvent-free systems**. The solvent-free system, i.e. the reaction mixture comprising only liquid organic substrates (such as liquid oil) without any organic solvent, if it is possible, has high volumetric performance and economical advantages over the organic solvent system especially for large scale production. It is also desirable for the synthesis of food-grade products since very stringent safety regulations concerning organic solvent usage have to be observed in food industry. Only *n*-hexane, acetone and ethanol are allowed in industrial food processing.

4-3 1-Monoacylglycerols

4-3-1 Importance of monoacylglycerols

1-monoacylglycerol (or 1-MAG) is one of the most common emulsifiers for food, cosmetics and pharmaceutical industries. Over 70% of the emulsifiers used for various food products in the world are 1-MAG. It facilitate the formation and stabilize the water in oil emulsions and therefore, they are essential ingredients of margarine and low-calorie spreads.

Industrial production method of 1-MAG is the chemical glycerolysis of oil as shown in CHAPTER 3. In fact, the products obtained by this chemical method contain only 30-50% 1-MAG. The product is distilled under high vacuum (2-4 mmHg) to increase the 1-MAG content and to remove the undesirable degradation by-products. After the molecular distillation, the product contains about 95% MAG.

4-3-2 Enzymatic syntheses of 1-MAGs

Enzymatic syntheses are very promising alternatives for chemical methods in lipid industry. They solve some serious problems encountered in chemical processes such as energy consumption, high reaction temperatures which cause degradation of product quality, toxic catalysts, etc. Glycerolysis of TAGs, direct esterification of glycerol and esterification of protected glycerol are the most practical approaches for 1-MAG synthesis.

Glycerolysis of TAGs: Lipase-catalyzed glycerolysis of TAGs is by far the enzymatic process which can compete in the future with the chemical glycerolysis for the commercial production of 1-MAG (Figure 4-3-1). The main advantages of the process are the low temperatures employed and the high yields of 1-MAGs (70-90%) obtained.

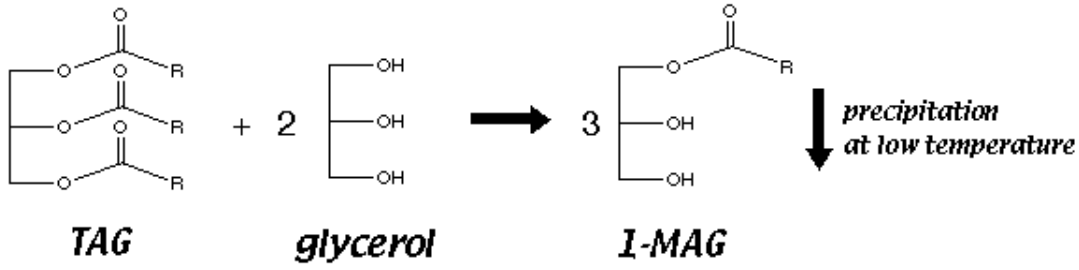

Figure 4-3-1: Synthesis of 1-MAG by solid-phase glycerolysis.

The principle of the method is the shift of reaction equilibrium towards the 1-MAG synthesis. 1-MAG has higher melting point than TAGs (i.e. 1-MAG solidifies easier than TAGs). Therefore, by lowering the reaction temperature at less than 10 ℃, the formed 1-MAGs precipitates, and then the equilibrium is shifted to MAG synthesis. No solvent is used and an optimum temperature program ensures high reaction rates and final 1-MAG yields. Various fats and oils such as beef tallow, palm oil, lard, and olive oil were used successfully.

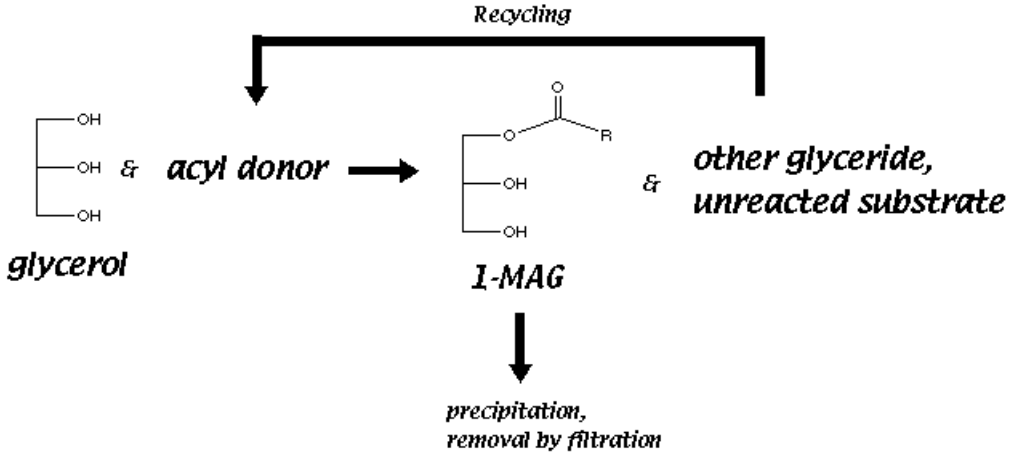

Figure 4-3-2: Synthesis of 1-MAG by esterification of glycerol.

Esterification of glycerol: Glycerol is reacted with various free fatty acids, fatty acid methyl esters, and vinyl esters dissolved in an organic solvent for the production of solid 1-MAG (Figure 4-3-2). The esterification is carried out in a reactor vessel and the filtered reaction media is continuously circulated into a second vessel (separator) in which 1-MAGs are separated at lower temperature and the reaction media with the unreacted substrate is reintroduced in the reactor vessel.

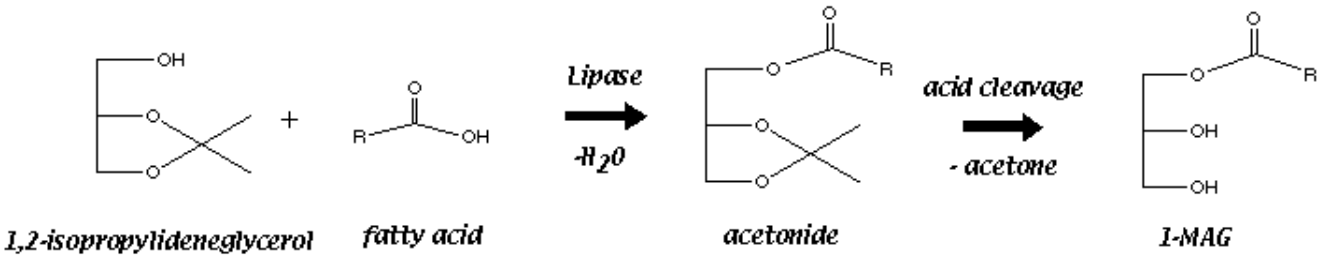

Figure 4-3-3: Synthesis of 1-MAG by esterification of protected glycerol, followed by deprotection.

Esterification of glycerol: Protected glycerol i.e. 1,2-isopropylideneglycerol, is acylated in solvent or non-solvent system (Figure 4-3-3). 1-MAG is finally obtained by the chemical cleavage of the acetonide by acid.

4-4 2-Monoacylglycerol

4-4-1 Use of 2-monoacylglycerol

Unlike 1-MAG, 2-monoacylglycerol (2-MAG) seems not to be suitable as an commercial product, because it very easily isomerizes to 1-MAG by acyl migration. However, it is of value as a synthetic intermediate of other lipid products. For example, "symmetric structured TAG" (described in the next CHAPTER) can be synthesized from 2-MAG.

4-4-2 Synthesis of 2-MAG

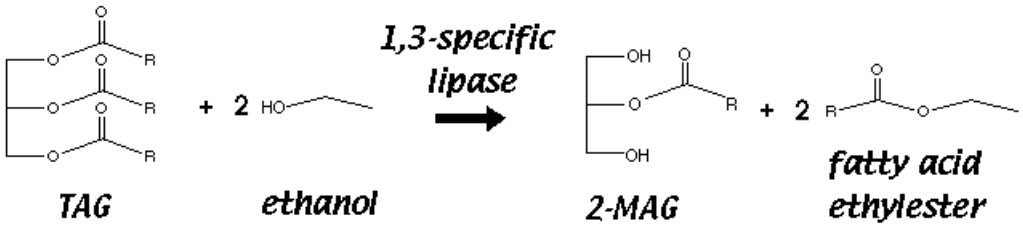

Figure 4-4-1: Synthesis of 2-MAG by deacylation of TAG with 1,3-position-specific lipase.

2-MAG can be prepared by deacylation of TAG by 1,3-position-specific lipase (Figure 4-4-1). For the deacylation, alcoholysis is better than hydrolysis, because acyl migration can be suppressed in alcohol rather than in water, and because the removal of ethanol is easier then water after the reaction. The TAG dissolved or suspended in alcohol is reacted with lipase. In this system, the alcohol acts both as the substrate and as the solvent, (like water in hydrolysis). Since the alcohol is in excess, the reaction proceeds almost completely, giving 1mol equivalent of 2-MAG and 2mol equivalent of fatty acid ethylesters.

4-5 1,3-Diacylglycerols

4-5-1 Applications of 1,3-Diacylglycerols

1,3-Diacylglycerols (1,3-DAG) has emulsifying properties and are used as surface active agents in food industry usually as mixtures with 1-MAG.

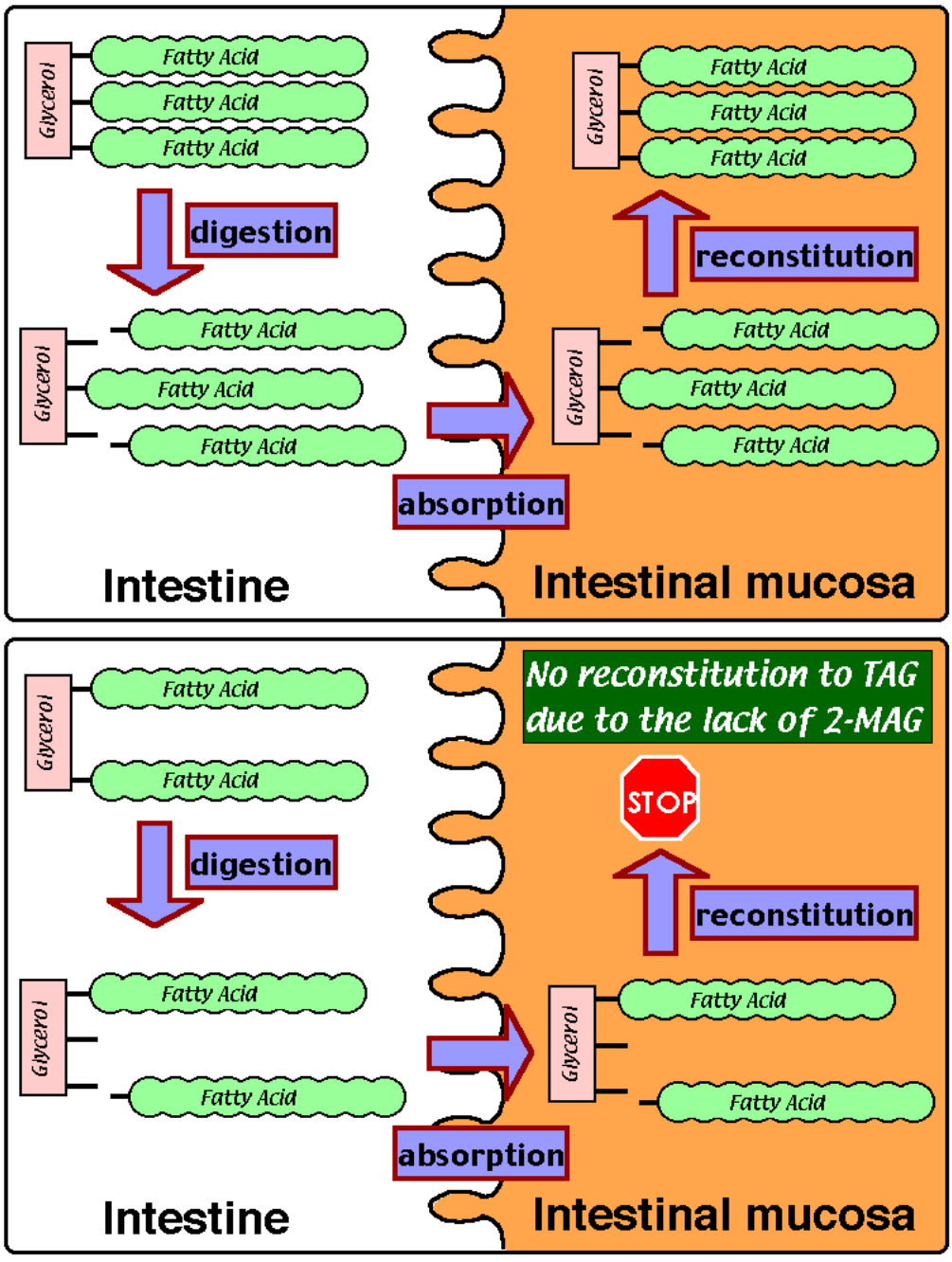

Figure 4-5-1: Digestion and absorption of TAG (upper) and 1,3-DAG (lower) in animal intestine.

Recently,1,3-DAG is used as a specialty oil, which prevents the accumulation of body fat. Although 1,3-DAG and TAG have almost the same energies (9 kcal/g), they are different in the mechanism of their metabolic fates. As shown in Figure 4-5-1, TAG is digested by 1,3-position-specific pancreatic lipase into fatty acids and 2-MAG. They are absorbed

through intestinal mucosa, and the 2-MAG is reesterified to reconstitute TAG. In contrast, digestion of 1,3-DAG with the lipase does not generate 2-MAG, thereby TAG is not generated, resulting in prevention of body fat accumulation (Figure 4-5-1). Featuring this unique property, KAO launched 1,3-DAG (Kenko Econa^{TM}) as a functional cooking oil.

4-5-2 Enzymatic synthesis

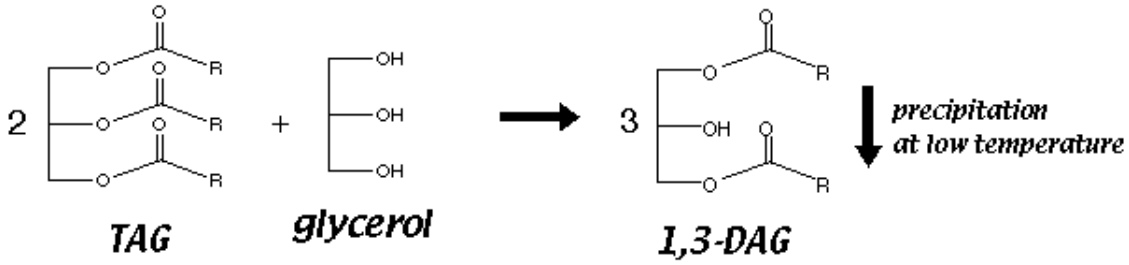

Figure 4-5-2: Synthesis of 1,3-DAG by solid-phase glycerolysis of TAG.

1,3-DAG with relatively high melting points (= high content of saturated fatty acid) can be obtained by enzymatic solid-phase glycerolysis of TAGs (Figure 4-5-2). Temperature program and glycerol to oil molar ratio are essential for the achievement of high 1,3-DAG yields. Similar to 1-MAG production, the driving force of the process is here also, the selective crystallization of 1,3-DAG. Bacterial lipase catalyzed the glycerolysis of hydrogenated beef tallow with 90% DAG yield from which 95% was 1,3-DAG.

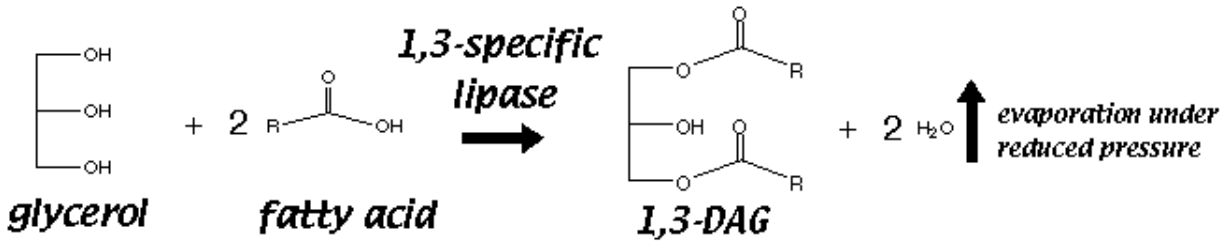

Figure 4-5-3: Synthesis of 1,3-DAG by direct esterification of glycerol with 1,3-specific lipase.

Alternatively, 1,3-DAG can be prepared in high yields by direct esterification of glycerol catalyzed by a lipase. One of the key point is the use of 1,3-position-specific lipase. Free fatty acids are used as acyl donors. To increase the yield, the generated water is removed by reducing the pressure of the reaction vessel. This method is industrially applied for the production of Kenko Econa^{TM}.