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Review

Lipase-Catalyzed Reactions for Fats and Oils in Non-Polar Solvent

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유기용매 내에서의 유지의 리파제 촉매반응

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Lipases are well known as the enzymes which catalyze the hydrolysis of ester bonds combining aliphatic chains and glycerol on mono-, di- and triglycerides. Their reactions are characterized by being heterogeneous and catalyzing the water-insoluble substrates. This property has been one of the hurdles which delayed the application of lipases in fats and oils industry. However, with the development of biological reaction system of which organic solvent is introduced in part or whole as the reaction media, enzymatic manipulation of fats and oils is attracting increasing attention from the academic and industrial sectors. Trials in two-phase system and reversed micellar system to produce fatty acids through enzymatic hydrolysis of triglycerides proved to be efficient in respect to volumetric productivity, fat hydrolysis rate, product separation, etc. In organic solvent system lipases have been found to have the ability to catalyze aminolysis, transesterification, esterification, thiotransesterification and oximolysis that are virtually impossible to catalyze in water. The organic solvent system is being extensively used in interesterifying glycerides to produce a fat with the modified physical and chemical nature.

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is the enzyme which catalyzes the hydrolysis of the esters of long-chain aliphatic acids from glycerol. Though lipases have been known as early as 1849 (1), they are not well understood as yet because the catalytic reaction of the enzymes is performed in heterogeneous reaction system containing insoluble substrates (2), and this property makes it difficult to analyze the kinetic results. It may also be the main reason why previous studies on lipases were mainly concerned with characterization of the enzymes rather than dealing with their industrial application. Their use, at best, have been limited to the *in situ* application for enhancing flavors in the dairy products (3,4).

With the development of such biological reaction systems as the use of organic solvents as a medium for the conversion by enzymes of poorly water-soluble compounds, the application of lipases in fats and oils industry has become a new focus, since the potential for application of lipases is great (5). Earlier subjects of research on the use of lipase as the possible industrial catalysts were primarily concentrated on the fatty acid production by hydrolyzing fats and oils (6). Under certain conditions, however, lipases can be shown to catalyze the formation of glycerides from free fatty acids and glycerols (7), since their reactions are reversible. The other field of great importance and of interest is the interesterification of triglycerides (8). Lipase can replace the fatty acid moieties of triglycerides with other fatty acid(s). The resulting product in that reaction is a fat with the modified physical and chemical properties. The production

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of cacao butter-like fat from olive oil and stearic acid or palmitic acid is a good example of products which can be obtained from enzymatic interesterification (9).

As a reflection of growing interest in lipase applications of fats and oils industry, this review article is intended to summarize the characteristics and the application field of the enzymes and to introduce the various types of reaction system in which water-immiscible organic solvents have been used as part or whole of the reaction medium. Concrete examples of lipase-catalyzed reactions are also described briefly.

Lipases

Lipases are found in pancreas, blood plasma, saliva, milk, in a number of lipid-producing plants (soybean, castor bean, peanut, etc), and in yeasts, molds and bacteria (10). A variety of lipase from different origins can be classified into 3 groups according to its specificities: non-specific, 1,3-specific and fatty acid-specific lipase (11). The first group of lipases catalyzes the release of any fatty acid at any position on glycerol molecule. With these enzymes, triglycerides can be completely hydrolyzed to free fatty acid and glycerol via di- and monoglyceride. The lipases from *Candida rugosa, Corynebacterium acnes and Geotricum candidum* are examples of enzymes of this type (12).

The second group shows distinct specificity to the outer 1- and 3-positions of glycerides, but does not show the stereospecificity (relative catalytic activity at the *sn*-1 and *sn*-3 positions on the glycerol moiety) (13). The lipases with the regiospecificity are found in *Aspergillus niger, Mucor miehei* and various *Rhizopus* species (14-16). If a 1,3-specific lipase is used to catalyze the interesterification, acyl exchange or migration is confined to the *sn*-1 and *sn*-3 position (12,17).

The third group of lipases catalyzes the release of a particular type of fatty acid from glyceride molecules. This fatty acid specificity is scarcely found in most extracellular lipases. However, *Geotricum candidum* secretes a lipase which has the characteristics to release preferentially long-chain fatty acid with a *cis* double bond in the 9-position (18). This lipase did not show the regiospecificity.

In contrast to the most ordinary enzymes, lipase is distinguished by its unique action at phase interface (19), the involvement of a lipid-water interface in the catalytic process. This interfacial activation was observed systematically by Sarda and Desnuelle (2) in studying the rate of hydrolysis of triacetin emulsified in gum arabic. When the concentration of added triacetin is low enough to be a true solution, the rate of hydrolysis is very slow, but it increases greatly as the concentration of substrate is increased to exceed to its critical concentration. Similarly, this type of stimulation could be produced without exogenous additives by merely increasing the total area of the interface (20,21). All the experimental facts strongly suggested that it was the adsorption of the enzyme at the interface that was important in addition to the normal enzyme-substrate binding. If the same weight of triolein was emulsified to give different interfacial areas, the rate of hydrolysis was fastest when the interfacial area is greatest (22).

Application fields of lipase

Various lipases, alone or in combination with other enzymes, have been used in dairy and other food processes, and lipases produced *in situ* by microorganisms are important in making foods palatable and acceptable. Manufacturers of lipases have suggested that the enzymes may be used in detergents, leather processing, pharmaceuticals, cosmetics, etc. For details of the industrial application of microbial lipases in those fields, see other review articles (23-26).

The new focus of currently intensive investigation on the use of lipases is in the mainstream of fats and oils processing; fatty acid production, glyceride synthesis and interesterification. The standard technology for fatty acid production is high temperature and pressure countercurrent steam splitting (27). This reaction can also be achieved enzymatically and have been the subject of many publications and patents (28-30). The rate and degree of hydrolysis varies with the reaction conditions, but under the certain conditions palm oil could be completely hydrolyzed within 3

hours (31).

All the lipase-catalyzed reactions were carried out at ambient pressure and at a relatively low temperature. Thus these procedures may prove to be economical because corrosion-proof vessels or equipments are not required and the fatty acids are much less corrosive. To secure the complete hydrolysis, the enzyme should have no specificity either to fatty acid or to its position on the glycerol molecule. An appropriate choice may be the lipase from *C. rugosa* because it is being produced cheaply by fermentation. Second area of potential application of the lipases is the reversal of the hydrolysis reaction (32,33), in order to synthesize the glycerides. By decreasing water activity, it is possible to shift the equilibrium position toward the synthesis. Formation of all the theoretically possible glyceride types on incubation of excess fatty acid and lipases obtained from *A. niger* or *C. rugosa* have been determined when water was removed from the reaction system. The products could be controlled by using the enzymes with the regiospecificity. If lipases derived from *Rhizopus delemar* or *A. niger* were used, only mono- and diglycerides were formed: triglyceride was not a product under these conditions (7). In general, the esterification process proceeds slowly but has the advantage of taking place at mild conditions and without having to resort to the use of fatty acyl halides.

Remaining one area is interesterification which is the process to exchange some of the fatty acids on the triglyceride molecule for another fatty acid. While ester exchange can be catalyzed chemically, the chemical interesterification suffers from the random distribution of the entire fatty acid pool at each position on the triglyceride (34). When the lipase is used as the catalyst, production of a variety of desirable glyceride mixtures may be possible due to the specificities for either fatty acid chain length or position (35). Under moderate reaction conditions, the reaction can still proceed at the rates that are acceptably rapid with regard to industrial processing time scales. Fats and oils with the modified physical properties, particularly their melting characteristics, attract commercial interest because of the great potential of their specific applications. For example, on reacting a mixture of palm oil midfraction and stearic acid in the presence of 1,3-regiospecific lipase from *Mucor miehei,* a particular product containing 1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol, 1,3-distearoyl-2-oleoyl-*rac*-glycerol and 1,3-dipalmitoyl-2-oleoyl-*rac*-glycerol has been obtained (9). Fractionation of the resulting mixture by a conventional technique gave in good yield a fat which has very similar to cacao butter both in physical properties and chemical composition.

Conventional reaction system for lipases-emulsion

In emulsion system lipase catalyzes only one reaction, hydrolysis, not only because alteration in catalytic properties of the enzyme is not afforded, but because other reactions in non-polar milieu are suppressed by hydrolysis (see below).

As mentioned previously lipase has essentially no activity at all on the dissolved substrates and the activity is enhanced markedly when the substrate is above its solubility limit in water, i.e., the velocity is a function of the surface of substrates offered to the enzyme. Accordingly, substrates must be dispersed in as fine an emulsion as possible. For substrates such as olive oil, shaking or stirring is not sufficient. Emulsification is done by blending or sonificating the mixture of water and lipids in the presence of such emulsifiers as polyvinyl alcohol, polyethylene glycol and gum arabic (36-38). Thus the step requires mechanical energy to increase the interfacial area and emulsifier to stabilize the resultant emulsion. Vigorous agitation of the emulsified mixture is also required during incubation to constantly renew the surface of the oil droplets, which increases somewhat the reaction rate. In addition the solution should contain sodium ions to suppress the enzyme inhibition by interfacial charge effects and calcium ions to accept a fatty acid which usually inhibits lipase (39,40). All these requirements have delayed the successful applications of lipases in emulsion system.

Lipase reaction in organic solvents system

In some cells at least half of the complement of enzymes is probably associated to varying degrees with membranes. In such case natural microenvironment of the enzymes is certainly less polar than bulk water, and investigations in a strictly aqueous solvent may lead to anomalous results. The use of non-aqueous solvent or mixture of such solvents with water may, therefore, be quite relevant to an understanding of how enzymes function *in vivo*. In this regard enzymes are increasingly investigated in solvent less polar than water (41-44). Aside from the above-mentioned biochemical aspects, improvement in the performance of some enzymes is expected when the reactions are carried out in non-polar solvents. This is more so when the enzymes catalyze the conversion of lipophilic substrates which are sparingly soluble in water. Several possible advantages for such reaction system are as follows. First, better solubility of lipophilic substrates in organic media could substantially reduce the volume of the reaction mixture needed to produce a given amount of product, resulting in the improvement of volumetric productivity. Product recovery from the solutions is often easy and cheap. Secondly, as the reaction environment contains relatively little water, thermodynamic equilibria of many reactions shift to the synthetic reaction, which is unfavorable in water. Synthesis of esters from carboxylic acids and alcohols or peptides from amino acids are good examples of synthetic products. Use of organic solvents as the reaction medium for the enzymatic transformation is further advantageous in the aspects of catalyst inhibition or microbial contamination. Reaction systems containing organic solvent thus far developed are two-phase system, reversed micellar system and organic solvent system (single organic phase).

Two-phase system

Two-phase system is composed of water and water-immiscible organic solvent. The aqueous phase contains the enzyme and any water-soluble cofactors or cosubstrates, and the organic phase contains the greater part of substrates and products (Fig. 1). It seems likely that in many cases the reactions occur in the aqueous phase, whereas reactions of the lipases which are active at the interface take place at the liquid-liquid interface. In order to increase the reaction rate in the two-phase system, it is essential to create sufficient interfacial area by agitating or stirring the reaction mixture. This approach has been proved to be successful with lipase whose catalytic rate has been proportional to the stirring speed (45). With the two-phase system the application of lipases has been carried out for the hydrolysis. We have tried lipase-catalyzed hydrolysis to produce fatty acids. If a reaction system composed of 20% water containing the free lipase and isooctane containing olive oil was stirred at 30 °C, degree of the hydrolysis reached over 92% within 48 hours for the substrate concentration of 10% (v/v) (46). An important point in the reaction was that the reaction rate was increased in proportion to olive oil content, suggesting that the enzyme in the two-phase system was kept from inhibition by substrates. The system was found to be particularly efficient for the conversion of fat (which is solid at room temperature by definition) because it dissolves in organic solvent. In aqueous system, however, the enzymatic hydrolysis of fat, e.g. tripalmitin, is of no practicality around the temperature (ca, 30 °C) which ensures the operational stability of most lipases, since the substrate cannot be emulsified at such a low temperature.

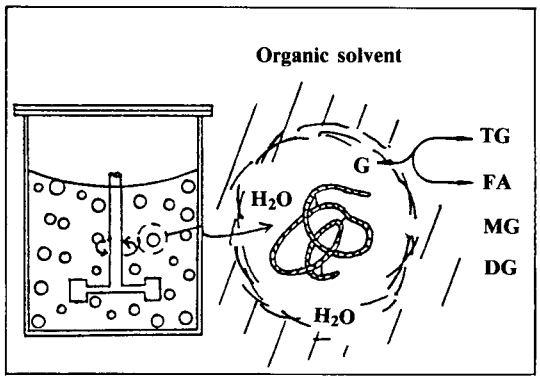

Fig. 1. Scheme showing two-phase system and lipase-catalyzed hydrolysis of triglycerides. (The form and size of water droplets are flexible).

Abbreriations: TG, Triglycerides; DG, Diglycerides; MG, Monoglycerides; G, Glycerols; FA, Fatty acids.

Despite some advantages, use of lipase in two-phase system cannot be extended to catalyze the esterification or the interesterification, since the location of the enzyme is not different from that in aqueous emulsion. Probably a real obstacle to use lipase even for hydrolysis in two phase system includes the small interfacial area. The observation that the water droplets formed by agitation or by stirring are much larger than the oil droplets formed by emulsification indicates that increase of the interfacial area is still desirable. However, vigorous agitation to reduce the droplet size may lead to the deactivation of the catalyst due to shear force (47). Making water in oil microemulsion by severe sonification or by other means in the presence of a surfactant may be a solution to secure enormous interfacial area, which is the basic concept of reversed micellar system.

Reversed micellar system

A relatively new approach of enzyme reaction in non-polar milieu is the micellar entrapment of enzymes in reverse micelles. Over the last decade, it has been shown that it is possible to solubilize the hydrophilic compounds in non-polar solvents with the help of surfactant and water (48-50). When a surfactant dissolves in organic solvent, the polar heads of the surfactant molecules are directed toward the interior of the spheroidal aggregate, forming a polar core and the aliphatic chains are directed toward the organic solvent (Fig. 2.). This is the 'reverse' of the situation in normal micelles in water. In the polar core of reverse micelles water can be solubilized, forming the water pool and in turn any hydrophilic compounds including biopolymers such as enzymes can be solubilized or entrapped in the water pool. The reverse micelles in which biopolymers are hosted are homogeneous and thermodynamically stable solutions. For a detailed analysis of these systems, see reference 51.

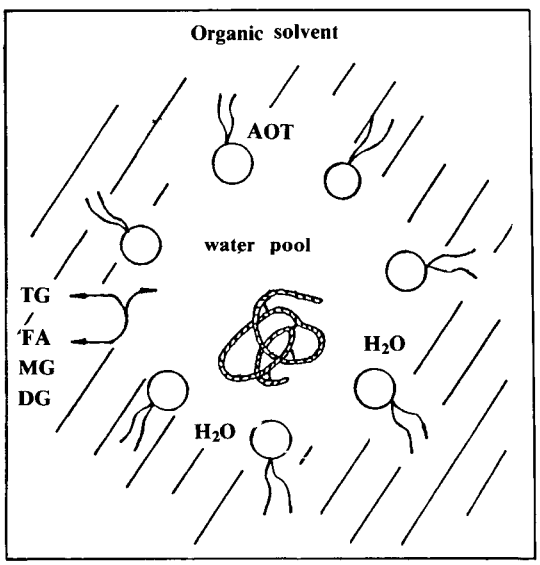

Fig. 2. Scheme showing reverse micellar system and lipase-catalyzed hydrolysis of triglycerides.

Abbreriations are the same as in Fig. 1. except AOT, bis (2-ethylhexyl) sodium sulfosuccinate.

Interest from biotechnologists has been focused over the last few years on the reverse micelles, since enzymes solubilized in the water pool of the micelles preserves nearly all their catalytic activity and stability. Thus more than twenty kinds of micelle-mediated enzymatic reactions have been studied (52, 53). Until now the best example of its technical use may be with lipase because the enzymes catalyzing the conversion of lipophilic substrates have the close biotechnological relevance in these micelles. We developed a novel assay method of lipases which has several different characteristics from those in conventional methods (54). First, the enzyme can be assayed under the conditions saturated with substrates. More important is in the respect that the kinetic results obtained are reproducible and comparable with the other studies because the system introduced a single liquid phase by solubilizing both the enzyme and substrate in organic solvent, and it is well characterized in physicochemical terms (55-57).

In the hydrolytic reaction of olive oil, lipase activity and stability in reverse micelles were highly dependent on R value (R value means the molar ratio of water to surfactant in the system) and the R values which maximized the initial velocity and stability were 10.5 and 5.5, respectively (58). Batchwise hydrolysis of olive oil by lipase from *C. rugosa* revealed that the substrate (5%, v/v) could be almost completely hydrolyzed at R value of 10 and at AOT concentration of 100 mM. At the end of the reaction, fatty acids produced could be

recovered with the high yield by adding water and acetonitrile, centrifugating the mixture and collecting the upper layer (59). This reaction system seems to be promising for the enzymatic hydrolysis of fats and oils in regard to the following aspects; 1) The catalytic efficiency is high with respect to the reaction rate and the inhibition by substrate and products, 2) The system requires little energy to promote the interfacial area and 3) Further process development may be simplistic because the system is chemically well defined. Though it is certain that reverse micelles may open a new way to the industrial application of lipase, a highest hurdle to the successful application may be a difficulty in recovering the enzyme, if necessary, from the reaction mixture at the end of the reaction. Appropriate enzyme reactors which are compatible with the organic solvent used and thus permit the continuous operation may contribute to the really fruitable application of reverse micelles, although it is yet to be developed (60).

The kinetics of lipase in reverse micelles deserves a mention in relation to the development of enzyme reactor. Effect of water on equilibria and rate parameters of hydrolytic reactions in the aqueous solution has been ignored by almost all authors, since the aqueous phase contains an excess amount of water to remain constant throughout the reaction (61). With the appearance of reaction systems containing minimum amount of water to secure the enzyme activity, this assumption becomes no longer valid. Lipase reaction in reversed micellar system is expected to be affected by the water concentration because its content in the system is usually below 1% (v/v) of the total reaction mixture and the water is dispersed uniformly and entrapped with the surfactant molecules. In fact, the initial water concentration affected significantly the equilibrium of hydrolytic reaction of lipase. The equilibrium fractional conversion of ester bond to fatty acid and alcohol moiety increased in proportion with the initial water content in reverse micelles (62). This result suggested that lipase reaction in the system should be regarded as two-substrate reaction with respect to water and ester bond. To infer the effect of water on equilibrium and rate parameters, Han *et al.* (62) derived the equations based on two substrate second-order reversible kinetics, with which the progress of lipase reaction in reverse micelles could be traced successfully.

Organic solvent system

In contrast to the previous concept that water is essentially required as the reaction medium in enzyme-catalyzed reactions, Klibanov and Zaks demonstrated somewhat an interesting experiment that lipase could catalyze the transesterification between tributyrin and heptanol in a 99% organic medium (63). Following studies reconfirmed that many enzymes, if not most, can work as catalyst in nearly anhydrous organic solvents (44, 64-66). The real meaning of this finding is not that enzymes are active regardless of the presence of water, but that they are active even in the presence of minimum of water which ensures the acquisition and maintenance of the catalytically active conformations of the enzymes. According to the Klibanov's reasoning (44), the minimum amount of water that enzymes really need is only a very thin layer of water around them, sometimes less than a monolayer. As long as enzymes are surrounded by the essential layer of water, the bulk of the water can be removed and replaced with organic solvents without adversely affecting the enzymes.

From the biotechnological viewpoint, the most striking feature of the use of enzymes in organic solvents is probably their stability to thermal treatment. According to Klibanov and Zaks' report (63), in toluene or decanal not only can the porcine pancreatic lipase withstand heating at 100 °C for several hours, but it exhibits much higher activity at that temperature than at 25 °C. However, this very interesting result has been criticized because of the lack of generality: It has neither been reproduced by any research, nor reconfirmed with another enzyme.

Another feature of enzymology in organic solvents is the ability of enzymes to catalyze reactions that are virtually impossible to catalyze in water. When placed in organic solvents, lipases can catalyze transesterification (67), esterification (68), aminolysis, thiotransesterification and oximolysis

(69). In aqueous solutions hydrolysis prevails and therefore the above-mentioned reactions do not occur to any appreciable extent. A good example is the lipase-catalyzed synthesis of a penicillin G precursor and other peptides (68). In dichloromethane lipase from *C. rugosa* could synthesize peptides by accepting, as an acyl-forming catalyst, N-protected amino acid esters as substrates and amines as nucleophiles during the deacylation step. The synthesis of peptides through aminolysis of N-protected amino acid ester was much faster than that through dehydration of the N-protected amino acid and an amino acid esters or amides.

The hottest issue of practical value on the use of lipase in organic solvent may be the interesterification to produce the triglycerides with the modified physical and chemical characteristics. Lipase from *R. delemar* immobilized with photocrosslinkable resin prepolymer could catalyze the replacement of oleic acid moieties at 1- and 3-position with stearic acids, in the reaction solvent, n-hexane (33). Macrae (9) reported the result of more practical value. By feeding the stock solution composed of a water saturated solution of refined palm oil mid-fraction (1 part) and stearic acid (0.4 parts) in petroleum ether into the packed bed reactor containing hydrated catalyst prepared from the 1,3-regiospecific *M. miehei* lipase and Hyflo Supercel, the mixture of triglycerides whose melting nature and triglyceride species are very similar to those of cacao butter could be produced.

Though promising in several aspects, this system also has some problems from the technological point of view. Suspension of enzymes in organic solvents indicates that the catalysis is solid-liquid phase reaction. In case of lipase which is active at interface, it seems likely that the catalytic efficiency is reduced drastically because of mass transfer of the catalyst to the substrate. This rationale becomes more obvious, considering that the object of single organic phase is to minimize the exposure of the enzyme to organic solvent in which substrates are dissolved. Yokozeki *et al.* (71) reported that incorporation of stearic acid into olive oil catalyzed by immobilized lipase reached about 40% within 10 hours, but it was at the expense of large consumption of the catalyst.

요 약

리파제는 모노, 디, 트리글리세리드 분자 내의 에스테르 결합을 가수분해 시키는 효소로 잘 알려져 있다. 그런데, 이 효소의 기질인 유지는 물에 용해되지 않아 그 반응이 불균일계에서 일어남으로 리파제 반응의 반응속도론적 해석이 곤란하였다. 이러한 성질은 유지공업에서 리파제를 산업적 촉매로 사용하는데 커다란 정해 요인이 되었었다. 그러나, 최근에 이상계, 역미셀계, 미수계와 같이 반응매질로 유기용매를 도입한 효소반응계가 개발됨에 따라 리파제를 이용한 유지의 전환에 대한 관심이 점증하는 추세에 있다. 리파제를 사용하여 유지를 가수분해시킴으로써 지방산을 생산하고자 할 때 효소반응계로 재래식의 에멀젼보다 이상계 또는 역미셀계를 사용하면 생산성, 굳기름의 가수분해 속도, 생성물 분리 등의 측면에서 전체 공정의 효율이 향상될 수 있었다. 한편, 미수계에서 리파제는 에멀젼에서는 불가능한 에스테르 교환반응, 글리세리드 합성, aminolysis, thiotransesterification 및 oximolysis 같은 반응을 촉매 할 수 있는 획기적인 특성을 나타냈다. 공업적 측면에서 이 반응계는 물리적 또는 화학적 특성(특히 융점)이 변형된 유지를 생산하고자 하는 에스테르 교환반응의 효소반응계로 널리 이용되고 있다. 앞으로 유기용매 내에서 효소의 안정성을 확보할 수 있는 수단 및 연속조작이 가능한 효소반응기의 개발에 관한 연구가 계속된다면 이러한 효소공정이 공업적 제조기술로 발전될 수 있을 것이다.

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