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Review Article

Lipase and Its Unique Selectivity: A Mini-Review

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Contrary to other solid catalysts, enzymes facilitate more sophisticated chemical reactions because most enzymes specifically interact with substrates and release selective products. Lipases (triacylglycerol hydrolase, EC 3.1.1.3), which can catalyze the cleavage and formation of various acyl compounds, are one of the best examples of enzymes with a unique substrate selectivity. There are already several commercialized lipases that have become important tools for various lipid-related studies, although there is still a need to discover novel lipases with unique substrate selectivity to facilitate more innovative reactions in human applications such as household care, cosmetics, foods, and pharmaceuticals. In this mini-review, we focus on concisely demonstrating not only the general information of lipases but also their substate selectivities: typoselectivity, regioselectivity, and stereoselectivity. We highlight the essential studies on selective lipases in terms of enzymology. Furthermore, we introduce several examples of analysis methodology and experimental requirements to determine each selectivity of lipases. This work would stress the importance of integrating our understanding of lipase chemistry to make further advances in the relevant fields.

1. Introduction

Since its beginning, the field of catalysis, particularly that related to biological molecules such as enzymes, has captivated both academia and industry. Researchers have historically discovered numerous enzymes and investigated their catalytic characteristics to explain complicated biological phenomena [1], by extension, to directly control or utilize them [2, 3]. Contrary to the use of solid catalysts (e.g., metals, oxides, sulfides, and halides), enzyme-mediated catalysis facilitates more sophisticated chemical reactions because most enzymes interact specifically with substrates and release selective products [4, 5]. Generally referred to as specificity or selectivity, these catalytic competencies are fundamentally determined by the stereostructure of the enzyme's active site periphery [6]. Catalytic environments structured by the shape of the active site periphery have naturally evolved to be favorable to catalyzing specific or selective reactions [7], and enzymes sometimes take advantage of interactions with cofactors (i.e., cooperative catalysis) including inorganic ions, prosthetic groups, and cosubstrates [8]. In other words, changes in the structure of an enzyme could lead to a significant alteration of its catalytic properties. Based on this information, numerous attempts are made to either find enzymes with the desired specificity and selectivity or to engineer enzymes by *de novo* design or directed evolution [9–12].

Lipases (triacylglycerol hydrolase, EC 3.1.1.3), which belong to a group of esterases (EC 3.1.1.X), are one of the best examples of enzymes with unique substrate selectivity [13]. There have been numerous lipases discovered in a wide variety of organisms, ranging from prokaryotes to eukaryotes [14–18], and researchers have mainly focused on their inherent substrate selectivities [19]. While the lipase definition distinguished from other esterases is still controversial, lipases are typically defined as carboxylesterases that can catalyze the cleavage or formation of water-insoluble acyl compounds (R–C=O) such as mono-, di-, and triacylglycerols containing relatively long-chain (more than C_6) fatty acid esters [20, 21]. Chromophoric or fluorophoric compounds, for instance, possessing an ester bond of fatty acid can be also used as selective substrates for the screening of lipases (Figure 1) [22]. In terms of structure, most

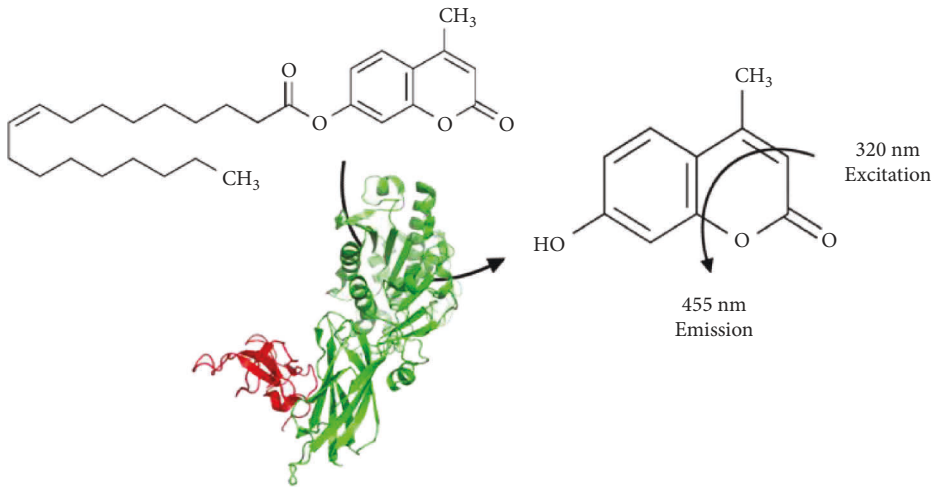

Figure 1: The hydrolysis of 4-methylumbelliferyl oleate (4-MUO) by lipase. When the ester bond of 4-MUO is hydrolyzed, oleate and fluorophoric 4-methylumbelliferone (4-MU) are simultaneously liberated. Fluorescence of 4-MU can be easily detected at an excitation wavelength of 320 nm and an emission wavelength of 455 nm using fluorometer.

eukaryotic lipases have an additional hydrophobic lid domain together with an α/β hydrolase fold and a Ser-Asp-His catalytic triad, and these lipases can be identified by the kinetic criterion of interfacial activation [23, 24]. Under minimum substrate concentration, the lid domain normally enveloping the active site periphery moves apart via conformational changes at the oil/water interface, making the active site more accessible to substrates [25, 26]. No interfacial activation occurs in other enzymes such as glycosidases, proteases, and nucleases, which react with water-soluble substrates. Most importantly, the key attribute of lipases among other aspects is substrate selectivity, which is subdivided into typoselectivity (i.e., preference for certain types of fatty acids), regioselectivity (i.e., distinguishment between the *sn*-1(3) and *sn*-2 positions), and stereoselectivity (i.e., differentiation between enantiomeric positions in acylglycerol species). Unlike other common properties of lipases, each lipase with a different protein structure exhibits a different selectivity in a greater or lesser degree [27]. These unique substrate selectivities are considered to be remarkable properties found in lipases, and it has been highlighted that there is a need to use them for further innovative reactions in human applications such as household care, cosmetics, foods, biodiesel, and pharmaceuticals [28–33]. There are already several commercialized lipases like Lipozyme® (developed by Novozymes A/S), and they have become important tools for various lipids-related studies and even industries [34]. Nonetheless, it still needs to develop more advanced lipases with unique substrate selectivity to facilitate more specific reactions.

For that reason, recent studies have consistently found novel unique lipases, revealed their selectivity precisely, and also developed relevant analysis methodologies. There are already published review articles covering lipase in terms of its catalytic nature, structure, analytical methodology, and industrial application. Unfortunately, most review articles focusing on the selectivity of lipases mainly highlight stereoselectivity, and there are still no articles covering all types of lipase selectivity and relevant analytical methodology. From this perspective, this mini-review is mainly devoted to three key selectivities (typoselectivity, regioselectivity, and stereoselectivity) of triacylglycerol lipases (no other classes of esterases like phospholipases or lipoprotein lipases). Furthermore, the essential studies on selective lipases were organized in terms of enzymology and analysis methodology. Such work will highlight the importance of organizing our understanding of lipase chemistry and provide rising researchers with practical insights to yield further advances in the relevant fields.

2. Typoselectivity of Lipase

The major substrates for lipase hydrolysis are commonly known as triacylglycerols with long-chain fatty acids. There are numerous kinds of triacylglycerols depending on the type of fatty acid ester groups attached to the glycerol backbone [35]. Fatty acids are carboxylic acids with an aliphatic hydrocarbon chain, which is either saturated or unsaturated [36]. Generally, natural fatty acids have an unbranched chain consisting of even-numbered carbon atoms (C_4–C_{28}) because of their biosynthesis from acetyl-CoA [37, 38], and their types can be classified in two ways: chain length and saturation/unsaturation (Figure 2). Firstly, fatty acids are divided into short-chain ($<C_6$), medium-chain (C_6–C_{12}), long-chain (C_{13}–C_{21}), and very long-chain ($>C_{21}$) types, according to aliphatic chain lengths (carbon number). The hydrophobicity of triacylglycerols with long chains is typically greater than that of triacylglycerols with short chains under identical chemical conditions. Secondly, when these fatty acids have all single bonds (C–C), they are sorted as saturated fatty acids ($CH_3(CH_2)_nCOOH$). Most hydrophobic fats such as lard and beef tallow, consist of saturated triacylglycerols. In vegetables and fishes with lower hydrophobicity, on the other hand, there are unsaturated triacylglycerols, which have fatty acids with one (monounsaturated fatty acids) or more (polyunsaturated fatty

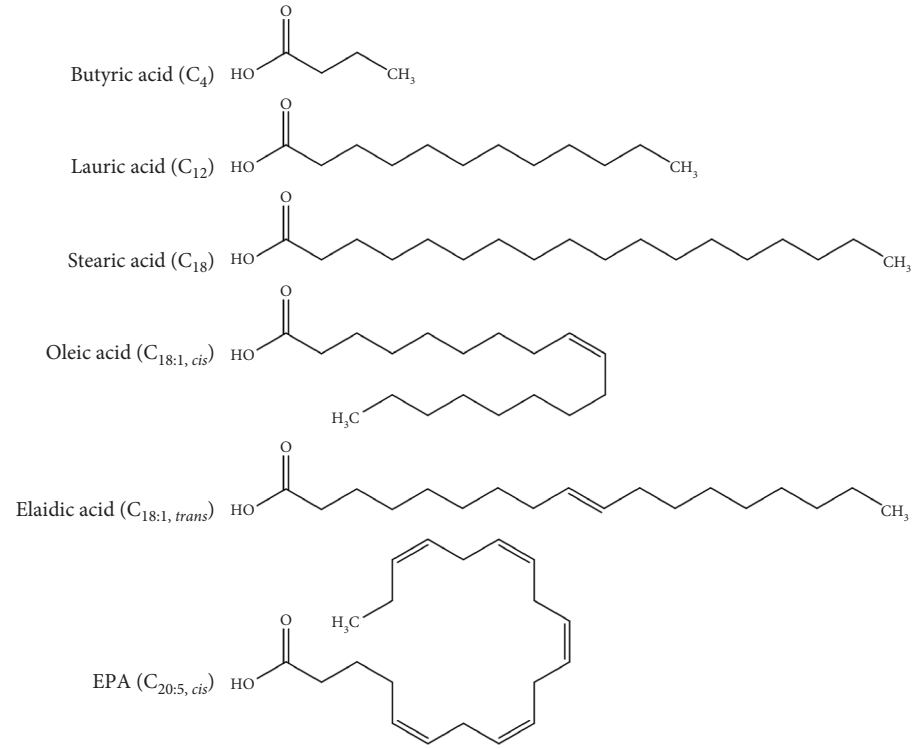

Figure 2: Examples of each type of fatty acid. Butyric acid is a minimum short-chain saturated fatty acid with four carbons (C_4), which can be hydrolyzed by lipases. Lauric acid is a medium-chain saturated fatty acid with twelve carbons (C_{12}), which is rich in coconut and palm kernel oil. Stearic acid is a long-chain saturated fatty acid with eighteen carbons (C_{18}), which is more abundant in animal fat. Oleic acid is a monounsaturated fatty acid with eighteen carbons (C_{18}) and one *cis* double bond in C_9. Elaidic acid is a *trans* geometric isomer of oleic acid. Notably, elaidic acid has almost the same topology as stearic acid. Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid with twenty carbons (C_{20}) and five *cis* double bonds in C_5, C_8, C_{11}, C_{14}, and C_{17}.

acids) carbon-carbon double bonds (C=C) in their aliphatic chains [39, 40]. These unsaturated fatty acids can also be subdivided by specific geometric forms (e.g., number, location, and *cis*/*trans* isomerism) of double bonds.

If so, can each lipase use all the aforementioned types of triacylglycerols as substrates? Of course not. Although lipases exhibit somewhat promiscuous behavior regardless of chain length and saturation/unsaturation [41], each can hydrolyze specific types of fatty acid esters preferentially or even exclusively [42]. This ability of lipases is called typoselectivity (a prefix "typo-" referring to shape, figure, or form) or fatty acid specificity, and this is the most basic property of lipases to be characterized. There are numerous reports about the typoselectivity of lipases under various reaction conditions. Lipase from *Rhizomucor miehei* (Lipozyme® RM IM) showed maximum activity for tricaprylin (C_8) and low activity for unsaturated triacylglycerols, whereas lipase from *Geotrichum candidum* is highly selective towards long-chain and unsaturated triacylglycerols [42]. *Candida antarctica* lipase B and *Thermomyces lanuginosus* lipase favored medium-chain fatty acid esters [42, 43]. Porcine pancreatic lipase, *Candida rugosa* lipase, and *Bacillus licheniformis* lipase were reported to prefer short-chain triacylglycerols to those with longer chains [42, 44]. Lipases from *Yarrowia lipolytica* and *Adansonia grandidieri* showed no specific typoselectivity [45, 46]. Most lipases can adopt unsaturated fatty acid esters with a double bond on *n*-9 (e.g., oleic and linoleic acids) as well as *n*-3 polyunsaturated fatty acids (e.g., α-linolenic, eicosapentaenoic, and docosahexaenoic acids) as substrates [47, 48]. In contrast, only a few lipases discriminate unsaturated fatty acid esters with an additional double bond on *n*-12 like γ-linolenic acid [49], probably due to the proximity of substituent groups to ester bonds. Additionally, *cis*/*trans* geometric isoforms of fatty acids are also discriminated against by the typoselectivity of lipases. It was reported that *Candida antarctica* lipase A preferentially catalyze elaidic acid ($C_{18:1,trans}$) over oleic acid ($C_{18:1,cis}$) [50].

Taking the evolution perspective, the fatty acid compositions in natural triacylglycerols considerably vary with origin, region, climate, and ecological environment, and it probably makes inherent lipases structurally suitable to use specific triacylglycerols as substrates. Pleiss et al. demonstrated that the typoselectivity of lipases is reflected in the shape and physicochemical properties of the active site peripheries, in which substrates approach a long hydrophobic scissile binding site in the binding pocket [51]. Because a small number of amino acids are arranged in the binding pocket, the length and hydrophobicity of the binding site correlate with substrate the chain length profile [51], indicating that the mutation of these residues is anticipated to alter the typoselectivity of lipases. Duncan et al. evolved *Rhizomucor miehei* lipase to have the typoselectivity for water-soluble short-chain (C_4) substates by random

mutagenesis [52]. Henrike et al. engineered *Candida antarctica* lipase A by rational mutagenesis of its binding tunnel, resulting in enhancing its typoselectivity for *trans* and saturated fatty acid esters [53].

To determine the typoselectivity of lipases, it is necessary to prepare various types of fatty acid esters such as triacylglycerols, methyl fatty acid esters, *p*-nitrophenyl fatty acid esters, and 4-methylumbelliferyl fatty acid esters [22, 43]. Chromophoric and fluorophoric substances with different chain lengths are attractive options because their liberated products are easy to analyze. Unfortunately, however, lipases may exhibit quite different typoselectivities between triacylglycerols (true substrates) and those alternative substrates; hence, researchers should interpret the overall data obtained from using both true and alternative substrates to determine the typoselectivity of lipases more precisely. Then, the typoselectivity can be expressed using the specificity constant ($1/\alpha$) for a particular substrate, as proposed by Rangheard et al. [54], as follows:

$$\frac{1}{\alpha} = \frac{\log\left([x]_t/[x]_0\right)}{\log\left([\text{reference}]_t/[\text{reference}]_0\right)}, \quad (1)$$

where $[x]_0$ and $[x]_t$ are molar concentrations of each substrate at reaction times 0 and t, respectively. The reference notes substrate showing the highest rate of catalysis, indicating its specificity constant is calculated as 1. The values for the other substrates are relatively calculated ranging from 0 to 1.

3. Regioselectivity of Lipase

Triacylglycerols, the primary substrates for lipases, are triesters composed of three fatty acid molecules (RCO_2H, $R'CO_2H$, and $R''CO_2H$) bound to all three hydroxyl groups (–OH) in a glycerol backbone. Homogeneous triacylglycerols such as tripalmitin (C_{16}), tristearin (C_{18}), and triolein ($C_{18:1,cis}$) are those in which the three fatty acids are chemically identical; however, naturally occurring triacylglycerols usually have different fatty acid components on each position of the glycerol moiety [55]. These acyl positions of triacylglycerols are especially defined by stereospecific numbering (*sn*), designating as *sn*-1 (RCO_2H), *sn*-2 ($R'CO_2H$), and *sn*-3 ($R''CO_2H$), respectively (Figure 3) [56], where it is often called α and α' to refer to primary hydroxyl groups (*sn*-1 and *sn*-3), and β to refer to secondary hydroxyl groups (*sn*-2). When one molecule of triacylglycerol is hydrolyzed by lipases, there are three possible reactions to 1,2-*sn*-diacylglycerol, 2,3-*sn*-diacylglycerol, or 1,3-*sn*-diacylglycerol depending on the hydrolyzed acyl positions (Figure 4). What would that actually result in? Surprisingly, most lipases are known to preferentially or exclusively hydrolyze the ester groups at the external positions (*sn*-1 and *sn*-3) over those at the internal positions (*sn*-2) [57]. This ability of lipases to distinguish between the *sn*-1(3) and *sn*-2 positions of triacylglycerols is called regioselectivity (a prefix "regio-" referring to region) or positional specificity. Compared with the typoselectivity, which is a relatively promiscuous property, the regioselectivity of lipases is

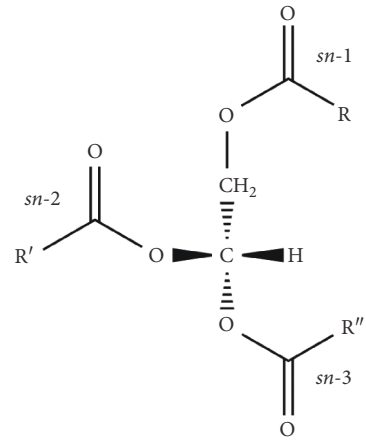

Figure 3: The Fisher projection of a triacylglycerol molecule with different acyl chains (R, R′, and R″) in each acyl position (*sn*-1, *sn*-2, and *sn*-3).

strictly demonstrated during catalysis. Hence, lipase reactions must therefore scrutinize not only what fatty acids are attached but also where the fatty acids are attached to triacylglycerols.

In terms of regioselectivity, lipases can be classified into two major groups based on the molar ratio of released 1,2(2,3)-*sn*-diacylglycerols to 1,3-*sn*-diacylglycerols during catalysis. One group is regiospecific lipases, which exclusively work on the specific acyl position of triacylglycerols. All reported regiospecific lipases exhibit *sn*-1,3 regiospecific properties, while no evidence has been found for *sn*-2 regiospecific lipases, which are expected to produce 1,3-*sn*-diacylglycerols solely. Pancreatic lipase, a lipid-digestive enzyme secreted from the mammal's pancreas, is the most representative *sn*-1,3 regiospecific lipase, converting triacylglycerols to 1,2(2,3)-*sn*-diacylglycerols at an early stage of catalysis and eventually to 2-*sn*-monoacylglycerols [58]. In addition to this, *sn*-1,3 regiospecific lipases have been identified, including *Candida antarctica* lipase B, *Rhizomucor miehei* lipase, *Chromobacterium viscosum* lipase, *Yarrowia lipolytica* lipase, and *Thermomyces lanuginosus* lipase [45, 57, 59]. These *sn*-1,3 regiospecific lipases can be used for various intriguing applications to regioselectively produce novel acyl compounds, some of which are detailed later in this article. On the other hand, some lipases work on any acyl positions of triacylglycerols. The group of these lipases is called nonregiospecific lipases, which produce 1,2(2,3)-*sn*-diacylglycerols as well as 1,3-*sn*-diacylglycerols as reaction intermediates, resulting in the complete breakdown of triacylglycerols to glycerols. However, the reaction rates catalyzed by nonregiospecific lipases are generally much faster at the *sn*-1,3 acyl positions than at the *sn*-2 acyl position (*i.e.*, *sn*-1,3 regioselective) [27, 60]. Only *Candida antarctica* lipase A, *Geotrichum candidum* lipase, and adipose lipase have been previously reported to show clear preferences for the *sn*-2 acyl position of triacylglycerols (i.e., *sn*-2 regioselective) [27, 61, 62]. It is noteworthy that these two lipases have the potential to be further evolved into strictly *sn*-2 regioselective or *sn*-2 regiospecific through

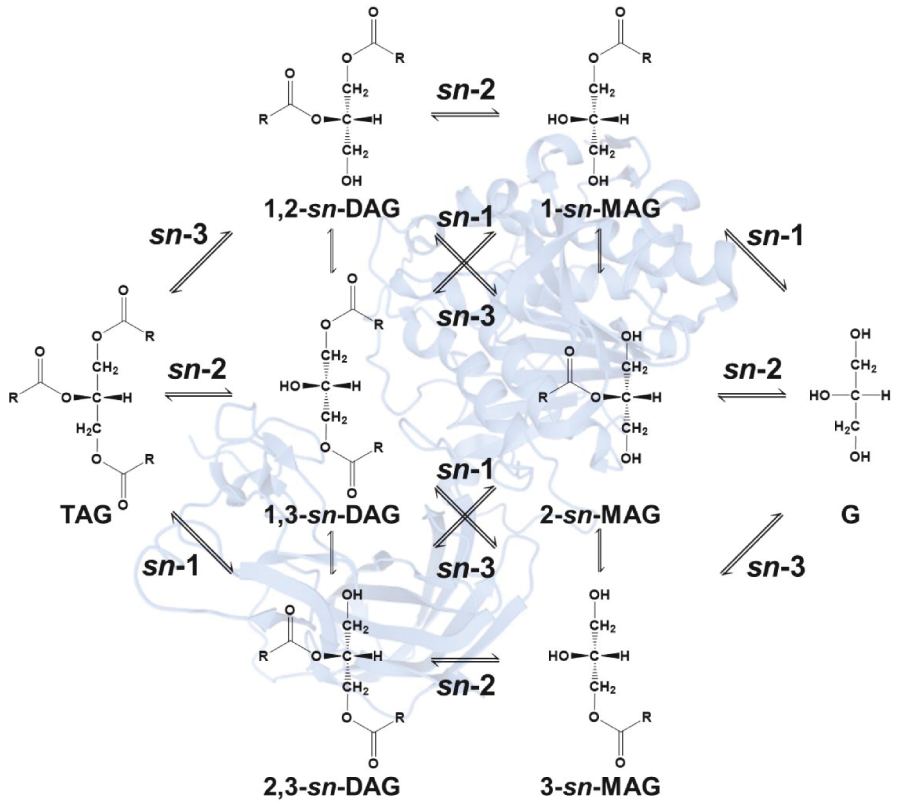

Figure 4: Lipase-catalyzed hydrolysis of a homogeneous prochiral triacylglycerol (TAG). From TAG, not only regioisomeric/enantiomeric diacylglycerols (DAG) but also monoacylglycerol (MAG) can be produced in accordance with the selectivity of lipase. Nonenzymatic acyl migrations are represented by vertical arrows between DAGs and MAGs. R fatty acid acyl moiety; G, glycerol.

advanced biotechnology. In addition, there are several reports on peculiar lipases, for example, an *sn*-1 (or *sn*-3) regioselective lipase from *Cordyceps militaris*, although more concrete evidence is needed [63].

When reviewing the research articles using the same lipase, it can be found that the derived regioselectivity of the lipase is often inconsistent with each other. The regioselectivity of lipases for triacylglycerols is fundamentally determined by the communication between the enzyme structure and the substrate structure; nevertheless, other extrinsic factors such as temperature, water content, organic solvent hydrophobicity, reaction medium, and immobilization, can induce conformational changes of the protein structure followed by affecting the regioselectivity [41]. The lipase from *Pseudomonas fluorescens* showed an *sn*-1,3 regiospecificity for triacylglycerols in oil-in-water emulsion medium [27], as compared to showing nonregiospecific properties in aprotic organic solvent medium with low water contents such as reversed micellar systems [64]. The free enzyme form of *Candida antarctica* lipase B is reported to be *sn*-1,3 regiospecific [57], whereas its immobilized form (Lipozyme® CALB manufactured by Novozymes A/S) is nonregiospecific. There is no doubt that the alteration of physicochemical conditions can cause conformational changes of lipases; however, how they affect their regioselectivity remains unclear. Hence, it is necessary to ensure the regioselectivity of the enzymes under the desired reaction conditions beforehand. Moreover, the unexpected production of 1,3-*sn*-diacylglycerols, 1(3)-*sn*-monoacylglycerols, or glycerols may be observed during a prolonged reaction of *sn*-1,3 regiospecific lipases [41]. This phenomenon is called "acyl migration," which is a nonenzymatic spontaneous movement of an acyl moiety between the *sn*-1,3 and *sn*-2 acyl positions of acylglycerol species (Figure 4), leading to difficulties in interpreting the data. Because the rate of acyl migration increases with the decrease of water activity in the reaction medium [41], it is important to construct a comprehensive kinetic model incorporating the parameters of acyl migration of diacylglycerol and monoacylglycerol regioisomers to analyze the lipase catalysis in organic solvent media appropriately [60].

Chromatographic techniques such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been traditionally used to determine the regioselectivity of lipases [65]. First of all, to fundamentally determine the regioselectivity, lipolytic products should be acquired from homogeneous triacylglycerols such as trioctanoin (C_8) and triolein ($C_{18:1,cis}$) [66]. In the screening steps, TLC analysis can be applied for a rapid resolution of nonvolatile regioisomeric diacylglycerols. Despite their isomeric properties, 1,3-*sn*-diacylglycerols generally tend to develop faster along a nonpolar mobile phase than 1,2(2,3)-*sn*-diacylglycerols since the secondary hydroxyl group (*sn*-2) is less exposed to the mobile phase [63, 64]. Each spot of lipid substances can be visualized using iodine vapors as color reagents [63], or directly detected

under ultraviolet (UV) radiation after phenyl carbamate derivatization of the exposed hydroxyl groups in diacylglycerols [64]. For in-depth quantitative analysis of all regioisomers of acylglycerol species, an evaporative light-scattering detector (ELSD) in conjunction with a reverse-phase high-performance liquid chromatography (HPLC) is a great choice because it can detect and quantify all regioisomers without any chemical pretreatment [65]. Unlike diacylglycerols, 2-*sn*-monoacylglycerols tend to be detected before 1(3)-*sn*-monoacylglycerols due to two primary hydroxyl groups (*sn*-1 and *sn*-3) being exposed to the mobile phase [67]. Additionally, Mendoza et al. proposed a UV spectrophotometric assay to screen *sn*-2 regiospecific lipases using 1,3-*O*-dioleoyl-2-*O*-*α*-eleostearic -*sn*-glycerol as a substrate [68]. The liberated *α*-eleostearic acids by putative *sn*-2 regioselective lipases show chromophoric properties, absorbing light of wavelength 272 nm. The regioselectivity of lipases is then qualitatively determined based on whether they are regiospecific or nonregiospecific. Furthermore, Matori et al. proposed that the regioisomeric excess of diacylglycerols virtually indicates the positional specificity index (PSI) of lipases under the presumption that the hydrolysis of diacylglycerols is negligibly slower than that of triacylglycerols and unexpected acyl migrations can be ignored under given conditions [69, 70]. The PSI is defined by the following equation:

$$PSI\,(\%) = \frac{[1,2\,(2,3)\mathrm{DG}] - ([1,3\mathrm{DG}] \times 2)}{[1,2\,(2,3)\mathrm{DG}] + ([1,3\mathrm{DG}] \times 2)} \times 100\,(\%), \quad (2)$$

where [1,2(2,3)DG] and [1,3DG] are molar concentrations of each regioisomer after reaction. If the PSI is 100, the lipase is determined as *sn*-1,3 regiospecific.

4. Stereoselectivity of Lipase

In the Fischer projection of a triacylglycerol molecule (Figure 3), it can be found that the secondary acyl carbon (*sn*-2) of the compound is capable of being an asymmetry center (also called a chiral center) within the framework of stereochemistry, indicating that triacylglycerols with different fatty acid moieties in the primary acyl positions (*sn*-1 and *sn*-3) are chiral compounds. The stereospecific numbering (*sn*) system is especially used to enable stereochemically identifying the acyl positions of triacylglycerols instead of the usual D/L or R/S nomenclatures in stereochemistry [56]. While other hydrolases such as proteases, phospholipases, glycosidases, and nucleases exclusively react with only one of the enantiomers of their substrates, lipases can interact with all enantiomers of the chiral acylglycerol species as well as prochiral triacylglycerols [66]. The hydrolysis of homogeneous prochiral triacylglycerols by lipases may release enantiomeric diacylglycerols (1,2-*sn*-diacylglycerols and 2,3-*sn*-diacylglycerols) and monoacylglycerols (1-*sn*-monoacylglycerols and 3-*sn*-monoacylglycerols). A racemic mixture of those products can also be designated as 1,2-*rac*-diacylglycerols and 1-*rac*-monoacylglycerols, respectively Figure 5(a). Then, can lipases recognize that the two primary acyl positions of acylglycerol species are stereochemically different? Rogalska et al., by using prochiral triacylglycerols as substrates, showed that most lipases produced each enantiomer of 1,2-*rac*-diacylglycerols in different amounts at the beginning of the catalysis (2–5% conversion) [27]. Choi et al. recently revealed that the lipase-catalyzed hydrolysis of triacylglycerols kinetically tends to follow a specific enantiomeric pathway [60]. These findings that most lipases are stereo-biased toward one enantioselective reaction over the other suggest that lipases can discriminate between enantiomeric positions in acylglycerol species. This ability of lipases is called stereoselectivity (a prefix "stereo-" referring to three-dimensional), which is the most important substrate selectivity of lipases.

It has been reported that the stereoselectivity of each lipase varies in either *sn*-1 or *sn*-3 enantioselective directions. Here are concise details regarding several well-known lipases and their stereoselectivities. *Candida antarctica* lipase B, *Chromobacterium viscosum* lipase, and gastric/pancreatic lipases from *Canis lupus* (dog) are reported to have an *sn*-3 stereoselectivity, preferentially liberating 1,2-*sn*-diacylglycerols from triacylglycerols [27, 64]. On the other hand, lipases from *Candida rugosa*, *Thermomyces lanuginosus*, *Pseudomonas fluorescens*, and *Rhizomucor miehei* are reported to have an *sn*-1 stereoselectivity, preferentially liberating 2,3-*sn*-diacylglycerols from triacylglycerols [27, 67]. The most remarkable case is that of pancreatic lipase extracted from porcine (*Sus scrofa*) pancreas, obviously exhibiting no stereobias with any triacylglycerols [27, 64, 66]. Based on this property along with its *sn*-1,3 regiospecificity, a porcine pancreatic lipase can be practically utilized for the analysis of fatty acid components in the *sn*-2 position of triacylglycerols [71]. However, it was also reported that the stereoselectivity of other pancreatic lipases from different mammals totally differs from each other [27, 72], in spite of the high homology of their protein structures (above 85% identity) (Figure 6). Gastric lipases from different mammals, in contrast, exhibited similar *sn*-3 stereoselective properties during the hydrolysis of triacylglycerols [27, 72]. Additionally, lipases are typically known to maintain their stereoselective properties irrespective of extrinsic factors, whereas several enzymes are susceptible to undergoing alterations in stereoselectivity. *Candida antarctica* lipase B, for example, can switch its stereoselectivity from *sn*-3 position to *sn*-1 position with the increase of the chain lengths from C_8 (trioctanoin) to C_{18} (triolein), although the enzyme especially has a strict *sn*-3 stereoselective preference for trioctanoin (100% enantiomeric excess at 5% conversion) [27]. Unfortunately, it is significantly more difficult to pinpoint common contributing factors determining the stereoselectivity of lipases than other substrate selectivities. Therefore, in order to understand why each lipase retains an individual stereoselectivity over a broad range of triacylglycerols, the stereoselectivity should be fundamentally connected to the enzyme-substrate chiral recognition mechanism based on the original three-dimensional structures of lipases. According to Rogalska et al., lipases possess putative enantiomorphic chiral recognition centers in their active sites, which display mirror-handedness during catalysis [27]. It has also been revealed

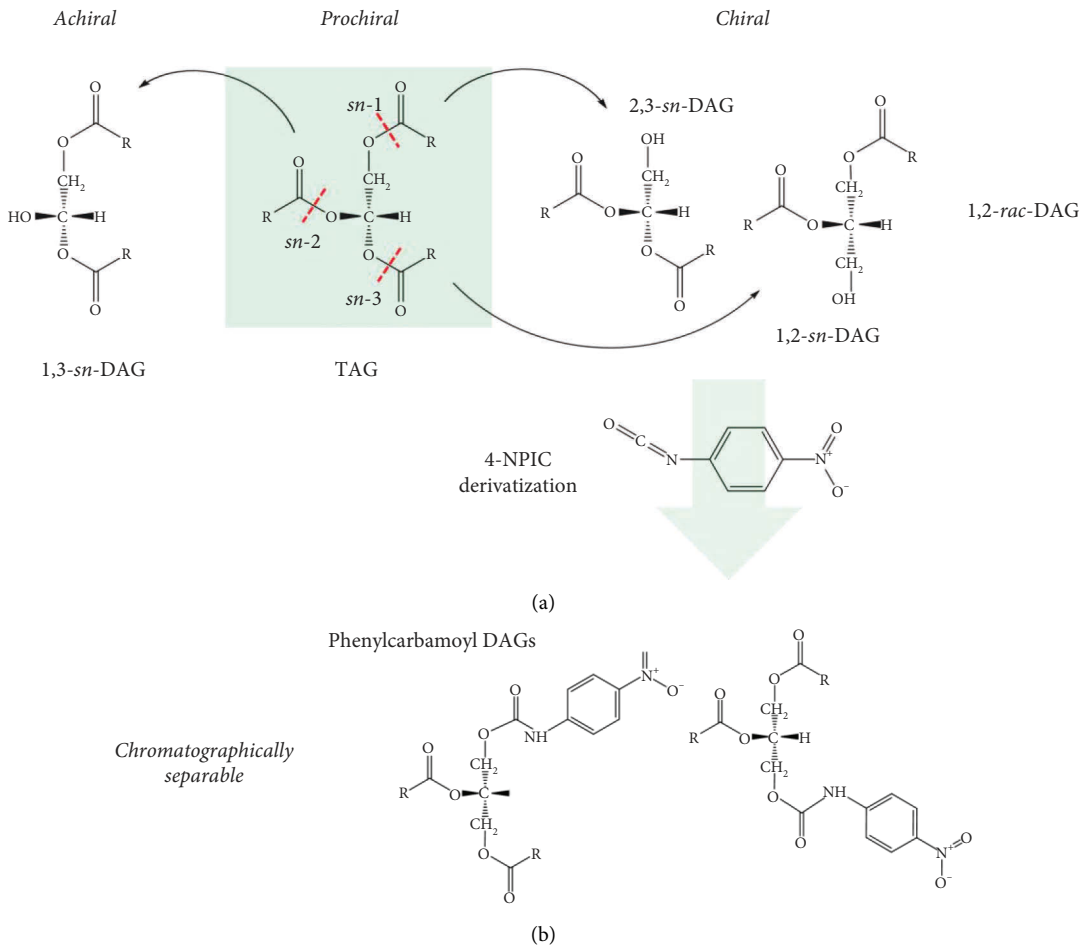

Figure 5: Chromatographic resolution of produced acylglycerol species during lipase catalysis. (a) Chiral status of putative diacylglycerols (DAG) liberated from a prochiral triacylglycerol (TAG) molecule by lipase. (b) Carbamate derivatization of chiral DAGs by using 4-nitrophenyl isocyanate (4-NPIC) for chromatographic resolution.

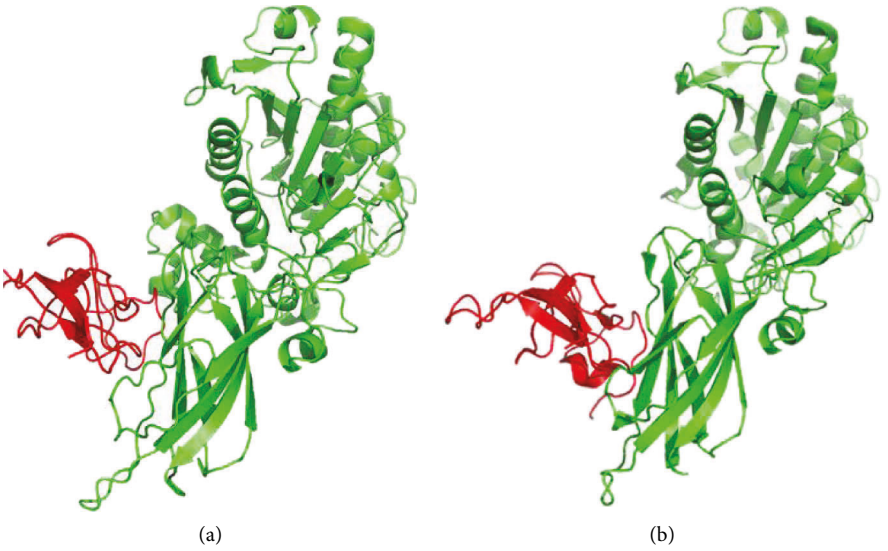

Figure 6: Three-dimensional protein structures of two pancreatic lipases from different origins. (a) Porcine pancreatic lipase (green) with colipase (red) (PDB ID, 1ETH). (b) Human pancreatic lipase (green) with colipase (red) (PDB ID, 1N8S). These lipases have 86.35% identity in amino acid sequence (some mutations included), which means that they are almost the same protein in structure.

that several structural factors–lipase/substrate steric complementarity, regional structural flexibility of lipase, hydrogen bonding state of tetrahedral intermediates in the active site, and electrostatic interactions with surface residues of lipase–play important roles in chiral recognition [73]. However, the comprehensive effects of these multiple factors on the stereoselectivity of lipases still need to be elucidated. We added several valuable references to help readers discuss this topic in greater depth [73–75].

The stereoselectivity of lipases has been investigated using either prochiral triacylglycerols or other acylglycerol analogs; however, homogeneous prochiral triacylglycerols with all identical acyl chains are basically recommended to determine the stereoselectivity of lipases independently of other substrate selectivities such as typoselectivity and regioselectivity [66]. It is critical to perform the hydrolysis of triacylglycerols under appropriate lipolytic conditions before analyzing the diacylglycerol and monoacylglycerol enantiomers generated by lipases. Ordinarily, to separate and quantify the acylglycerol enantiomers, the enantiomers are required to be derivatized with isocyanate compounds and subsequently introduced into the chiral stationary phase (CSP) columns like CHIRALPAK® (developed by Daicel Co.) [27, 64, 66]. The exposed hydroxyl groups in the acylglycerols can react with isocyanate groups (R–N=C=O) in the presence of catalysts, resulting in carbamoyl acylglycerol derivatives (Figure 5(b)). These carbamate derivatives with additional chromophoric or fluorophoric groups are able to be resolved by CSP columns coated with carbamate adsorbents and detected by each type of detector. On the other hand, it is highly challenging to separate the acylglycerol enantiomers in their original chemical forms by chromatographic resolution, unlike the regioisomers. Although there are several analytical methodologies previously described for the direct separation of enantiomeric acylglycerol species without any pretreatment, most of the cases are practically inapplicable for precisely determining the stereoselectivity of lipases due to low resolution and analytical inefficiency [76–79]. In a recent study, a novel methodology based on using an HPLC-ESLD equipped with a chiral stationary phase, CHIRALPAK® IA, was proposed for directly resolving triolein and its seven hydrolysis products in a relatively short amount of time [67]. This method is expected to be more practically advantageous than other methods to determine the stereoselectivity of lipases.

The stereoselective properties of lipases for triacylglycerols are evaluated using enantiomeric excess (ee) derived from the amounts of 1,2-*sn*-diacylglycerols and 2,3-*sn*-diacylglycerols as follows:

$$ee\,(\%) = \frac{[1,2\mathrm{DG}] - [2,3\mathrm{DG}]}{[1,2\mathrm{DG}] + [2,3\mathrm{DG}]} \times 100\,(\%), \quad (3)$$

where [1,2DG] and [2,3DG] are molar concentrations of each diacylglycerol enantiomer at a specific time after the reaction. If the *ee* value is either positive or negative, the lipase is thought to be *sn*-3 or *sn*-1 stereoselective, respectively. When the *ee* value is close to zero, the lipase is considered to have no stereobias. Notably, the amounts of diacylglycerols continuously change not only by the formation from triacylglycerols but also by the disappearance due to subsequent hydrolysis into monoacylglycerols; hence, it is necessary to measure the amounts of diacylglycerols at an early stage of catalysis (usually 2–5% conversion), in which no monoacylglycerols are formed [27]. The overall aspects of enantiomeric excess change in the time course of hydrolysis were often investigated to precisely describe the stereochemistry of lipase during every step of the reaction. Furthermore, several attempts have been made to model the hydrolysis of triacylglycerols in terms of reaction kinetics, contributing to an in-depth understanding of the stereoselectivity of the lipases [80–82]. Choi et al. recently established the kinetic model for the lipase-catalyzed hydrolysis of triacylglycerols based on the Ping-Pong Bi–Bi mechanism, and validated their model using three lipases and triolein substrates [60]. This model is the only one to demonstrate the integral stereoselectivity of lipases, which includes the entire hydrolytic process as well as nonenzymatic spontaneous acyl migration.

5. Conclusion and Future Perspective

The biocatalysts, also called enzymes, that basically play an essential role in living organisms have now infiltrated deeply into human lives, becoming an increasingly necessary part of every day. Moreover, numerous synthetic processes catalyzed by chemical catalysts have been substituted for biocatalysis involving the implementation of biocatalysts in an effort to reduce carbon footprints. An advantage of biocatalysis is the remarkable selective properties of enzymes, which can provide substantial benefits. Lipase-catalyzed biocatalysis is an attractive choice for designing any type of innovative reaction because of its peculiar substrate selectivity (typoselectivity, regioselectivity, and stereoselectivity) as well as promiscuity, though the original lipolytic characteristics of lipases discovered are already used in various ways in real life. The substrate selectivity of lipases can be especially exploited for practical purposes of either selective hydrolysis or esterification to synthesize high value-added functional compounds. Enzymatic interesterification between two types of triacylglycerols are the most representative technique to produce reconstructed triacylglycerols (i.e., structured lipids) with a high selectivity [31]. Chemical interesterification using sodium methoxides (alkaline) as catalysts, which is relatively inexpensive and convenient to use, is also used to rearrange the fatty acids in the triacylglycerol molecule; however, it lacks selectivity to control the distribution of fatty acids in a desired position. Lipases can be used to produce functional fatty acid derivatives (e.g., emulsifier, texturizer, odorant, nutraceuticals, and pharmaceuticals) [16]. Our research team, for example, has recently been working to synthesize novel multifunctional emulsifiers through regioselective acylation catalyzed by immobilized lipase [83, 84]. Surprisingly, an immobilized lipase (Novozym® 435) consistently used in those studies regioselectively synthesized erythorbyl fatty acid esters (at the C-6 hydroxyl group of erythorbic acid) or tripeptidyl fatty acid esters (at ε amine group of lysine). Likewise, a significant advancement has been made in the field of lipases

over the past three decades. However, in comparison with other hydrolases, the fundamental information of lipases is still poorly understood. How to develop a special lipase with the desired substrate selectivity still remains an ultimate task for the relevant researchers. Therefore, these topics should be the major focus of future studies, and we anticipate that both the theoretical and technical knowledge of the substrate selectivity of lipases covered in this mini-review would provide insights and encourage the studies.

Data Availability

The data used to support this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

J.Y.P. and K.M.P. wrote the manuscript. J.Y.P. and K.M.P. reviewed the first draft of the article and revised the draft. All authors discussed the contents of the manuscript and approved the submission.

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