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Review

Increase in stability of *Fusarium heterosporum* lipase

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Abstract

Thermostable lipases are useful for their industrial application. The present review focuses on the increase in thermostability of 1,3-positionally specific lipases from *Fusarium heterosporum* and *Rhizopus oryzae*. The *Saccharomyces cerevisiae* carrying *F. heterosporum* lipase cDNA produced two kinds of lipases, A and B. Lipase B was composed of one polypeptide (301 amino acids), and lipase A was composed of two polypeptides (275 and 26 amino acids) generated by the cleavage between Arg275 and Asp276 by a protease. Lipase B was more thermostable than lipase A, so it revealed that the C-terminal peptide (26 amino acids) is important for the stability. To produce only the thermostable lipase in *S. cerevisiae*, a mutant lipase (R275A, Arg275 → Ala) was constructed. The C-terminal peptide of R275A lipase was not cleaved, and its thermostability increased to the level of lipase B. When acidolysis of tripalmitin (PPP) with oleic acid (OA) was conduced at 50 °C using immobilized R275A lipase, the half-life of R275A lipase preparation was 370 d, which was significantly longer than those of industrially available immobilized lipases. *R. oryzae* lipase was also synthesized as prolipase (366 amino acids), which was then converted to mature lipase (269 amino acids) by the cleavage of the N-terminal prosequence (97 amino acids) by a protease. Prolipase was more stable against heating than mature lipase, and *S. cerevisiae* carrying a mutant lipase gene (Arg−29 → Ala) produced only the thermostable lipase.

Keywords: *Fusarium heterosporum*; Lipase; C-terminal peptide; Stability; Immobilized lipase

1. Introduction

Lipases [EC 3.1.1.3] hydrolyze the ester bonds of triacylglycerols (TAGs), and also catalyze esterification and transesterification (acidolysis, alcoholysis, and interesterification) in a non-aqueous system. So far, lipases have been used as digestives, for the production of flavors, diagnostic reagents, ingredients of detergent, catalysts of optical resolution, and so on. In addition, a great deal of attention is now focused on the application of lipases to production of modified lipids [1–10], purification of useful materials [11], and production of biodiesel fuel which is a subject of this issue [12–14].

Commercially available microbial lipases can be classified into several families based on the homologies of their primary structures, (i) *Burkholderia cepacia* [15]; (ii) *Pseudomonas fluorescens* [16]; (iii) *Candida antarctica* [17]; (iv) *Candida rugosa*[18]; (v) *Rhizomucor miehei* [19,20]. Lipases of the same family are similar not only in their three-dimensional structures but also their enzymatic properties. For example, many lipases in *B. cepacia* family act on polyunsaturated fatty acids (PUFAs) as strongly as C18 saturated and unsaturated fatty acids, and are effective for the complete digestion of PUFA-containing oils [3,4]. *C. antarctica* lipase possesses broad fatty acid specificity, and acts on PUFA strongly [21]. The

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immobilized enzyme is commercialized, and effective for the production of PUFA–TAGs [22] and alcoholysis of TAGs with short-chain alcohols [23]. Lipases of *C. rugosa* family act on PUFA very weakly, and have been applied to industrial production of PUFA-rich oil [12]. The final group, *R. miehei* family, includes industrially useful enzymes. They are 1,3-positionallly specific, and are used for production of cocoa fat substitute [24], 1,3-dioleoyl-2-palmitoyl-glycerol (OPO, commercial name, Betapol) [25], and diacylglycerol [26]. In addition, lipases in this family are widely used for production of various kinds of structured lipids [8–10]. But unfortunately, the lipases are comparatively unstable against heating. We thus aimed to create a thermostable 1,3-positionally specific lipase. This review deals with structure analysis on the stability of *Fusarium heterosporum* lipase, creation of the stable mutant lipase, and its application to production of structured lipids at high temperatures.

2. Lipase from *F. heterosporum*

2.1. Enzymatic properties of F. heterosporum lipase

A filamentous fungus producing a lipase was isolated from soil [27], and the strain was identified as *F. heterosporum* from its appearance and taxonomical characteristics [28]. The lipase purified from the culture filtrate was a monomeric protein with a molecular weight of 31 kDa, and the positional specificity for TAG was 1,3-specific [28]. The lipase was more stable against heating than 1,3-positionally specific *Rhizopus oryzae* (former name, *Rhizopus delemar*) lipase [29], which is commercially available and effective for the production of structured lipids. The fatty acid specificity of *F. heterosporum* lipase in hydrolysis was similar to that of *R. oryzae* lipase [30,31].

2.2. Structure of F. heterosporum lipase

Cloning of a *F. heterosporum* lipase cDNA was first performed to determine the structure of the enzyme. A cDNA library was constructed according to Okayama–Berg method [32,33], and the positive clones containing 1.3 kbp lipase cDNA fragment were isolated from the library by colony hybridization with an oligonucleotide probe corresponding to the N-terminal amino acid sequence [34]. The open reading frame consisted of 999 bp, and the deduced amino acid sequence contained 16 amino acid residues of a putative signal peptide, 16 residues of a propeptide, and 301 residues of a mature lipase. The enzyme was homologous to lipases from *R. oryzae* (32% homology) [35], *Rhizopus niveus* (32%) [36], *R. miehei* (32%) [19,20], and *Thermomyces lanuginosa* (39%) [37], and to mono- and diacylglycerol lipase from *Penicillium camembertii* (38%) [38] (Fig. 1). Comparison of the primary structure of *F. heterosporum* lipase with those of the other homologous lipases showed that the C-terminus of *F. heterosporum* lipase was longer than that of the other lipases by about 34 amino acid residues. Though we could not understand the meaning of this finding when *F. heterosporum* lipase cDNA was cloned, the longer C-terminus was clarified to play an important role afterwards.

The X-ray analysis of *R. miehei* lipase showed that Ser144, Asp203, and His257 are the residues of the catalytic triad, and Ser82 is involved in the oxyanion hole stabilizing the tetrahedral intermediates [39–41]. These residues are conserved in all homologous lipases, suggesting that the catalytic triad of *F. heterosporum* lipase is composed of Ser144, Asp198, and His256, and that Ser82 involved in the oxyanion hole (Fig. 1) [34]. The catalytic triad of *R. miehei* lipase is not exposed to solvent, but is buried under a short surface helix referred to as the lid. The displacement of this helix was postulated to be the structural basis of the oil–water interfacial activation of the lipase. From the homology of *R. miehei* lipase, *F. heterosporum* lipase was assumed to possess a lid composed of residues 85–91.

3. Increase in stability of *F. heterosporum* lipase

3.1. Expression of F. heterosporum lipase by Saccharomyces cerevisiae

Expression of the lipase cDNA is inevitable for clarifying the relationship between the structure and function of lipase. Hence, *F. heterosporum* lipase cDNA was expressed using *Saccharomyces cerevisiae*. The expression plasmid of *F. heterosporum* lipase was constructed by ligating the cDNA (coding a signal peptide, a propeptide, and a mature lipase) between the *GAP*

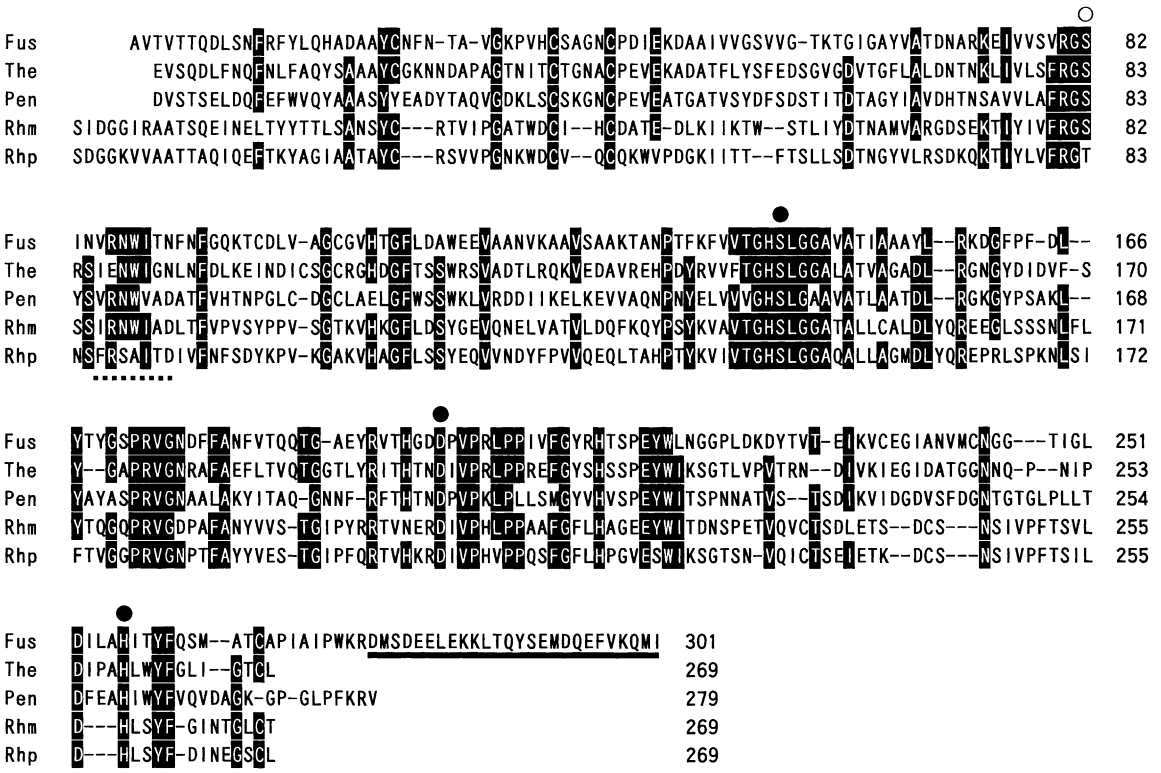

Fig. 1. Comparison of deduced amino acid sequence of *F. heterosporum* lipase with sequences of other fungal lipases. The deduced amino acid sequences of enzymes from *F. heterosporum* (Fus), *T. lanuginosa* (The), *P. camembertii* (Pen), *R. miehei* (Rhm), and *R. oryzae* (Rhp) are aligned. Dashes indicate gaps introduced into the sequences so that the maximum homology may be obtained. Identical amino acid in all or four of five enzymes are shown with white-on-black letters. Closed circles and an open circle indicate amino acid residues involved in the catalytic triad and oxyanion hole, respectively. The sequence presumed to form the helical lid is underlined with a broken line. The C-terminal peptide is indicated with a thick line.

(the gene coding glyceraldehyde-3-phosphate dehydrogenase of *S. cerevisiae*) promoter and its terminator [42]. *S. cerevisiae* strains transformed with the resulting plasmid, pYGF2, secreted the lipase into the culture supernatant, and the productivities were dependent on the host strains and medium compositions. *S. cerevisiae* SH2041[pYGF2] was most superior among the strains tested, and the lipase was produced effectively in a medium containing 3% yeast extract, 1% peptone, and 4% sucrose; the production attained 78 U/ml (amount of lipase protein, 39 μg/ml).

The lipase was purified from the culture supernatant of *S. cerevisiae* SH2041[pYGF2], and the activity was separated into two fractions (lipases A and B) at the final purification step, SP-Sephadex C-50 ion-exchange chromatography. Lipase B was significantly more stable against heating than lipase A [43]. When the thermostability of lipase was expressed in the term of T_m value (the temperature at which heating for 30 min results in 50% decrease of activity), the values of lipases A and B were 66.2 and 80.0 °C, respectively (Table 1). Furthermore, stabilities against various organic solvents of lipase B were superior to those of lipase A. However, optimum temperature, activities in the presence of organic solvents, specific activities, fatty acid specificities, and K_m values for TAG were closely similar between two lipases.

3.2. Structures of lipases A and B

The molecular weights of lipases A and B estimated by SDS-PAGE were 31 and 34 kDa, respectively, and

Table 1
Thermostability of *F. heterosporum* and *R. oryzae* lipases

Lipase	Deletion size (residues)	Thermostability	
		T_m (°C)[a]	Δ T_m (°C)[b]
F. heterosporum lipase			
Lipase A		66.2	−13.6
Lipase B		80.0	+0.2
R275A		79.8	
R275A/D293A		61.1	−18.7
R275A/D293E		69.9	−9.9
R275A/D293N		60.1	−19.7
R275A/D293K		58.2	−21.6
R275A/Y289A		70.9	−8.9
R275A/D279A		66.5	−13.3
R275A/D279E		77.9	−1.9
R275A/D279N		78.0	−1.8
R275A/D279K		75.7	−4.1
R275A/Δ299	2	77.6	−2.2
R275A/Δ297	4	73.4	−6.4
R275A/Δ295	6	65.3	−14.5
R275A/Δ293	8	64.1	−15.7
R275A/Δ290	11	63.0	−16.8
R275A/Δ288	13	60.1	−19.7
R275A/Δ282	19	60.5	−19.3
Δ275	26	60.2	−19.6
R. oryzae lipase			
Prolipase		74.4	−5.4
Δ28prolipase		59.1	−20.7

[a] The temperature at which heating for 30 min results in 50% decrease in activity (100 mM acetate buffer, pH 5.6).

[b] The difference between the T_m values of the mutant and R275A lipases.

the lipase A preparation contained an additional band of <10 kDa [43]. The N-terminal amino acid sequence of lipase B was Ala-Val-Thr-Val-Thr---, which was identical to that of mature lipase estimated from the cDNA. Meanwhile, analysis of lipase A detected two N-terminal sequences: one was the same as that of lipase B, and the other was Asp-Met-Ser-Asp-Glu---, which coincided with the sequence starting from the position 276 (Fig. 1, underlined with thick line). These results showed that lipase B constituted of one polypeptide (301 amino acids) and that lipase A was composed of two polypeptides (position 1–275 and 276–301, Fig. 2).

Based on these results, the post-translational process was estimated as follows. After lipase B was produced, the peptide bond between Arg275 and Asp276 was cleaved by a trypsin-like protease, and lipase B was converted to lipase A in which the peptide of 26 amino acids (C-terminal peptide) still interacted with the remaining peptide of 275 amino acids (N-terminal large peptide). Because the cleavage of the C-terminal peptide was incomplete in *S. cerevisiae*, two lipases were detected in the culture supernatant. Lipase B was more stable against heating than lipase A, showing that the C-terminal peptide tightened the lipase structure before being cleaved. Furthermore, these facts suggested that the C-terminal peptide participated in the stability, but did not in the catalytic activity. Since the cleavage of the C-terminal peptide was complete in the original strain *F. heterosporum*, thermostable lipase B was not observed in the culture supernatant. *S. cerevisiae* harboring wild-type lipase cDNA produced two lipases with different thermostabilities. To produce only the thermostable lipase by *S. cerevisiae*, a mutant lipase, R275A (Arg275 → Ala), was constructed (Fig. 2). The production level of R275A protein was almost the same as that of the wild-type enzyme. The C-terminal peptide of R275A lipase was not cleaved by protease, and the thermostability of the lipase (T_m = 79.8 °C) was increased to the level of lipase B [43].

4. Amino acid residues contributing to the stability of *F. heterosporum* lipase

The mutant lipases were constructed to specify the amino acids in the C-terminal peptide of *F. heterosporum* lipase, which were responsible for the stabilization of the enzyme [44]. *S. cerevisiae* bearing R275A gene produced only thermostable lipase; thus R275A gene was used as the template for mutating of all amino acids in the C-terminal peptide to Ala. Mutation of Asp293 to Ala decreased the T_m value to 61.1 °C (Table 1), which was the same as that of the lipase without the 26 amino acid C-terminal peptide (Δ275 lipase, T_m = 60.2 °C). Mutations of Asp279 and Tyr289 to Ala decreased the T_m to 66.5 and 70.9 °C, respectively. Asp293 and Asp279 were more important for the lipase stability than Tyr289, and had a negative charge. Thus, Asp 293 and Asp279 were further replaced with Glu, Asn, and Lys. The thermostability were remarkably decreased by the mutation of Asp293 to the neutral and positive charged amino acids, Ala, Asn and Lys (Table 1). However, the

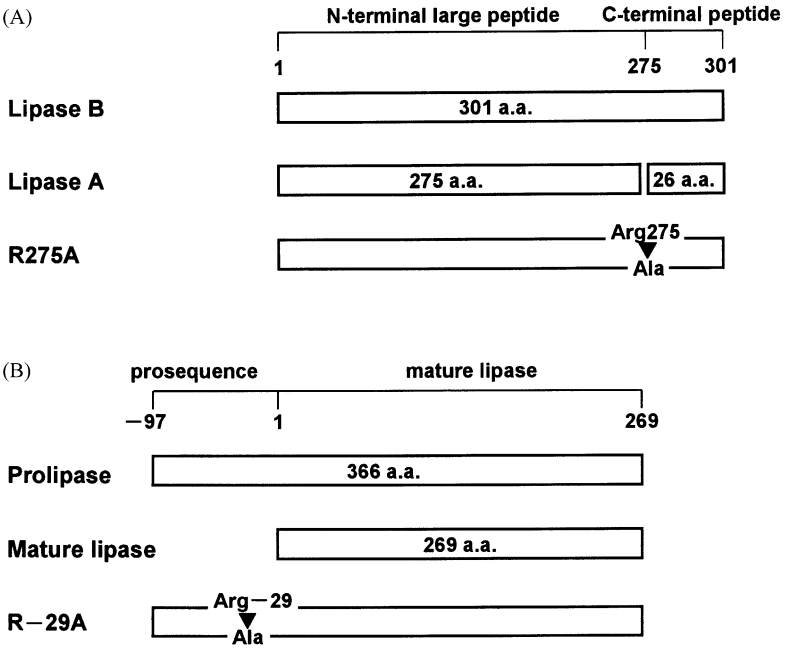

Fig. 2. Schematic structures of (A) *F. heterosporum* lipases and (B) *R. oryzae* lipases. Amino acid residues (a.a.) are numbered from the N-terminus of mature lipase, and the prosequence is indicated by negative numbers.

thermostability of the lipase from the D293E mutant was not decreased to the same extent as that of the lipase from the D293A, D293N, and D293K mutants. Negative charge of Asp293 therefore was considered to be essential for the thermostability, and these results suggested that the lipase with the C-terminal peptide was stabilized by an ionic bond between the negative charge of Asp293 and positive charge of an amino acid of the N-terminal large peptide. Meanwhile, replacement of Asp279 with Glu, Asn, or Lys did not significantly decrease the thermostability, indicating that the negative charge of Asp279 might not affect the stability, although the side chain of Asp was important.

The thermostability of the lipase gradually decreased with increasing the deletion size from the C-terminus (Table 1), and a 13 amino acid deletion decreased the stability to the level of the lipase not having the C-terminal peptide (Δ275 lipase). These results suggested that the 13 amino acid region from the C-terminus participated in the lipase stability. In addition, the production of the mutant lipase correlated well with the lipase stability, showing that the C-terminal peptide also influenced the lipase productivity. However, the kinetic constants, K_m value for olive oil, of these mutant lipases were not significantly different from that of the R275A lipase. These findings revealed that the catalytic activity was not influenced by the amino acid or deletion mutations of the C-terminal peptide.

5. Application of thermostable R275A lipase

Human milk fat contains 20–25% palmitic acid (PA), and about 70% of this fatty acid is esterified to the 2-position of TAG [45,46]. In addition, the main component of the milk dienoic TAG is OPO. Pancreatic lipase hydrolyzes TAG to free fatty acids and 2-monoacylglycerol, and the absorption of free PA liberated from the outer positions is lower than that of free unsaturated fatty acids [47]. It has been, therefore, hypothesized that fat absorption is higher in infants fed fat with PA at 2-position of TAG than the 1,3-positions [48]. For this reason, OPO has been used as an ingredient in infant formula, and can be produced by acidolysis of tripalmitin (PPP) with oleic

acid (OA) using immobilized 1,3-positionally specific lipase. The high melting point of PPP requires thermostable 1,3-positionally specific lipase for its production. It was thus attempted to use the thermostable R275A lipase for the production of OPO [49].

Screening test of carriers showed that an anion exchange resin, Dowex WBA, was suitable for immobilizing thermostable R275A lipase. When PPP was acidolyzed at 50 °C with two-fold of OA using 8 wt.% immobilized R275A lipase, the OA content in TAGs reached >50 mol% after 24 h, and the OPO content was 36 mol%. The durability of immobilized R275A lipase was compared with those of other 1,3-positionally specific lipases [49], and the acidolysis of PPP with OA was repeated by transferring the lipase to a fresh substrate mixture every 24 h. The stability of the immobilized preparation was precisely evaluated by investigating the decrease in the reaction velocity, which was determined from the OA content in TAGs after 3-h reaction (Fig. 3). The degree of acidolysis by R275A lipase after 80 cycles was 87% of that at the first cycle reaction (half-life, 370 d), and its stability was higher than those of immobilized *R. miehei* lipase (Lipozyme IM60, Novozymes) and *F. heterosporum* wild-type and *R. oryzae* lipases immobilized on Dowex WBA. While the reaction was continued for 220 cycles using immobilized R275A lipase, the acidolysis degree after 24 h was 88% of that at the first cycle.

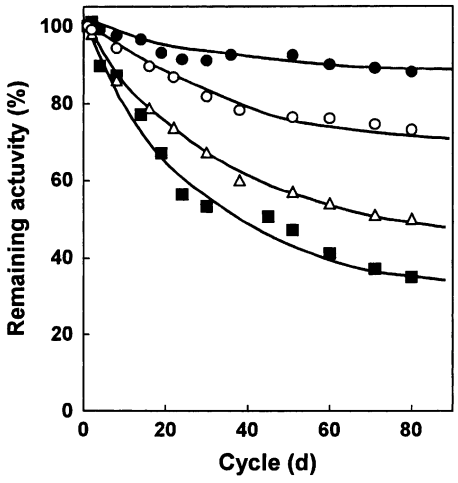

Fig. 3. Stability of various immobilized lipases. Acidolysis was conducted at 50 °C in a mixture of 30 g PPP/OA (1:2, (w/w)) and 2.4 g immobilized lipase with shaking, and was repeated by transferring the lipase to a fresh substrate mixture every 24 h. Three grams of the reaction mixture was taken out after 3 h, and OA content in TAGs was analyzed. The OA content is expressed relative to that in the first-cycle reaction. (●), *F. heterosporum* R275A lipase; (○), *F. heterosporum* wild-type lipase; (△), *R. oryzae* lipase; (■), *R. miehei* lipase.

6. Structure and stability of *R. oryzae* lipase

R. oryzae lipase was synthesized as prolipase (366 amino acids, Fig. 2), which was then converted to mature lipase (269 amino acids) by the cleavage of the N-terminal prosequence (97 amino acids). Because prolipase was more stable against heating than mature lipase, the N-terminal prosequence was essential for the stability of the proteins [50]. The prosequence of *F. heterosporum* lipase (16 amino acids) did not influence the stability of the lipase, but the C-terminal peptide was important for the stability. Furthermore, the prosequence of *R. oryzae* lipase was essential for in vivo folding and secretion of active lipase [50–52]. Since the deletion of the C-terminal peptide of *F. heterosporum* lipase caused the decrease of the lipase amount in the culture supernatant, the C-terminal peptide also may participate in the folding of the enzyme. Therefore, the prosequence of *R. oryzae* lipase and the C-terminal peptide of *F. heterosporum* lipase were considered to have similar functions; stabilization and folding.

When the wild-type cDNA (containing the prosequence and mature lipase sequence, gifted from Dr. M.J. Haas, USDA, ARS, ERRC, PA, USA) was expressed in *S. cerevisiae*, the strain produced two lipases; one was identical to prolipase ($T_m = 74.4$ °C, Table 1), and the other was an unstable lipase carrying a 28 amino acid region of the C-terminus of the prosequence and a mature sequence of 269 amino acids (Δ28prolipase, $T_m = 59.1$ °C). The unstable lipase was assumed to be generated by cleaving peptide bond between Arg−29 and Asp−28 (the prosequence is indicated by the negative number) by a trypsin-like protease [51]. To produce only the thermostable lipase by *S. cerevisiae*, a mutant R−29 A lipase (Arg−29→Ala) was constructed (Fig. 2). The protease no longer cleaved the resulting mutant lipase, and *S. cerevisiae* carrying the lipase gene produced only the thermostable lipase (unpublished data). The specific activities, fatty acid specificities, and K_m values for

TAGs (lipase activity) were not significantly different between prolipase, Δ28prolipase, and R−29 A lipase, but the esterase activities of those enzymes were different [53]. Prolipase had the highest esterase activity towards *p*-nitrophenyl laurate (C12), whereas Δ28prolipase (the same enzyme as r28ROL in [53]) had the highest activity towards *p*-nitrophenyl caprylate (C8) and stearate (C18).

We attempted the thermostable R−29 A lipase to an oil processing. A suitable support for immobilization of *R. oryzae* wild-type lipase (mature lipase) was Dowex WBA, which was the same as that used for immobilization of *F. heterosporum* lipase [54]. To investigate the stability of immobilized R−29 A lipase, acidolysis of PPP with two weight parts of OA was repeated at 50 °C by transferring the enzyme to a fresh substrate mixture every 24 h. The acidolysis proceeded as efficiently as that with *F. heterosporum* lipase. But, contrary to our expectation, the stability of immobilized R−29 A lipase (half-life, 60 d) did not exceed the stability of immobilized wild-type enzyme. This result may be explained as follows: mature lipase was stabilized by the immobilization to the same level as that of R−29 A lipase; or R−29 A lipase as well as mature lipase was stabilized to the same level by the immobilization.

7. Conclusion

Stable enzymes have several advantages, such as a long period storage, employment in the reaction at high temperatures, a long life of immobilized preparation, and so on. Stability of several enzymes has been increased so far by introducing a mutation based on the tertiary structure of stable homologous enzymes [55,56] or screening from the random mutation library [57]. *Pseudomonas aeruginosa* lipase was randomly mutated to obtain a thermostable lipase, but the stability increased by only 3 °C compared with the wild-type enzyme [58]. We succeeded in significant stabilization of *F. heterosporum* lipase and *R. oryzae* lipases based on the fact that the lipase without post-translational modification was more stable than mature lipase. This success was achieved by exact studies on the process of mature lipase production. Strategy described here was a new method that has not been reported yet, and may become a model for stabilization of other enzymes, especially homologous lipases to *F. heterosporum* and *R. oryzae* lipases.

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