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Efficient Hydrolysis of Tuna Oil by a Surfactant-Coated Lipase in a Two-Phase System

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A surfactant-coated lipase (SCL) prepared by mixing *Candida rugosa* lipase with emulsifier in ethanol was used to hydrolyze tuna oil in a two-phase aqueous−organic system. Both enzyme (SCL) and substrate (tuna oil) were soluble in the organic phase, and the hydrolysis could occur with water molecules from the aqueous phase. This hydrolysis could promptly proceed compared to that catalyzed by native lipases which only occurred at the interface between the two phases. Michaelis−Menten kinetics in the two-phase reactions showed that the K_m value of the SCL was half that of the native lipase, while the maximum velocity (V_{max}) was 11.5 times higher. The hydrolysis method resulted in enrichment of n-3 polyunsaturated fatty acid (n-3 PUFA) content in glyceride mixtures from 26.4% to 49.8% and DHA from 19.1% to 38.9%. The SCL acted as an efficient hydrolytic catalyst for tuna oil.

KEYWORDS: Tuna oil; surfactant-coated lipase; *Candida rugosa*; polyunsaturated fatty acid; kinetics

INTRODUCTION

The n-3 series of polyunsaturated fatty acids (n-3 PUFA), especially *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), possesses various physiological functions (*1*, *2*), and its medical applications have attracted growing attention (*3*). However, dietary supplementation of long-chain n-3 PUFA is hampered because commonly available edible oils contain low concentrations of these acids. Most fish oils contain only moderate levels of DHA and EPA, and microbial oils containing high levels of either EPA or DHA can be produced via fermentation but it is relatively costly to do so (*4*). Therefore, it is important to enrich the content of n-3 PUFAs in fish oil.

All cis n-3 PUFAs are prone to a partial destruction by oxidation, cis−trans isomerization, or double-bond migration and are also susceptible to polymerization owing to the pH and high temperature required in conventional chemical hydrolysis processes (*5*). Enzymatic reactions, which proceed efficiently under ordinary temperature and pressure, are expected to be useful for high-level processing of PUFA-containing oils and their related compounds (*3*).

Lipase (triacylglycerol hydrolase, EC3.1.1.3) promoted hydrolysis of fish oil triacylglycerols is also commonly practiced, generally with an emphasis on DHA enrichment for infant formula development (*5*, *6*). However, the economic competence of the enzymatic process depends on improving the activity and the stability of biocatalysts as well as the cost of downstream separation (*7*).

Surfactant-coated enzymes have been extensively studied as promising biocatalysts for synthesis reactions in anhydrous organic solvents (*8*−*10*). Mori et al. were the first to report the application of a surfactant-coated enzyme hydrolytic catalyst for lipophilic esters in two-phase systems comprising an organic solvent and aqueous buffer (*11*). Since the surfactant-coated enzymes are soluble in various organic solvents, including some polar ones, but are insoluble in aqueous solution, these enzymes can be used as biocatalysts in a two-phase system provided that the coated enzymes solubilize or at least disperse well in the reaction medium.

The lipase from *C. rugosa* is a relatively cheap and widely used commercial enzyme. It demonstrates discrimination against longer chain PUFAs and has been used to prepare concentrates form fish and tuna oils in hydrolysis reactions (*12*, *13*). This work evaluates the kinetics of surfactant-coated *C. rugosa* lipase (SCL-Cr) by hydrolyzing tuna oil in a two-phase system. Furthermore, whether the enzyme selectivity for enrichment of PUFAs in glyceride mixtures of tuna oil by SCL-Cr is maintained was also investigated as a model reaction (**Figure 1**).

MATERIALS AND METHODS

Materials. Lipase (type-VII) from *C. rugosa* was obtained from Sigma Chemical Co. (LA). The nonionic surfactant sorbitan monostear-

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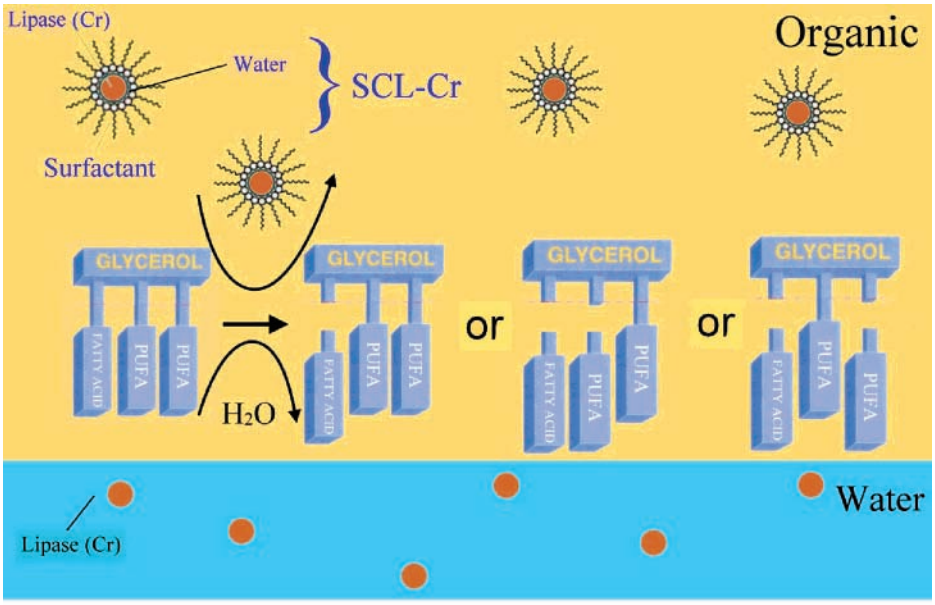

Figure 1. Schematic illustration of surfactant-coated *C. rugosa* lipase as catalyst for hydrolysis of tuna oil enrichment of PUFAs in a two-phase system. Key: SCL-Cr, surfactant-coated *C. rugosa* lipase; lipase (Cr), *C. rugosa* lipase; PUFA, polyunsaturated fatty acid; yellow phase, organic phase; blue phase, water phase.

ate, commercial name Span 60, and fatty acid methyl ester (FAME) standards were purchased from Sigma Chemical Co. (LA). Tuna oil was donated from a local canned tuna factory in Chia-yi, Taiwan. All the other chemicals used in the investigation were analytical grade and obtained commercially.

Preparation of SCL. This study used modifications of the methods of Yoshiaki et al. (*14*) and Isono et al. (*15*). A typical SCL preparation was as follows: 100 mg of lipase added to 50 mL of 0.1 M phosphate buffer solution (pH 5.0) was mixed with 400 mg of Span 60 which was dissolved in 5 mL of 95% ethanol. The solution then was agitated with a magnetic stirrer at 800 rpm and 4 °C for 1 h. Subsequently, the mixture was sonicated at 4 °C for 5 min with an ultrasonic bath. The mixture then was left to stand overnight. The mixture was centrifuged at 10 000 rpm and 4 °C for 10 min. The supernatant was filtrated through a 0.45 μm membrane, and the protein content was determined. The enzyme content of SCL was calculated on the basis of the result of elemental analysis of the surfactant, lipase, and the lipase−surfactant complex. The precipitate obtained from centrifugation was lyophilized to yield SCL-Cr.

Protein Content in SCL. The protein content in SCL was determined using the method of Lowry (*16*). *C. rugosa* lipase served as a standard.

Coverage of SCL with Surfactant. The fraction of lipase not coated with surfactant in SCL was determined using the method of Yoshiaki (*14*). This fraction was determined by filtering the SCL supernatant through a 0.45 μm membrane. The fraction of lipase remaining in the filtrate was measured using the method of Lowry (*16*). The fraction of lipase coated by surfactant was estimated by subtracting the fraction of lipase remaining in the filtrate from the total lipase. The fraction of lipase coated by the surfactant is termed the coverage of SCL with surfactant. The protein content was determined using the mothod of Lowry (*16*).

Hydrolysis of Fish Oil (*17*). Hydrolysis of fish oil was performed in an organic and aqueous two-phase system. The procedure was as follows: 10 mL of isooctane, 2 mL of phosphate buffer (0.1 M), and 3 g of fish oil were placed in a screw-cap test tube and preincubated at 40 °C for 30 min. The reaction then was initiated by adding SCL (the lipase content was around 100 mg) or free lipase at 40 °C and shaking at 150 rpm. The concentration of fatty acids was determined at various times.

Determination of Hydrolysis Conversion (*17*). After a given time, aliquots of the reaction mixture were withdrawn and the enzyme was inactivated by heating at 90 °C for 15 min. The solvent was evaporated under a vacuum, and the residue was dissolved in 5 mL of an ethanol/ether mixture (1:1 v/v). The fatty acids produced were determined by titrating the sample with 0.5 N KOH in ethanol, using phenolphthalein as an indicator. The initial millimolar of ester bonds was determined from conversion of hydrolysis was determined by the following equation:

$$\text{conversion} = \frac{\text{millimolar of free fatty acids}}{\text{millimolar of ester bonds included in fish oil}}$$

Separation of Acylglycerols and Free Fatty Acid after Enzymatic Hydrolysis (*18*). After addition of the required amount of 0.5 N KOH required to neutralize fatty acids released during hydrolysis, the mixture was transferred into a separation funnel and the soap was filtered out. The filtrate was concentrated at 45 °C by rotary evaporator and then passed through a bed of anhydrous sodium sulfate.

Methylate from Tetramethylammonium Hydroxide (TMAH) (*19*). A 0.1 g amount of fish oil, 3 mL of ether, and 1 mL of 20% TMAH/CH_3OH were added into a screw-cap test tube. The mixture was shaken for 10 min. A 4 mL volume of water was added to stop the reaction, and then pentadecanoic acid methyl ester was added. Pentadecanoic acid methyl ester was used as the internal standard. The supernatant was collected and dehydrated using anhydrous sodium sulfate. Finally, the methylates were filtered with a 0.45 μm membrane for gas chromatograph analysis.

Determination of the Composition of FAME. A gas chromatograph (G-3000, Hitachi, Japan) equipped with a column (SP-2330, 30 × 0.25 mm i.d.; J & W Scientific, Folsom, CA) and a flame-ionization detector was used to measure the FAMEs' compositions. The temperatures of both the injector and detector were set to 250 °C. The column was held at 160 °C for 5 min, and then the temperature was increased to 240 °C at 4 °C/min. The temperature then was maintained at 240 °C for 8 min. The flow rate of the carrier gas (nitrogen) was 1.5 mL/min, and the split ratio was 100:1 (*20*). FAMEs were identified by comparing their retention times against standards. Retention times were measured 3−5 times to minimize the experimental error.

Statistical Analysis (*21*). All experiments were performed in triplicate. Moreover, Statistical Analysis Systems (SAS) were done with the software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined using Duncan's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Some variables affecting the features of SCL were correlated to assess the yield (Y), protein content (PC), and coverage (C)

Table 1. Various Features of Surfactant-Cated *C. Rugosa* Lipase Prepared in This Study[a]

	Y (%)	PC (%)	*C* (%)	ϵ
SCL-Cr	74.18 ± 1.52	8.67 ± 0.32	32.13 ± 1.41	3.99 ± 0.01

[a] Definitions: CL-Cr, surtactant-coated *C. rugosa* lipase; *Y*, yield (%); PC, protein content (%); *C*, coverage (%). $\epsilon = Cl((Y)PC) - 1 = S/L$.

of SCL in terms of the ratios of surfactant (*S*) and lipase (*L*). These correlations are useful for checking the accuracy of the experimental data. *Y*, *C*, and PC are defined as follows:

$$Y = \frac{\text{weight of SCL}}{\text{total weight of surfactant and lipase}} \quad \text{(a)}$$

$$C = \frac{\text{weight of lipase in SCL}}{\text{total weight of lipase added during preparation of SCL}} \quad \text{(b)}$$

$$\text{PC} = \frac{\text{weight of lipase in SCL}}{\text{weight of SCL}} \quad \text{(c)}$$

From eqs a−c, the following correlation is obtained:

$$\frac{C}{Y(\text{PC})} = \frac{L+S}{L} = 1 + \frac{S}{L} \quad \text{(d)}$$

$$\epsilon = \frac{\text{C}}{Y(\text{PC})} - 1 = \frac{S}{L} \quad \text{(d*)}$$

As can be seen in Table 1, the yield of the SCL-Cr was 74.18% and the protein content and coverage were 8.67% and 32.13%, respectively. Furthermore, the ϵ (4) was close to the original ratio of surfactant and enzyme.

Degree of Hydrolysis. Following 160 h of 40 °C reaction, progress curves for enzymatic hydrolysis of surfactant-coated and native *C. rugosa* lipase were compared (**Figure 2**). The curve, which was typical, displayed a fast reaction rate followed by a slow one. Enzyme hydrolysis of fish oil (*10*), palm oil (*22*), and olive oil (*7*) produced similar progress curves. The result demonstrates that the degree of hydrolysis reached the maximum value (68.3%) at 17 h for SCL-Cr. The hydrolysis reaction then began to slow, which might be ascribed to enzyme deactivation, product inhibition, and/or limitation by chemical equilibrium. Furthermore, this investigation finds that the degrees of hydrolysis of the surfactant-coated and native *C. rugosa* lipases were 71.7% and 55.5% at 160 h, respectively. It shows statistically significant differences of the degree of hydrolysis. Restated, the former exceeded the latter by 1.3 times. Therefore, SCL-Cr is more suitable than the native one for catalyzing the hydrolysis of tuna oil in a two-phase system.

Kinetics of Hydrolysis. The SCL system is suitable for kinetic measurements because both lipase and substrates are homogeneously soluble in the organic phase, compared with the native lipase. This work studied Michaelis−Menten kinetics in a two-phase system. The kinetic parameters were determined on the basis of the Lineweaver−Burk plot. Furthermore, **Figure 3** illustrates a Lineweaver−Burke plot of the data. The intercepts and slopes of the Lineweaver−Burk plots yielded K_m and V_{max} values, and the obtained results are listed in **Table 2**. The K_m value of the SCL was half that of the native lipase, while the maximum velocity (V_{max}) was 11.5 times higher. There were significant differences in the K_m and V_{max} of SCL and native lipase. In 2003, Wu et al. indicated that the K_m of the lipase coated with glutamic acid didodecyl ester ribitol amide hydrolytic olive oil was half that of the native lipase while V_{max} was 1.4 times higher (*23*). As the K_m value reflects the affinity between the enzyme and substrate, a lower K_m value of the SCL indicates an increased affinity between them, possibly suggesting that the active site of the coated enzyme was exposed in a way more suitable for combination with the substrate. The increase in V_{max} implies an increased rate of reaction between the enzyme and substrate, probably due to diffusion becoming less limited as the coated lipase and substrate were solubilized in the same

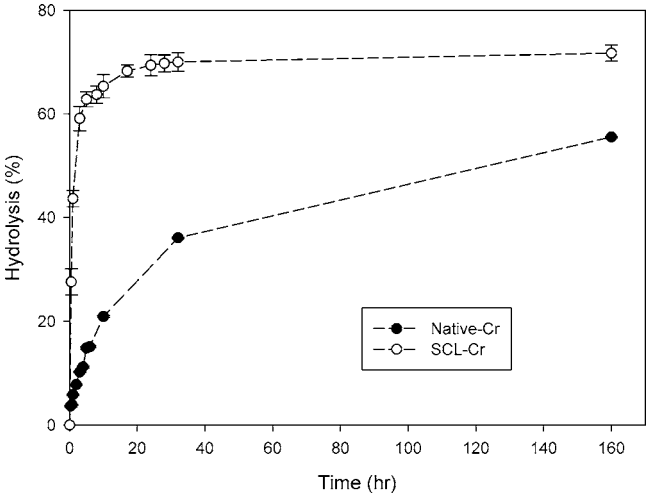

Figure 2. Degree of hydrolysis of tuna oil hydrolysis catalyzed by surfactant-coated and native *C. rugosa* lipase at 40 °C for 160 h. Key: SCL-Cr, surfactant-coated *C. rugosa* lipase; native-Cr, *C. rugosa* lipase.

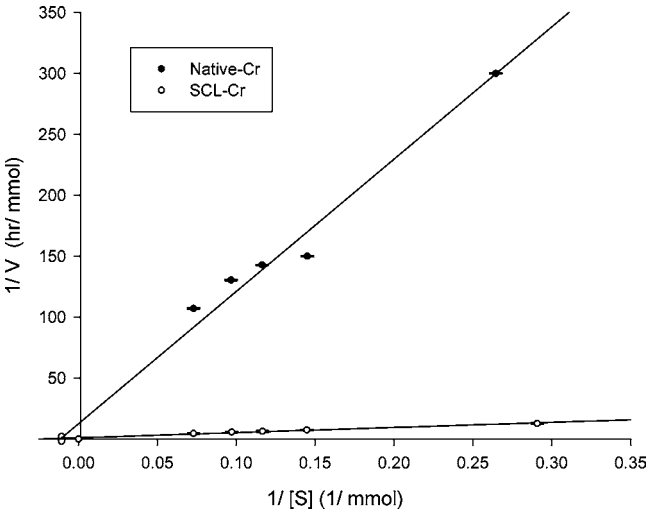

Figure 3. Lineweaver–Burk (double reciprocal) plots for surfactant-coated and native *C. rugosa* lipases hydrolysis with tuna oil as a substrate at 40 °C. Key: SCL-Cr, surfactant-coated *C. rugosa* lipase; native-Cr, *C. rugosa* lipase.

Table 2. Kinetic Parameters of Native and Surfactant-Coated *C. rugosa* Lipase[a]

param	native-Cr	SCL-Cr
K_m (mmol)	92.478[b]	41.150[a]
V_{max} (mmol/h)	0.08519[b]	0.979[a]
V_{max}/K_m[b] (h)	0.00092[b]	0.02379[a]

[a] Letters indicate significant differences ($P < 0.05$) for native-Cr and SCL-Cr. Definitions: SCL-Cr, surfactant-coated *C. rugosa* lipase; native-Cr, *C. rugosa* lipase. [b] The V_{max}/K_m ratio gave another indication of substrate to enzyme affinity (i.e., the larger the ratio, the better substrate/enzyme affinity).

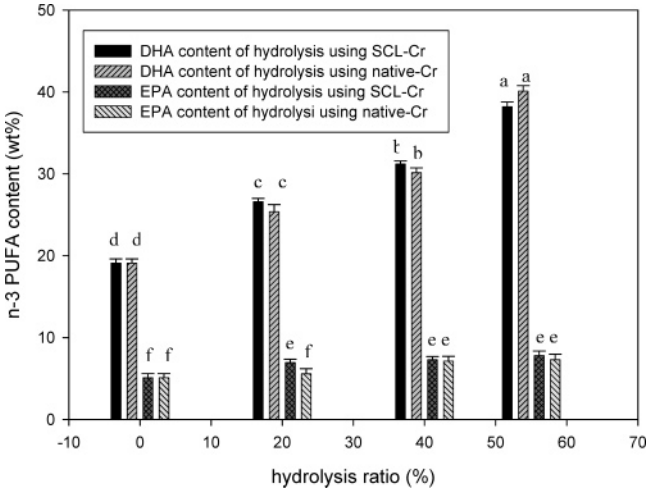

Figure 4. Concentration of DHA and EPA in glyceride mixtures (wt %) during hydrolysis of tuna oil by surfactant-coated and native *C. rugosa* lipase. Statistical differences were analyzed by ANOVA ($p < 0.05$), subset a > b. Key: SCL-Cr, surfactant-coated *C. rugosa* lipase; native-Cr, *C. rugosa* lipase.

Table 3. Major Fatty Acid Components of Original Tuna Oil and the Hydrolyzed (55%) Glyceride Mixtures by Surfactant-Coated and Native *C. rugosa* Lipase[a]

FAME	tuna oil	55% hydrolysis with SCL-Cr	55% hydrolysis with native-Cr
C14:0	4.5 ± 0.47^{a}	2.4 ± 0.42^{b}	2.2 ± 0.57^{b}
C16:0	24.5 ± 0.51^{a}	13.5 ± 0.39^{b}	11.3 ± 0.63^{c}
C16:1	6.24 ± 0.48^{a}	4.58 ± 0.38^{b}	4.24 ± 0.61^{b}
C18:0	6.5 ± 0.59^{a}	4.7 ± 0.53^{b}	4.8 ± 0.45^{b}
C18:1	12.9 ± 0.55^{a}	8.5 ± 0.43^{b}	8.2 ± 0.49^{b}
C20:5	5.1 ± 0.34^{b}	7.3 ± 0.49^{a}	7.8 ± 0.62^{a}
C22:6	19.1 ± 0.54^{b}	38.9 ± 0.67^{a}	40.1 ± 0.57^{a}
n-3 PUFA	26.4 ± 0.7^{b}	49.8 ± 1.2^{a}	51.5 ± 2.1^{a}

[a] Values represent means of duplicates ± standard deviation. The values do not total 100% because minor fatty acids are not reported. Values in the rows with different letters (a–c) are significantly different ($P < 0.05$). Definitions: SCL-Cr, surfactant-coated *C. rugosa* lipase; native-Cr, *C. rugosa* lipase; n-3 PUFA, n-3 polyunsaturated fatty acid; FAME, fatty acid methyl ester.

organic phase. The V_{max}/K_m ratio provided another indication of the affinity between the substrate and enzyme. The substrate/enzyme affinity rose with increasing V_{max}/K_m ratio.

Concentration of n-3 PUFAs. Figure 4 shows the change in the DHA and EPA contents of the glyceride fractions during the course of hydrolysis of tuna oil by surfactant-coated and native *C. rugosa* lipase. The DHA of the glyceride mixtures was consistently enriched throughout the reaction. There are no significant differences of the discriminability between SCL and native lipase. EPA was only enriched during the first 40% of hydrolysis, and then there was no significant change during the remainder of the reaction. This indicates strong discrimination by the lipase against DHA but only moderate discrimination against EPA.

Component of the Hydrolyzed Glyceride Mixtures. The compositions of the major fatty acids in the glyceride mixtures after 55% hydrolysis of tuna oil with SCL-Cr are shown in **Table 3**. Following 55% hydrolysis, the n-3 PUFA content of the surfactant-coated and native *C. rugosa* lipase fractions increased by approximately 2 times compared to the original tuna oil, giving a final concentration of approximately 50%. Most of the enrichment was due to DHA, which increased over 2-fold, whereas the EPA content increased by 1.5 times. The enrichment of n-3 PUFAs was compensated by a reduction in the content of $C_{14}-C_{18}$ saturated and monounsaturated fatty acids, indicated in **Table 3** by an approximately 2-fold decrease in the content of palmitic acids. Recently, a number of studies have demonstrated that lipases can be used to enrich the long-chain n-3 PUFAs in fish oil, by selective hydrolysis of the TG (*24*−*26*), selective esterification of fish oil FFA (*27*), or selective transesterification with low-molecular weight alcohols (*28*). Lipase from *C. rugosa* had been verified to be particularly effective (*24*, *25*). This could be partly because of the recently discovered fatty acid chain-length selectivity of lipase from *C.* rugosa, showing higher activity with C_{18} or shorter fatty acids than with C_{20} or C_{22} acids (*4*, *9*, *19*). Both surfactant-coated and native *C. rugosa* lipase displayed similar results, and the present experiments found no significant differences between them in terms of the concentrating n-3 PUFAs and DHA. As expected, the substrate selectivity of the SCL was not altered by the coating surfactant and showed high activity in a two-phase system.

Conclusion. The model reaction presented here demonstrates that the coating surfactant provided lipophilicity to lipase but did not alter the substrate selectivity to enrich the n-3 PUFA of the lipase. The SCL acts as an efficient catalysis for the hydrolysis of tuna oil in the two-phase aqueous−organic system and enriches the content of DHA 2-fold more than originally in the glyceride mixtures. The above results display the great potential that exists for the commercial application of SCL-Cr, a relatively cheap commercial enzyme.

ABBREVIATIONS USED

SCL, surfactant-coated lipase; V_{max}, maximum velocity; n-3 PUFA, n-3 polyunsaturated fatty acid; DHA, *cis*-4,7,10,-13,16,19-docosahexaenoic acid; SCL-Cr, surfactant-coated *C. rugosa* lipase; FAME, fatty acid methyl ester; *Y*, yield; PC, protein content; *C*, coverage; TMAH, tetramethylammonium hydroxide.

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