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Esterification and Hydrolytic Activities of *Candida rugosa* Lipase Isoform 1 (LIP1) Immobilized on Celite 545, Duolite A7, and Sephadex G-25

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The esterification and hydrolytic activities of free and immobilized *Candida rugosa* lipase isoform 1 (LIP1) were investigated. Esterification activity was determined by reacting caprylic acid with glycerol in the presence of molecular sieves (30%, w/w), and the volume of 1.0 M NaOH consumed by the reaction products upon titration was used to calculate esterification activity. Caprylic acid was also reacted with cottonseed oil, and the amount of caprylic acid incorporated after 12 h of reaction was determined. Results indicated that LIP1 had little esterification activity, which was not significantly improved upon immobilization. Hydrolytic activity was determined by incubating triacylglycerol emulsion (15%, w/w) with the respective lipases for 60 min, and the reaction products were titrated against 0.5 M NaOH. LIP1 showed hydrolytic activity comparable to Lipozyme RM IM. The hydrolytic activity improved significantly upon immobilization. Immobilization on Celite 545 produced the highest increase in hydrolytic activity.

KEYWORDS: Celite 545; Duolite A7; immobilization; LIP1; Lipozyme RM IM; Sephadex G-25

INTRODUCTION

Immobilized lipases are preferred for transesterification because they are stable and reusable and also offer easy separation of the enzyme from reactants and products. Immobilization of lipases can also affect their selectivity and chemical and physical properties (1) and creates non-aqueous conditions around the enzyme, which is necessary for ester synthesis or esterification (2). Commonly used immobilization methods include simple adsorption of the lipase to the surface of a solid support, covalent bonding of the enzyme to a solid support, encapsulation, and entrapment. Adsorption is the most commonly used method because of its simplicity. It is accomplished by mixing an aqueous solution of the lipase with the solid support or by precipitating the aqueous lipase solution onto the support using acetone, ethanol, or methanol. The mixture is filtered, and the immobilized enzyme is freeze-dried to reduce its moisture content.

Candida rugosa lipase isoform 1 (LIP1) is one of the seven isoforms of *C. rugosa* lipase (CRL). Its use in the synthesis of structured lipids has become attractive because of its ability to significantly incorporate acyl chains at the *sn*-1,2,3 positions of the triacylglycerol (TAG) backbone (3). The nonspecific incorporation of acyl chains results in increased TAG diversity, which improves the preponderance of β' crystals that impart a

smooth mouthfeel to margarine. A previous study in our laboratory (4) reported the inability of LIP1 to significantly incorporate stearic acid into canola oil. The aim of this study, therefore, was to immobilize LIP1 on Celite 545, Duolite A7, and Sephadex-G25 to enhance its activity for possible application in the synthesis of *trans*-free structured lipids. The activities of the immobilized lipases were compared to those of the free lipase (LIP1) and Lipozyme RM IM.

MATERIALS AND METHODS

Materials. Caprylic acid, triacylglycerol, glycerol, Celite 545, Sephadex G-25, and Duolite A7 were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized Lipozyme RM IM was purchased from Novo Nordisk A/S (Bagsværd, Denmark), and unimmobilized LIP1 (genetically engineered and expressed in *Pichia pastoris*) was donated by Dr. Jei-Fu Shaw of the Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei, Taiwan. Cottonseed oil was donated by Archer Daniels Midland Co. (Valdosta, Georgia). Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA). All other chemicals used were of analytical or HPLC grade.

Immobilization Procedure. LIP1 was immobilized on three different carriers (Celite 545, Sephadex G-25, and Duolite A7) as described (5). Briefly, LIP1 (3 g) was dissolved in 60 mL of 10 mM sodium phosphate buffer (pH 6) and mixed with 12 g of carrier. A volume (240 mL) of cold acetone ($-20\text{ }^{\circ}\text{C}$) was added, and the mixture was stirred for 30 min at room temperature ($20\text{ }^{\circ}\text{C}$). The mixture was filtered (suction) and washed with 60 mL of cold acetone ($10\text{ }^{\circ}\text{C}$), dried in an oven ($25\text{ }^{\circ}\text{C}$) for 72 h, and stored at $4\text{ }^{\circ}\text{C}$. Protein content was determined by

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Table 1. Hydrolytic Activity of Lipases

lipase	protein (wt %)	moisture (wt %)	activity (μmol $\text{min}^{-1} \text{mg}^{-1}$)	specific activity [$\mu\text{mol min}^{-1}$ (mg of protein) $^{-1}$]
Lipozyme RM IM	0.87	4.34	$0.04 \pm 0.00 \text{ d}^a$	$4.02 \pm 0.14 \text{ e}$
LIP1	5.89	3.38	$0.26 \pm 0.00 \text{ a}$	$4.32 \pm 0.00 \text{ d}$
LIP1 [Celite 545]	0.58	1.12	$0.13 \pm 0.00 \text{ c}$	$22.84 \pm 0.20 \text{ a}$
LIP1 [Duolite A7]	1.03	7.94	$0.14 \pm 0.00 \text{ b}$	$13.75 \pm 0.00 \text{ b}$
LIP1 [Sephadex G-25]	2.29	8.70	$0.13 \pm 0.00 \text{ c}$	$5.79 \pm 0.05 \text{ c}$

^a Values having the same letter in each column are not significantly different at $\alpha_{0.05}$.

means of a bicinchoninic acid (BCA) protein assay kit (Sigma Chemical Co., St. Louis, MO), and water content was measured on a Mettler Toledo moisture analyzer (Columbia, MD).

Esterification Activity. Glycerol (1 g) and caprylic acid (1.27 g) were incubated with each lipase (10%, w/w) for 1 h in screw-cap test tubes placed in an orbital shaking (200 rpm) water bath. The reaction temperature was 60 °C. A total of 30% (w/w) of molecular sieves (4 Å in diameter, 8–12 mesh) was added to increase the rate of reaction by absorbing water produced as a reaction byproduct. After the reaction was stopped, 1 mL of methanol was added and the content of the screw-cap test tubes was titrated against 1.0 M sodium hydroxide (NaOH) with 1% phenolphthalein as an indicator. The esterification activity was determined by the following expression:

$$\text{activity} = (VM)/(Wt) \quad (1)$$

where the product of V (the difference in titer values between the blank and samples) and M (molarity) is the amount (in moles) of caprylic acid consumed as a result of esterification after reaction time t (in minutes). W is the amount of lipase (in milligrams) used in the reaction. Esterification activity is defined as the amount of caprylic acid (in micromoles) consumed per minute per milligram of lipase in the reaction. All reactions were performed in duplicates.

Hydrolytic Activity. Lipase hydrolytic activity was assayed by preparing 200 mL of 0.33 M (or 15%, w/v) triacrylin emulsion using 10 mM sodium phosphate buffer (pH 6). Gum Arabic (5%, w/v) was used as an emulsifier. An aliquot (10 mL) of the emulsion was incubated with 500 mg of each lipase at 60 °C for 1 h. The reaction products were titrated against 0.5 M NaOH, with 1% phenolphthalein as an indicator. The difference in titer values between samples and the blank was used to calculate the amount of caprylic acid released. Hydrolytic activity was defined as the amount (in micromoles) of caprylic acid released per minute per milligram of lipase and was calculated from eq 1 above. All reactions were performed in duplicates.

Acidolysis Reaction. Structured lipids were synthesized in duplicates by reacting 1 g of cottonseed oil with 0.3 g of caprylic acid in screw-cap test tubes contained in an orbital shaking (200 rpm) water bath. The amount of each lipase used was 10% by total weight of reactants. The reactions were carried out for 12 h at 40, 50, and 60 °C. TAG bands were separated from other lipid classes by means of thin-layer chromatography as previously described (4). The TAG bands were scrapped off and converted to fatty acid methyl esters (FAMES) using the boron trifluoride (BF_3) method. Briefly, the TAG bands were

hydrolyzed at 90 °C in screw-cap test tubes by reacting with 2 mL of 0.5 M NaOH in methanol for 10 min, followed by 14% BF_3 in methanol for another 10 min. FAMES were extracted with 2 mL of hexane and analyzed in parallel with a FAME standard (Supelco 37 component FAME mix, Supelco, Bellefonte, PA), using an Agilent Technology 6890N gas chromatograph as previously described (4). The different amounts of FAMES were analyzed and integrated by an online computer, and the amount of caprylic acid incorporated was recorded.

Statistical Analyses. SAS Statistical Software, version 9.1 (SAS Institute, Cary, NC), was used for statistical analyses. Duncan's multiple comparison test was used to determine significant differences between treatments.

RESULTS AND DISCUSSION

Protein contents of the lipases were determined and expressed as percentage weight of the respective lipase preparations. Because the same amount of free LIP1 was used for immobilization on the solid supports, the effectiveness of the immobilization process can be evaluated by comparing the protein contents of the immobilized lipases to that of the free lipase (LIP1). From **Table 1**, it can be seen that immobilization on Sephadex retained the most protein and Celite 545 retained the least. Because this study was limited with regard to the determination of specific proteins present in the free and immobilized LIP1 preparations, it was assumed that protein content was fairly representative of the LIP1 content in these lipase preparations. The water contents of the lipases are also given in **Table 1**.

The hydrolytic activities of the lipases were determined by titrating reaction products against 0.5 M NaOH, and the amount (in micromoles) of caprylic acid released per minute per milligram of lipase preparation calculated. LIP1 lipase preparations showed significantly higher hydrolytic activities than Lipozyme RM IM (**Table 1**). In terms of specific activity (determined by dividing lipase activity by the protein content of the lipase used), the value for free LIP1 [$4.32 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$] was comparable to but significantly different from that of Lipozyme RM IM [$4.02 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$]. Our results also showed that the specific activity of LIP1 was significantly improved upon immobilization. The specific activities for the immobilized lipase preparations were $22.84 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$ for LIP1 [Celite 545], $13.75 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$ for LIP1 [Duolite A7], and $5.79 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$ for LIP1 [Sephadex G-25].

With regard to esterification activity, the free and immobilized LIP1 preparations showed no activity, whereas Lipozyme RM IM showed significant activity [$0.09 \mu\text{mol min}^{-1} (\text{mg of lipase})^{-1}$]. A similar pattern was observed in the acidolysis reaction involving caprylic acid and cottonseed oil (**Table 2**). The amount of caprylic acid incorporated after 12 h of reaction was significantly higher for reactions catalyzed by Lipozyme

Table 2. Incorporation of Caprylic Acid into Cottonseed Oil

lipase	reaction temperature					
	40 °C		50 °C		60 °C	
	Inc. ^a (mg %)	spec Inc. [Inc. h^{-1} (mg of protein) $^{-1}$]	Inc. (mg %)	spec Inc. [Inc. h^{-1} (mg of protein) $^{-1}$]	Inc. (mg %)	spec Inc. [Inc. h^{-1} (mg of protein) $^{-1}$]
Lipozyme RM IM	$15.01 \pm 1.07 \text{ a}^b$	$1.11 \pm 0.08 \text{ a}$	$21.8 \pm 2.22 \text{ a}$	$1.61 \pm 0.16 \text{ a}$	$17.11 \pm 0.72 \text{ a}$	$1.26 \pm 0.03 \text{ b}$
LIP1	$1.83 \pm 0.33 \text{ b}$	$0.02 \pm 0.00 \text{ b}$	$1.76 \pm 0.37 \text{ b}$	$0.02 \pm 0.00 \text{ b}$	0 b	0 b
LIP1 [Celite 545]	$0.75 \pm 0.33 \text{ b,c}$	$0.08 \pm 0.04 \text{ b}$	$1.27 \pm 0.11 \text{ b}$	$0.14 \pm 0.01 \text{ b}$	0 b	0 b
LIP1 [Duolite A7]	$1.69 \pm 0.09 \text{ b,c}$	$0.10 \pm 0.01 \text{ b}$	$1.94 \pm 0.04 \text{ b}$	$0.12 \pm 0.00 \text{ b}$	$0.82 \pm 0.86 \text{ b}$	$0.05 \pm 0.03 \text{ b}$
LIP1 [Sephadex G-25]	$0.40 \pm 0.28 \text{ c}$	$0.01 \pm 0.01 \text{ b}$	$0.76 \pm 0.34 \text{ b}$	$0.02 \pm 0.01 \text{ b}$	$0.97 \pm 0.77 \text{ b}$	$0.03 \pm 0.01 \text{ b}$

^a Abbreviations: Inc., incorporation of caprylic acid into cottonseed oil; spec Inc., specific incorporation (incorporation per hour per milligram of protein). ^b Values having the same letter in each column are not significantly different at $\alpha_{0.05}$.

RM IM (15.01–21.8 wt %) compared to reactions catalyzed by the free and immobilized LIP1 lipases (0–1.94 wt %). Our results also indicate that the immobilized LIP1 preparations showed higher specific incorporation [Inc. h^{-1} (mg of protein) $^{-1}$] than the free LIP1, even though these were not significantly different. It was highest for LIP1 [Celite 545] [0.14 Inc. h^{-1} (mg of protein) $^{-1}$] at 50 °C. The effect of the reaction temperature was significant in this study. Specific incorporation, in the amount (mg %) of caprylic acid incorporated into cottonseed oil per hour per milligram of protein, increased between 40 and 50 °C and decreased between 50 and 60 °C for all lipases. The reaction at 50 °C may therefore be suitable for the activities of these lipases under the reaction conditions used in this study.

It is notable that the results of this study did not meet our initial expectations. A previous study in our laboratory (3) showed significant oleic acid incorporation when LIP1 was used as the biocatalyst, but this level of fatty acid incorporation was not replicated in this study or the one (4) that preceded it. This discrepancy can be attributed to the selectivity of lipases (1). Lipases could be regiospecific or have preference for fatty acids based on carbon chain length, saturation, degree of unsaturation, or the type of acyl donor used. We are convinced on the basis of the above three studies that LIP1 has a preference for the unsaturated oleic acid over the saturated fatty acids: caprylic (this study) and stearic (4). LIP1 has also been reported (6) to show a preference for medium-chain fatty acids (C_8 – C_{10}), but its esterification activity with regard to caprylic acid ($\text{C}_8:0$) was not significant in this study.

This study has shown that LIP1, whether free or immobilized, had no significant effects on the esterification process compared to Lipozyme RM IM under the experimental conditions described here. LIP1 however showed significant hydrolytic ac-

tivity, which may be applicable in the hydrolysis of fats and oils to produce glycerol and fatty acids.

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