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ORIGINAL PAPER

Enzymatic Interesterification of Palm Stearin and Coconut Oil by a Dual Lipase System

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Abstract Enzymatic interesterification of palm stearin with coconut oil was conducted by applying a dual lipase system in comparison with individual lipase-catalyzed reactions. The results indicated that a synergistic effect occurred for many lipase combinations, but largely depending on the lipase species mixed and their ratios. The combination of Lipozyme TL IM and RM IM was found to generate a positive synergistic action at all test mixing ratios. Only equivalent amount mixtures of Lipozyme TL IM with Novozym 435 or Lipozyme RM IM with Novozym 435 produced a significant synergistic effect as well as the enhanced degree of interesterification. The interesterification catalyzed by Lipozyme TL IM mixed with thermally inactivated immobilized lipase preparations indicated that the carrier property may play an important role in affecting the interaction of two mixed lipases and the subsequent reactions. A dual enzyme system, consisting of immobilized lipases and a non-immobilized one (Lipase AK), in most cases apparently endows the free lipase with a considerably enhanced activity. 70% Lipase AK mixed with 30% immobilized lipase (Lipozyme TL IM, RM IM and Novozym 435) can achieve an increase in activity greater than 100% over the theoretical value when the reaction proceeds for 2 h. The co-immobilization action of the carrier of the immobilized lipases towards the free lipase was proposed as being one of the reasons leading to the synergistic effect and this has been experimentally verified by a reaction catalyzed by a Lipase AK-inactivated preparation. No apparently synergistic effect of the combinations of Lipozyme TL IM and RM IM was observed when the dual enzyme systems applied to the continuous reaction performed in a packed bed reactor. In brief, this work demonstrated the possibility of increasing the reaction rate or enhancing the degree of conversion by employing a dual lipase system as a biocatalyst.

Keywords Enzymatic interesterification · Dual lipase system · Synergistic effect · Interesterification degree · Lipases

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Introduction

As ubiquitous enzymes, lipases (EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications [1, 2]. Lipases have surprising flexibility to catalyze the acylation and deacylation of a wide range of natural and unnatural substrates, which find a number of attractive applications in organic chemistry, pharmaceuticals, cosmetics and leather processing, etc. [3, 4]. However, from an industrial point of view, enzymatic modification of bulky oils and fats still represents one of the most promising technologies in terms of processing scale and practical applications [5, 6]. As one of a few commercialized approaches, enzymatic interesterification has been intensively studied [7]. The investigations cover enzyme evaluation, protocol development, fatty acid specificity, reaction optimization and packed bed reactions. These efforts have provided a lot of useful information for the better understanding of the interaction among lipase, substrates and operation parameters, and the development of practical techniques [8]. However, either in batch reaction or continuous operation, a single lipase is generally employed as the biocatalyst. Little attention has been given to dual or multiple lipase systems to reveal their synergistic or antergic effects [9].

Generally speaking, multiple enzyme systems have been assigned to successive reactions [10–12] or assistant

actions to the main reactions by removing byproducts or enzyme pre-treatment [13, 14]. The former approach, also called combinatorial biocatalysis, can obviate some separation steps and simplify purification procedures. The latter operation could deplete the inhibiting byproducts of the enzyme for the main reaction, which would facilitate the accumulation of desired products. These investigations are not only scientifically interesting, but also result in a significantly higher enzymatic turnout than that of the enzyme employed alone [13]. This concept has been applied to crude enzyme systems (consisting of multiple enzymes), by which Barthomeuf and Pourrat [15] showed a better performance for the production of high-content fructo-oligosaccharides using crude fructosyl transferase from *Penicillium*-containing glycosidase. The utilization of combined non-specific and 1,3-specific lipases has been widely used for structured lipid production [16]. However, these consecutive syntheses were usually performed in two steps, accompanying the purification of intermediate products, in which the two types of lipases acted as biocatalysts separately. Obviously, this differs conceptually from a dual enzyme system, in which two biocatalysts acted simultaneously, with possible interactions.

The primary aim of this work was to take a close look at the possible interaction of two simultaneously occurring lipases in a dual lipase-mediated interesterification, and explore the possibility of achieving an improved reaction. As a typical system for margarine fat studies [17], palm stearin (PS) has a relatively simple fatty acid composition (oleic + palmitic > 85%) and coconut oil (CO) enriches the composition with medium-chain fatty acids (Table 1). Their blend shows a concentrated distribution of triglyceride (TG) profiles (Fig. 1a) and was therefore chosen as a model system in this study. 1,3-Specific lipases from different sources, existing in either free (Lipase from *Pseudomonas flourescens*) or immobilized form (Lipases from *Thermomyces lanuginosa, Rhizomucor miehei* and *Candida antarctica* B), were employed as biocatalysts for enzymatic interesterification. The synergistic effects of dual enzymes were evaluated by the comparison of the experimental observation of individual lipase reaction systems and systems with a mixture of two enzymes. This work attempted to present new observations and a consideration of the enzymatic interesterification mediated by a dual lipase system.

Table 1 Fatty acid composition of palm stearin and coconut oil (wt%) and blend oil (PS/CO 7/3, *w*/*w*)

Fatty acid	Palm stearin	Coconut oil	Blend oil (mol%)	Blend oil (wt%)[a]
Caprylic	0.07	8.02	4.16	2.46
Capric	0.00	5.88	2.50	1.76
Lauric	0.48	47.00	17.59	14.44
Myristic	1.29	18.39	6.87	6.42
Palmitic	52.38	9.00	37.89	39.37
Stearic	4.94	2.80	3.72	4.30
Oleic	33.44	7.01	22.28	25.51
Linoleic	7.19	0.12	4.46	5.07
Linolenic	0.20	1.78	0.59	0.67

Means of triplicate determinations

[a] The fatty acid compositions of the blended oils were calculated by mass balance based on the average molecular weight of palm stearin and coconut oil

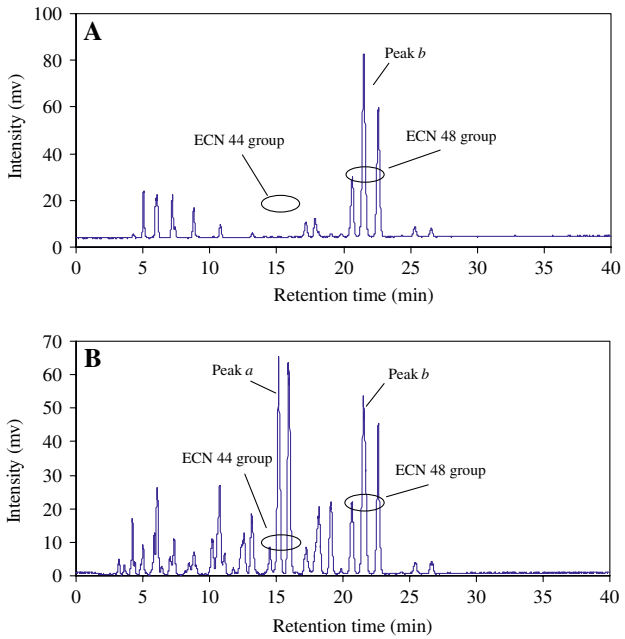

Fig. 1 HPLC chromatograms of the triglyceride profiles of palm stearin/coconut oil (7:3) blend before (**a**) and after 5 h enzymatic interesterification (**b**) catalyzed by a dual lipase system consisting of 70% (wt%) Lipozyme TL IM and 30% Lipozyme RM IM. The changes of relative contents of central peak of ECN (equivalent carbon number) 44 (*Peak a*) and 48 (*Peak b*) groups with reaction evolution, which also represent the corresponding major component of representative group, were employed as an index to monitor reaction progress

Experimental Section

Materials

Palm stearin and coconut oil were purchased from Karlshamn AB, Karlshamns, Sweden. Lipozyme TL IM (*Thermomyces lanuginosa*), Lipozyme RM IM (*Rhizomucor miehei*), and Novozym 435 (*Candida antarctica B*) lipases were kindly provided by Novozymes A/S, Bagsvaerd, Denmark, while Lipase AK Amano 20 (*Pseudomonas*

flourescens) was donated by Amano, Nagoya, Japan. Fatty acid methyl esters used as standards were procured from the Sigma Chemical Co. (St Louis, MO, USA). Tricaprin, trilaurin, trimyristin, tripalmitin, and tristearin used as the references for ECN determination were from Nu-Chek Prep, Inc. (Elysian, MN, USA) and with a minimum purity of 99%.

Enzymatic Interesterification

The enzymatic interesterification was conducted in a 60-mL screw-capped brown bottle and thermostated by a water bath at 60 °C. A typical reaction employed 20 g of palm stearin/coconut oil (7/3, *w*/*w*) blend, and the total amount of enzyme load was 9% (wt%) of the substrate for either sole or dual lipase systems. The reaction was initiated by the addition of lipase and agitated continuously by magnetic stirring at 300 rpm. All batch reactions were conducted under the above conditions unless stated elsewhere. Twenty-microliters of aliquots were withdrawn at desired intervals and dissolved in 1 mL of hexane assigned for HPLC analysis.

To evaluate the effects of the carriers of immobilized lipases on enzyme activity, thermal inactivation of Lipozyme TL IM, Lipozyme RM IM and Novozym 435 was carried out in a vacuum oven. The immobilized lipases were kept at 160 °C for 24 h under a vacuum. The resulting preparations were kept at room temperature for 2 h to partly recover lost moisture. The inactivated lipases were employed for interesterification of PS and CNO under the same conditions as used for active lipase to assay the residual activity. After 16 h no detectable reaction was observed for all 3 inactivated lipases, indicating the inactivated immobilized lipases can be treated as a carrier. The resulting preparations were therefore used as a replacement for an unavailable blank carrier for evaluation.

Lipozyme TL IM was used as a model to examine the effect of carrier properties on the immobilized lipase-catalyzed reaction. Equal amounts of inactivated preparation and Lipozyme TL IM were mixed for enzymatic interesterification and compared with a solely Lipozyme TL IM-catalyzed reaction. The same dosage of Lipozyme TL IM for all tests was used for comparison.

The effects of the carriers of the 3 immobilized lipases on the blended free enzyme were evaluated by the comparison of the Lipase AK-inactivated preparation blend-catalyzed interesterification with the reaction catalyzed by the same dosage of Lipase AK without inactivated immobilized lipase addition.

All reactions were performed in triplicate, and the means were used for evaluation of the results.

Determination of Fatty Acid and Triglyceride Compositions

Palm stearin and coconut oil were methylated by the boron trifluoride-methanol method [17]. Fatty acid methyl esters were analyzed on a Hewlett-Packard 5830A GC system equipped with a 25-m fused silica capillary column (25QC2/BPX/0.25 μm film, I.D. 0.22 mm) (Scientific Glass Engineering, Melbourne, Australia) and a flame-ionization detector (FID), as well as a HP 7671A auto-sampler. The injection temperature was set at 250 °C and helium was employed as the carrier gas at a flow rate of 40 mL/min. Oven temperature programming was as follows: starting at 70 °C and held for 2 min; the temperature was increased to 210 °C at a rate of 10 °C/min and maintained at 210 °C for 5 min; and following the second increase with a rate of 40 °C/min to 250 °C and held at 250 °C for another 2 min. Fatty acid methyl ester peaks were identified by the comparison of retention times with standards.

The triglyceride compositions of materials and products were determined with a Hitachi-Merck HPLC Series 7000 (Hitachi-Merck, Japan), conjugated with a PL-ELS 2100 evaporative light scattering detector (ELSD) (Polymer Laboratories, Shropshire, UK). The reverse phase column employed was a Supelcosil LC-18 (250 mm × 4.6 mm) (Supelcosil Inc., Bellefonte, PA). The ELSD was operated at an evaporating temperature of 70 °C and a nebulizing temperature of 50 °C with air as the nebulizing gas. Acetone and acetonitrile acted as the mobile phases by a gradient elution, beginning with an equal amount of the two solvents (50/50) and ending with 70% acetone and 30% acetonitrile. The mobile phase flow rate was 1.5 mL/min. The TG peaks were identified by the comparison of retention times with authentic triglyceride standards. Area percentages were used as weight to quantify the triglyceride composition. All measurements were conducted in triplicate.

Setup for the Evaluation of the Extent of Interesterification and the Synergistic Effect

The triglycerides of oils and fats in the HPLC chromatogram can be, in general, classified by the equivalent carbon number (ECN). The ECN depends mainly on the carbon number and the degree of unsaturation of the three bound fatty acids, of which the value is equal to the total carbon number of acyl groups with the subtraction of the number of double bonds. The triglycerides with the same ECN were able to be eluted as adjacent but separate peaks by HPLC, forming a so-called ECN group of TGs in the chromatogram (Fig. 1). For example, OOP, POP and PPP

can be eluted as a group of peaks of ECN 48 in the order as shown in Fig. 1.

As shown in Fig. 1a, the mixture of palm stearin and coconut oil exhibits characteristic TG profiles, namely, the TGs with medium chain length fatty acids are located in the retention time range of 5–10 min and the ECN 48 group of TGs of 20–23 min. According to individual HPLC analysis for PS and CO, the former are dominated by the triglycerides from the coconut oil, while the dominant ECN 48 group is represented by characteristic peaks of palm stearin [17]. Interesterification leads to the rearrangement of acyl groups within or inter-triglycerides, resulting in the changes of relative contents of TG profiles or the generation of new triglycerides [18]. The comparison of Fig. 1a and b revealed that a significant change before and after reaction is the appearance of the ECN 44 group of triglycerides (almost undetectable in the starting materials) and an evident decrease in the relative contents of the ECN 48 group of TGs. Furthermore, these two groups of triglycerides always occupy the major mass portion (>55%) during the reaction evolution. Thus, the change of relative contents of the two groups could be viewed as an index denoting the reaction evolution. Representatively, Peaks a and b are the major component belonging to respective groups. Therefore, the degree of interesterification (ID) can be simplified as

$$\mathrm{ID} = \frac{\text{area of Peak a}}{\text{area of Peak b}}. \quad (1)$$

To quantitatively evaluate the reaction performance of a dual lipase system, herein we defined the synergistic effect coefficient (SEC) as:

$$\mathrm{SEC}(\%) = \frac{\mathrm{ID_{AB}} - (x_\mathrm{A} \times \mathrm{ID_A} + x_\mathrm{B} \times \mathrm{ID_B})}{x_\mathrm{A} \times ID_\mathrm{A} + x_\mathrm{B} \times ID_\mathrm{B}} \times 100\% \quad (2)$$

where $\mathrm{ID_A}$ and $\mathrm{ID_B}$ denote the individual ID of the reaction when Lipase A and B act solely as the biocatalyst. $\mathrm{ID_{AB}}$ is the corresponding reaction degree catalyzed by the enzyme mixture of lipase A and B with the mass fraction of x_A and x_B. For comparison, the total enzyme load of dual lipases is always the same as the amount of a single lipase in this work. Clearly, the value of SEC can be positive, if the combination effect of two lipases is ameliorative; thereby, the value can also be negative, if the effect is deteriorative. Therefore, this definition can be used to characterize a dual enzyme system qualitatively and quantitatively.

Results and Discussion

Synergistic Effects of Lipozyme TL IM and RM IM

Figure 2 presents the time courses of interesterification of PS and CO catalyzed by Lipozyme TL IM and RM IM alone and their mixtures with different proportions. Compared with Lipozyme RM IM, Lipozyme TL IM achieved a better initial rate and a higher final degree of interesterification after 24 h, which agreed with the previous observation that Lipozyme TL IM shows a better performance for interesterification [19]. Before 4 h, the ID of dual lipases catalyzed reactions ranked between Lipozyme TL IM- and RM IM-mediated reactions, and at an identical time, the ID decrease with the increase of the RM IM proportion in the two lipase mixtures. Provided that the two lipases act separately with little interaction, this observation is reasonable because the introduction of RM IM to TL IM should result in a lower total reaction rate. Interestingly after 6 h, the dual enzyme systems, especially for the mixture of 70% TL IM and 30% RM IM, obtained a higher reaction degree (0.93 at 24 h) than the system with just Lipozyme TL IM (0.89 at 24 h).

To evaluate the synergistic effects of dual lipase systems more accurately, the theoretical ID (calculated from individual IDs of the two lipases and their proportions in the mixture) as well as the SEC of two enzyme mixtures with different ratios are depicted in Fig. 3. It is clear that for the mixtures of Lipozyme TL IM and RM IM, at any test ratio, the experimental degrees of interesterification were higher than the corresponding theoretical values. The strength of the synergistic effects was also shown to be associated with

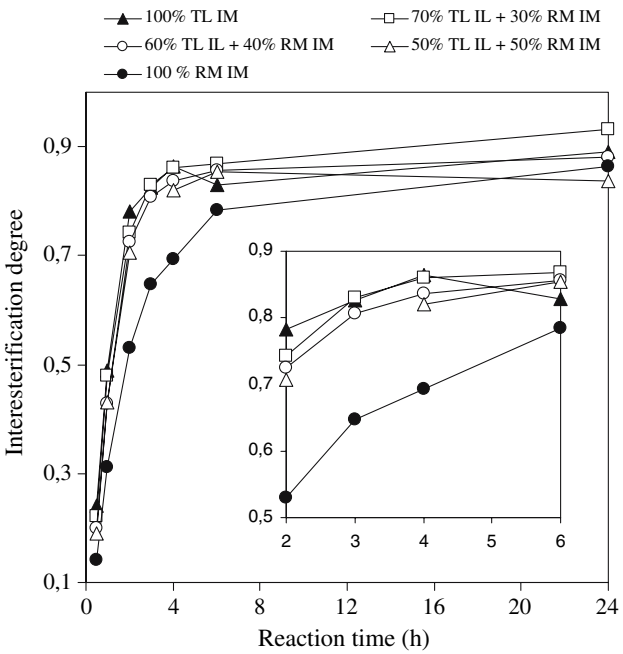

Fig. 2 Time course of enzymatic interesterification catalyzed by Lipozyme TL IM, Lipozyme RM IM, or their mixtures with different proportions. The inset is an enlarged part of the time range of 2–6 h. The batch reactions were conducted in triplicate at 60 °C with magnetic agitation. The enzyme load, either for an individual or dual lipase system was 9% (wt%) of the substrate

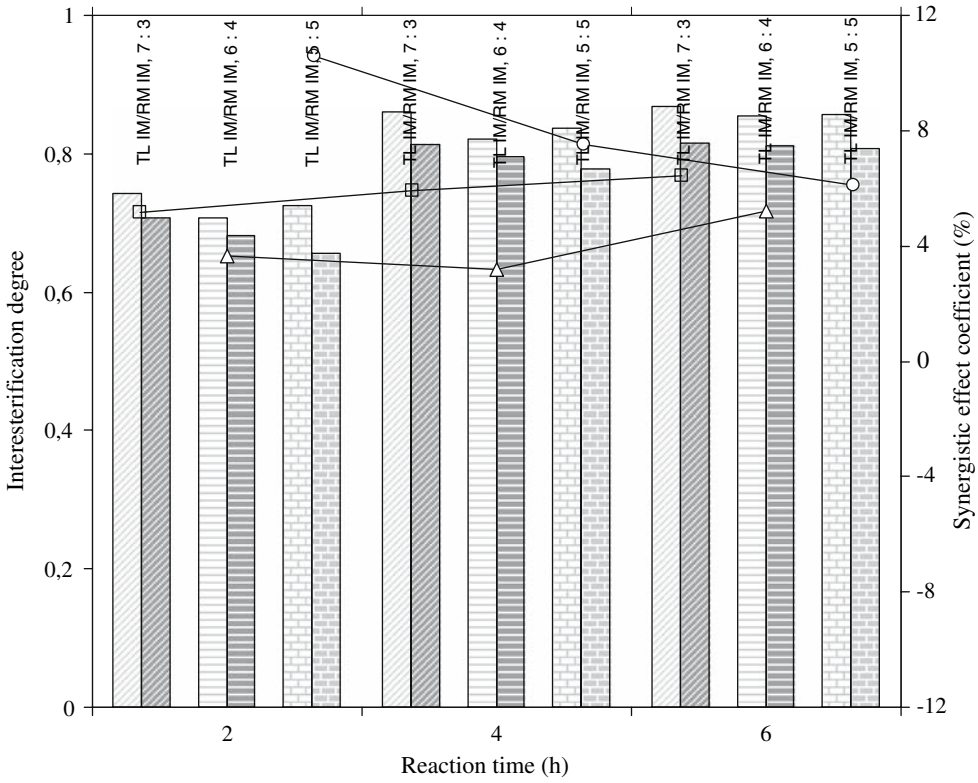

Fig. 3 Synergistic effects of dual enzyme systems of Lipozyme TL IM and RM IM mixed with different ratio (TL IM/RM IM). The reaction conditions were the same as in Fig. 2. The *bars* with a *light color* represent the experimental ID and those with a *dark color* are the corresponding theoretical values calculated by the weighted ID sum of two-combined lipases when they catalyze interesterification individually. Synergistic effect coefficients (*open squares*, *open triangles* and *open circles*) were calculated with Eq. (2)

the ratio of two mixed lipases (Fig. 3). The SEC values of TL IM–RM IM (60:40) varied in the range of 3–5 during the time course and TL IM–RM IM (50:50) around 5.5. While TL IM–RM IM (70:30) at 2 h exhibited 10% reaction amelioration over the expected value. However, with the reaction progress this synergism comes to be closer as indicated in Fig. 3. Overall, a synergistic effect between two immobilized lipases from *Thermomyces lanuginose* and *Rhizomucor miehei* seems to be operative, even though these positive impacts are not very significant.

Interplay of Novozym 435 and Lipozyme TL IM and RM IM

To better understand the synergistic effect of a dual immobilized lipase system, we conducted similar reactions employing the dual enzyme combinations of Lipozyme TL IM–Novozym 435, and Lipozyme RM IM–Novozym 435 with different mixing ratios (Table 2). The results shown in Table 2 demonstrate that a positive interaction between two mixed lipases does not always happen. The results also indicate that the interactive effect differs with different lipase combinations and also depends on the mixing ratio. The negative SEC values of Lipozyme TL IM–Novozym 435 at the ratio of 7/3 and 3/7 at the early stage of the reaction demonstrate an antergic effect possibly occurred in this dual lipase system at the test ratios. Similar phenomena have been observed for another dual lipase system of Lipozyme RM IM–Novozym 435 at the mixing ratio of 7/3. Interestingly, the dependency of the interactive effects on the mixing ratio of two lipases is experimentally repeatable.

It is worth noting that a significantly synergistic effect on enzymatic interesterification during the whole time course occurred, when equal amounts of Lipozyme TL IM–Novozym 435 or Lipozyme RM IM–Novozym 435 were in the mixture employed as biocatalyst. In comparison with the corresponding single enzyme system, the dual lipase system not only showed a faster reaction, but also achieved a higher degree of interesterification (Table 2).

Effects of Carriers on Lipozyme TL IM-Catalyzed Interesterification

The effects of the carriers of 3 immobilized lipases (the inactivated preparations) on Lipozyme TL IM-catalyzed interesterification are depicted in Fig. 4. Compared with sole Lipozyme TL IM-catalyzed interesterification, the contributions of co-existent inactivated Lipozyme TL IM and RM IM to the enhancement of the reaction were significant at any monitoring time. Inactivated Lipozyme TL IM generally produced over 15% ID enhancement for an identical reaction time; and the inactivated Lipozyme RM IM gave 50% enhancement of ID at 1 h and a 21% increase

Table 2 Synergistic effects of enzymatic interesterification of palm stearin and coconut oil catalyzed by dual lipase systems

	Ratio of two lipases	Lipozyme TL IM: Novozym 435					Lipozyme RM IM–Novozym 435			
		10:0	7/3	5:5	3:7	0:10	10:0	7:3	5:5	3:7
2 h	[a]ID	0.7819	0.3388	0.7820	0.4912	0.4675	0.5306	0.4143	0.5691	0.4760
	[b] SEC (%)	–	−50.73	24.39	−12.57	–	–	−18.96	14.10	−2.12
4 h	[a]ID	0.8291	0.6557	0.8763	0.7263	0.6967	0.7847	0.6979	0.8398	0.8797
	[b]SEC (%)	–	−26.10	14.85	−1.40	–	–	−3.06	18.29	24.50
8 h	[a]ID	0.8311	0.8826	0.9318	0.8567	0.9023	0.8012	0.8788	0.9277	0.9543
	[b]SEC (%)	–	3.54	7.51	−2.75	–	–	5.64	8.92	9.46

[a] Means of triple batch experiments. 9% of substrate was used as the dosage of biocatalyst for either single or dual lipase systems and all other conditions were identical

[b] The synergistic effect coefficient (SEC) was calculated by Eq. (2)

at 4 h, respectively. However, the inactivated Novozym 435 showed a different behavior, i.e. a positive contribution before 2 h and a negative effect at 4 h and 6 h as well as a ID close to that with just Lipozyme TL IM at 8 h.

It should be pointed out that, after inactivation processing at higher temperatures, the structure of Lipozyme TL IM could have changed, as the color changed to brown. The resulting preparation showed good mixing with Lipozyme TL IM, behaving as a hydrophobic carrier. In contrast, the structure of the carrier of Novozym 435 seems to change little (No color change observed). Aggregation was observed when mixed with Lipozyme TL IM, showing a more hydrophilic property. No apparent change for inactivated Lipozyme RM IM could be seen (due to the original brown color) and a good mixing with Lipozyme TL IM was also observed. These observations are not only able explain the results in Fig. 4 but also support our assumption that the dispersion state of immobilized lipases may affect dual lipase-catalyzed reactions. A hydrophobic carrier could be well dispersed in the oil blend and help the dispersion of the co-existent lipase, while a hydrophilic carrier is not good for dispersion in a hydrophobic reaction mixture and also induces the aggregation of co-existent immobilized lipase. This might also help us understand why the synergistic effect was always observed for the Lipozyme TL IM–RM IM dual system (Fig. 3) but not always for the Lipozyme TL IM–Novozym 435 system (Table 2), if one considers the hydrophobic property of the RM IM carrier differing from the carrier of Novozym 435.

Dual Enzyme System of Free and Immobilized Lipases

To examine the effects of different occurring forms of enzymes in a combining system, Lipase AK Amano 20 (powder) was mixed with Lipozyme TL IM at the ratio of 7/3, 5/5 and 3/7, and employed for enzymatic interesterification of palm stearin and coconut oil, respectively (Fig. 5). After 4 h, a visible enhancement of the degree of interesterification by a dual lipase system was obtained and compared with either immobilized or free lipase applied alone. The reaction catalyzed by Lipase AK alone is very slow and the degree of interesterification is less than 0.5 after 8 h. Lipozyme TL IM alone yielded an ID of 0.83 after 8 h. In contrast, all dual lipase systems produced a degree of reaction of over 0.9, the ID of the system with 70% Lipase AK/30% Lipozyme TL IM amounted to as much as 0.95. It is known that the enzyme in its free form is, in general, physical aggregates of enzyme protein (in most cases with accompanying oligosaccharides), existing

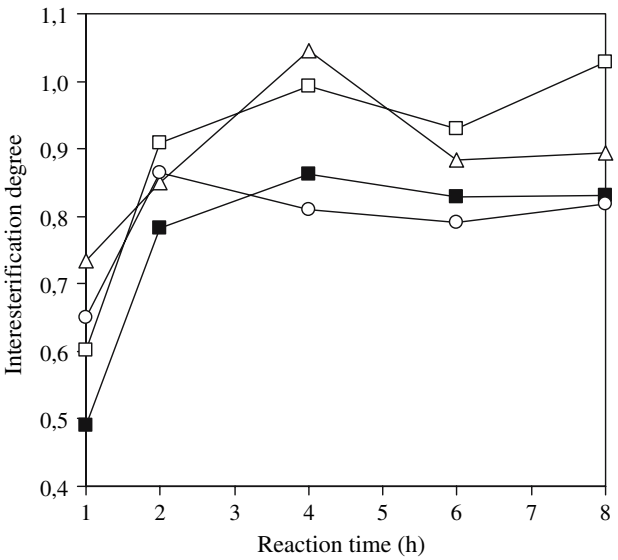

Fig. 4 Effects of the carriers of immobilized lipases on Lipozyme TL IM catalyzed interesterification. The interesterification was conducted with 10 g substrate (7/3, PS/CNO) at 60 °C and with agitation at 300 rpm. The biocatalyst consisted of 9% (wt% of substrate) Lipozyme TL IM and the same amount of inactivated Lipozyme TL IM (*open squares*), RM IM (*open triangles*) and Novozym 435 (*open circles*). The Lipozyme TL IM without inactivated immobilized lipase (*filled squares*) was used as a control

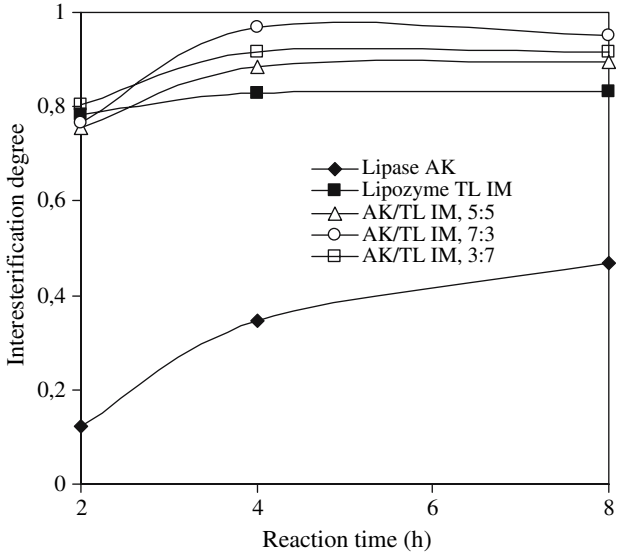

Fig. 5 Synergistic effects of free (Lipase AK) and immobilized (Lipozyme TL IM) lipases. The total amount of free or immobilized enzyme as well as their mixtures are 9% (wt%) of the substrate

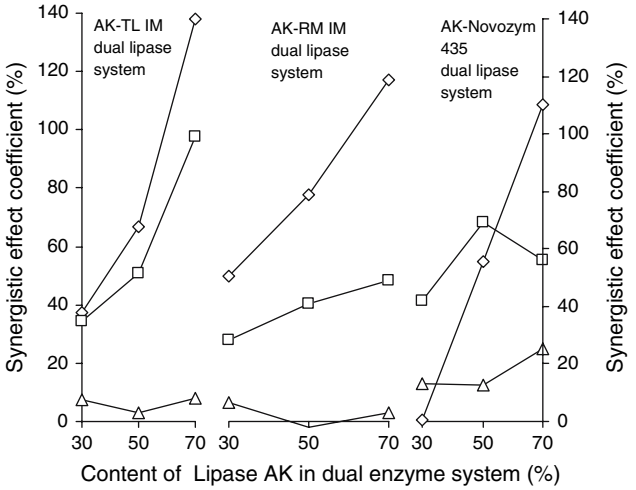

Fig. 6 Synergistic effects of Lipase AK and different immobilized lipases mixed with different proportions. The data shown are the corresponding results at 2 h (*open diamonds*), 4 h (*open squares*) and 8 h (*open triangles*)

as a supramolecular structure held together by noncovalent bonding [20]. In terms of its properties, the free enzyme (here Lipase AK) is hydrophilic. While the substrate in this study, the blend of palm stearin and coconut oil, is hydrophobic in nature and has a certain amount of viscosity at the operating temperature (60 °C). Therefore, a good dispersion of free Lipase AK in the oil (substrate) and a sufficient access of the oil molecules to the enzyme aggregates existing in a supramolecular structure are theoretically impossible, and thus it has a low apparent activity. However, in a dual lipase reaction system, the support of the immobilized lipase can possibly also act as a carrier to adsorb co-existing free enzyme. In other words, in this dual lipase reaction system, the immobilized lipase, besides acting as a biocatalyst, at the same time may play the role of "immobilizing" the co-existing lipase in free form. It is known that immobilization usually leads to a significant enhancement of the specific activity of an enzyme because the aggregated enzyme molecules in powder form could be re-distributed or organized and located on the surface of the carrier thus having a greater specific surface area, which facilitates the efficient interaction between enzyme and substrate [21]. Based on the above assumption, one can understand the synergistic effect between free Lipase AK and immobilized TL IM.

Figure 6 summarizes the synergistic effects of a dual enzyme system composed of Lipase AK and three different immobilized lipases with differing mixing ratios. From the presentation in Fig. 6, three observations can be generalized. Firstly, for all three immobilized lipases and in most cases, a markedly positive impact has been exerted on Lipase AK. Secondly, with reaction progress, the synergistic effect gradually recedes, which agrees with the observation that the dual enzyme system consists of two immobilized lipases. The last and also the most interesting observation is that, with the increase of the free lipase proportion in the dual enzyme system, the synergistic effects generally become greater with few exceptions [AK (50%) -Novozym 435 (50%) at 4 h]. Seventy percent of Lipase AK with 30% immobilized lipase (for all three immobilized lipases) can achieve >100% activity enhancement over the theoretical value at 2 h; while the SEC values for 50% Lipase AK dual enzyme systems varied in the range of 65–75% at 2 h (Fig. 6). The general synergistic effects of Lipase AK with different immobilized lipases, from another angle, supported the assumption that the synergism possibly comes mainly from the "assisted immobilization" of the immobilized enzyme for the co-existing free enzyme.

The "assisted immobilization" effect of the immobilized lipase towards the co-existing free enzyme has been experimentally verified by the reactions catalyzed by the mixture of Lipase AK and the inactivated immobilized lipases (Fig. 7). At 4 h and thereafter, the Lipase AK mixtures with the inactivated Lipozyme TL IM and RM IM yielded an over 15% average conversion increase compared with sole free lipase system. The contribution of the inactivated Novozym 435 was much more significant, achieving a 60% increase at 6 h and the enhancement at 8 h came to as much as 84%. The differences are most likely associated with the properties of the carriers. As mentioned above, the structure of the silica granules of Lipozyme TL IM is destroyed during thermal processing, possibly accompanied by the break of hydrophilic group

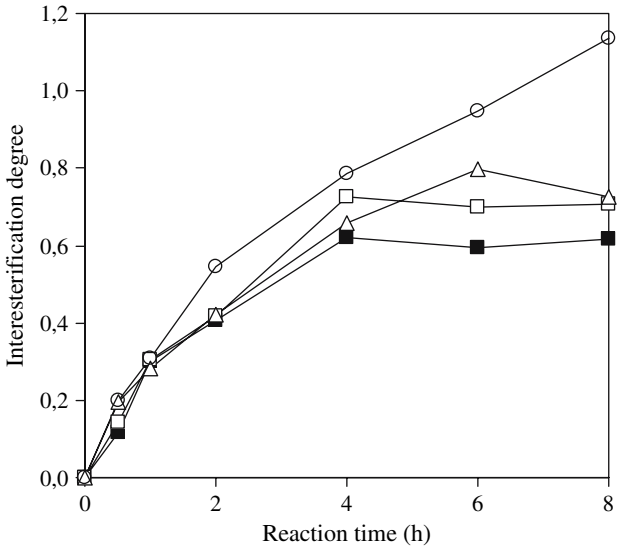

Fig. 7 Effects of the carriers of immobilized lipases on free Lipase AK catalyzed interesterification. The interesterification is conducted with 10 g substrate (7/3, PS/CNO) at 60 °C and with agitation at 300 rpm. The biocatalyst consists of 9% (wt% of substrate) Lipase AK and the same amount of inactivated Lipozyme TL IM (*open squares*), RM IM (*open triangles*) and Novozym 435 (*open circles*). Lipase AK without inactivated immobilized lipase (*filled square*) is used as a control

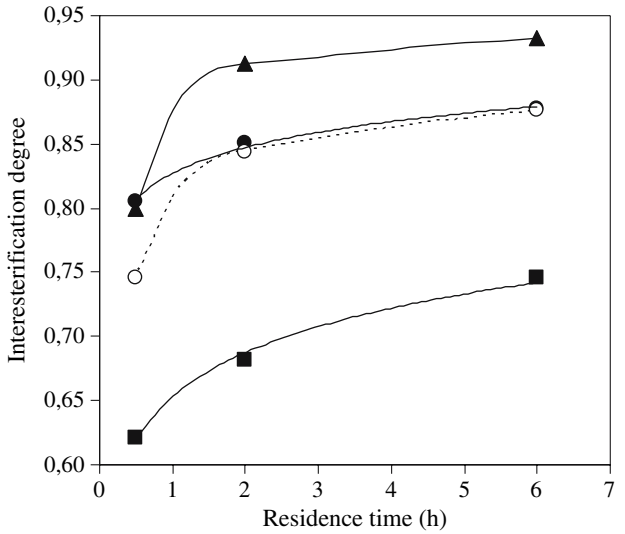

Fig. 8 Enzymatic interesterification of palm stearin and coconut oil (7/3) in a packed bed reactor filled with just Lipozyme TL IM (*filled triangles*) or just Lipozyme RM IM (*filled squares*) or a mixture of the two (7:3, *w/w*) (*filled circles*). The theoretical interesterification degree of the dual lipase system (*open circles*) was calculated by the weighted ID sum of Lipozyme TL IM and RM IM acting solely as the biocatalyst

and resulting in a dramatic loss of the capacity to adsorb protein. Regardless of the structural change of Lipozyme RM IM, the ion-exchange resin itself is not a good absorbent for protein loading (the high density of lipase in commercial Lipozyme RM IM is loaded by ion-exchange). However, as a kind of macroporous polymer, the carrier of Novozym 435 possesses a stable structure with very large specific area and is capable of immobilizing co-existed free lipase efficiently. Therefore, this result also implies that the carrier of Novozym 435 could be a good support for lipase immobilization by simple physical adsorption.

Dual Lipase Systems in Continuous Operation

Figure 8 depicts the reaction evolutions of the interesterification performed in a packed bed reactor employing Lipozyme TL IM, Lipozyme RM IM and their mixture with a ratio of 7/3 as biocatalyst, respectively. At the initial stage of the reaction, a synergistic effect of the dual lipase system was observed (at 0.5 h the experimental ID is 0.81, significantly higher than the theoretical value, 0.62). However, after 2 h the experimental ID almost overlapped the theoretical values; no apparently synergistic or antergic effect was observed. Similar results were observed for the dual lipase system of 50% Lipozyme TL IM and 50% RM IM applied in a packed bed reaction (data not shown). These results indicated that a dual lipase system in a batch reaction exhibited a better synergistic effect than in continuous operation, which is probably due to an ameliorative mass transfer by convection in the batch reaction but which is lacking in a continuous operation.

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