Plackett-Burman Design for Determining the Preference of *Rhizomucor miehei* Lipase for FA in Acidolysis Reactions with Coconut Oil

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ABSTRACT: The preference of lipase (EC 3.1.1.3) from *Rhizomucor miehei* in the incorporation of 11 FA, ranging from C10:0 to C22:6, into coconut oil TAG during acidolysis was studied by applying the Plackett–Burman experimental design. Enzymatic acidolysis reactions were carried out in hexane at 37°C for 48 h with coconut oil (0.1 M) and a mixture of 11 FA at a TAG to FA molar ratio of 1:1. Lipase was used at the 5 wt% level. The incorporation of FA into coconut oil TAG was determined by GC. The lipase showed preference for long-chain saturated FA for incorporation into coconut oil TAG. The FA with 18 carbon atoms showed a high incorporation rate (18:0 > 18:1 > 18:3). The lipase showed the least preference for the incorporation of 12:0, which occurs in maximal concentration (46%), whereas the most preferred FA, 18:0, occurs at a very low concentration (<2%) in coconut oil. The overall preference of lipase for the incorporation of different FA into coconut oil TAG was 18:0 > 18:2, 22:0 > 18:1, 18:3, 14:0, 20:4, 22:6 > 16:0 > 12:0 ≫ 10:0.

Paper no. J10136 in *JAOCS 79,* 555–560 (June 2002).

KEY WORDS: Acidolysis, coconut oil, fatty acids, lipase, Plackett-Burman design, *Rhizomucor miehei.*

Lipases (TAG hydrolases EC 3.1.1.3) from various sources preferentially hydrolyze acylglycerols. As this reaction is reversible, it can also be used for the synthesis of such compounds (1). Various lipases show degrees of regiospecificity, FA selectivity, or stereospecificity (2,3). Owing to one or more of these specificities, lipases find application as biocatalysts for the modification of TAG of naturally occurring fats and oils to improve their physical and nutritional properties. Such modifications are attained by interesterification reactions involving acidolysis or transesterification, resulting in the synthesis of structured lipids (4). Some lipases show preference for specific FA based on their chain length or degree of unsaturation (5,6).

The specificity of lipase as a biocatalyst in the hydrolysis of fatty esters and in the esterification of FA has been studied extensively (7–10). The most widely studied lipase in this regard is that from *Geotrichum candidum*, which shows specificity for long-chain FA with a *cis* double bond in the C-9 position (10). It is generally accepted that the expression of lipase specificity for or against a given FA is more pronounced in esterification than in hydrolysis reactions (11).

Lipase specificity may be due to the structural features of the substrate, such as FA chain length, unsaturation, stereochemistry, physicochemical factors at the interface, and differences in the binding site of the enzyme (12). Although a few kinetic models have been proposed to determine lipase specificity toward selected FA (9,13), they are generally based on the reaction of alcohol with FA and hence cannot be applied to complex systems such as TAG present in oils and fats. Acidolysis reactions involving TAG and FFA have been employed for the incorporation of specific FA into TAG of oils and fats (14,15). Generally, in such reactions the positional specificity of the lipases has been explored. However, results of several studies indicate that the lipase also shows substrate specificity in terms of the TAG species and the acyl donor. It is observed that when the same FA (acyl donor) is incorporated into different TAG (14) or when different acyl donors are used for the same TAG, the level of incorporation achieved is different (15). In preliminary experiments we observed differential rates of incorporation of stearic acid and n-6 and n-3 PUFA when structured lipids were prepared from coconut oil TAG using *Rhizomucor miehei* lipase-catalyzed acidolysis reactions.

Coconut oil is one of the few vegetable oils that has a very high content of medium-chain TAG, and therefore it is used in infant formula and parenteral and enteral nutrition. However, it has a very high level of saturated FA, 12:0, 14:0 and 16:0, which are known to be atherogenic (16). Moreover, coconut oil contains negligible amounts of PUFA, which could lead to EFA deficiency if this oil is consumed as the sole source of dietary fat (17). Therefore, it would be advantageous to modify the FA composition of coconut oil by replacing some of its saturated FA with less- or nonatherogenic FA and PUFA. However, in such a system, hydrolysis of FA from the TAG releases FA of coconut oil into the medium, which also compete with the acyl donor of interest for incorporation into TAG (esterification). Hence, a composite study to assess the specificity of lipase toward a range of naturally occurring FA needs to be carried out for selecting ideal FA in interesterification reactions for the modification of lipids. However, this would require a large number of experiments. The use of statistical designs, such as the Plackett–Burman (PB) experimental design, has the advantage of limiting the number of experiments that are to be performed in the selection of suitable substrates in esterification reactions.

The PB experimental design (18) has been successfully used for screening suitable alcohols for preparing anthranilic acid esters (19) and for selecting organic acids for esterification of α-terpineol (20). Because the PB design is a fractional

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factorial design, it allows analysis of $(N - 1)$ variables in N experiments. It can be applied efficiently to systems where the interactions between the variables are small when compared to their impact on the process being studied (21).

In the present investigation, the PB design was used to study the preference of lipase from *R. miehei* toward FA ranging from C10:0 to C22:6, with varying degrees of unsaturation, for their incorporation into coconut oil TAG.

MATERIALS AND METHODS

Materials. Immobilized lipase from *R. miehei*, IM60 (Lipozyme) was a gift from Novo Nordisk Bioindustrial Inc. (Danbury, CT). The FA—caprylic (10:0), lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidonic (20:4), behenic (22:0), and docosahexaenoic (22:6) acids—were obtained from Nu-Chek-Prep (Elysian, MN). Refined coconut oil was obtained from a local supermarket. Pentadecanoic acid was obtained from Sigma Chemical Company (St. Louis, MO). All chemicals and organic solvents used in the experiment were of analytical grade, and solvents were distilled before use.

Methods. (i) Interesterification reaction. The reaction mixture consisted of the FA mixture and coconut oil taken at a molar ratio of 1:1. The mixture of FA, each taken at either 0.015 or 0.002 M in 3 mL *n*-hexane based on the experimental design, added up to 0.1 M. Immobilized lipase IM60 was added at 5 wt% of reactants and incubated for 48 h at 37°C in an incubator with a shaker speed of 160 rpm.

(ii) Analysis of the products. At the end of the incubation period, the reaction mixture was separated from the lipase by decanting and passage over anhydrous sodium sulfate. An aliquot of this solution was applied on preparative TLC plates coated with silica gel G and developed in petroleum ether/diethyl ether/acetic acid (80:20:1, by vol). Bands were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in methanol. The bands corresponding to TAG were scraped and eluted using chloroform/methanol (2:1, vol/vol). The TAG were saponified using methanolic potassium hydroxide, and the FFA were methylated with boron trifluoride in methanol (22). The FAME were extracted with hexane, dried over anhydrous sodium sulfate, and analyzed by capillary GC (Shimadzu 14B, fitted with FID) on a fused-silica capillary column (0.25 mm × 25 m, Parma bond FAP-DF-0.25; Macherey-Nagel GmbH Co., Duren, Germany). The injector and detector temperatures were kept at 210 and 250°C, respectively. The initial temperature of the column was 160°C, and it was programmed to increase at a rate of 6°C/min to 250°C. The individual FA were identified by comparing the retention times to standards obtained from Nu-Chek-Prep (22).

RESULTS

The TAG of unmodified coconut oil contained a mixture of eight FA. Lauric acid was predominant (45.81%), followed by myristic (23.31%), palmitic (10.94%) and oleic (10.12%) acids. Other FA, such as 8:0, 10:0, 18:0, and 18:2, were observed in small quantities (2.02, 3.69, 1.56, and 2.53%, respectively). In this study, TAG of coconut oil were subjected to enzymatic acidolysis with a mixture of 11 FA in the presence of *R. miehei* lipase. The resulting interesterification generated modified TAG of coconut oil, with different levels of the added FA in addition to the already existing ones. In such a reaction, the major as well as minor FA of coconut oil that were displaced may or may not have been reesterified to the glycerol backbone in competition with the FA (acyl donors) added to the system. Hence, the FA composition of the modified TAG showed differences from that of the coconut oil.

The PB experimental design is a statistical experimental design used largely to determine the significance of important variables in a multivariable experiment, such as the one described in the present work. This design was employed to infer the significance of incorporation of the 11 FA into TAG of coconut oil through interesterification. This design is also known as the balanced incomplete block (21). An equimolar concentration (0.1 M) of coconut oil and a mixture of 11 FA (0.1 M) were taken for this reaction. Each variable (FA) was tried at two concentration levels, "**+**" denoting a high level of 0.015 M and "**–**" denoting a low level of 0.002 M (Table 1) as required by the PB design. The matrix itself was con-

TABLE 1
Matrix of the Plackett–Burman Design for 11 Variables at Two Levels[a]

Run number	Acid										
	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:4	22:0	22:6
1	+	–	+	–	–	–	+	+	+	–	+
2	+	+	–	+	–	–	–	+	+	+	–
3	–	+	+	–	+	–	–	–	+	+	+
4	+	–	+	+	–	+	–	–	–	+	+
5	+	+	–	+	+	–	+	–	–	–	+
6	+	+	+	–	+	+	–	+	–	–	–
7	–	+	+	+	–	+	+	–	+	–	–
8	–	–	+	+	+	–	+	+	–	+	–
9	–	–	–	+	+	+	–	+	+	–	+
10	+	–	–	–	+	+	+	–	+	+	–
11	–	+	–	–	–	+	+	+	–	+	+
12	–	–	–	–	–	–	–	–	–	–	–

[a]– = Low, 0.002 M; + = high, 0.015 M.

structed by taking six "+" signs and six "–" signs in a defined order and writing them as a column. The next column was generated from the first by moving the elements of the column by one position and placing the last element in the first position. A third column was generated from the second similarly, and so on. A row of minus signs was then added to get the last run (*N*th experiment) to complete the design.

Eleven FA (11 variables) were used in the 12 ($N = 12$) experiments carried out. In every run (represented by a row except the 12th), six FA were at a high level and five were at a low level, adding up to a total FA concentration of 0.1 M. Since each FA possesses the potential to be incorporated into any of the TAG of coconut oil, up to an upper limit of 7.5%, the experimental yield for the incorporation of that FA into coconut oil was determined in all 12 experimental runs by product analysis. In total, there were 11 experimental yields corresponding to 11 FA. These experimental yield determinations also included the content of the eight FA originally present in coconut oil. The change in composition of each FA under consideration indicated the extent of incorporation into the coconut oil. Corresponding theoretical yields were also evaluated.

A series of mathematical operations, described as follows, were performed to ascertain the extent of incorporation and thereby the specificity of the lipase for certain FA. The coefficient values necessary for evaluating the theoretical yields for the 11 FA from the percent incorporation (experimental yield) of each acid were determined by

$$A_i = \frac{1}{N}\sum_{i=0}^{N} X_i\, K_i \qquad [1]$$

where A_i = coefficient value, X_i = experimental yield (incorporation), K_i = coded value of each acid in a column corresponding to respective experimental yields X_i, and N = number of experiments. This was repeated to obtain a set of coefficients from all the experimental yields of the corresponding incorporated FA. Table 2 shows the set of coefficients obtained. The predicted yield is given by

$$Y_i = \sum_{i=0}^{N} A_i\, K_i \qquad [2]$$

where A_i is coefficient values determined as above and K_i is the coded value in each row. For $i = 0$, a dummy level of 1 was used, and the coefficients obtained were called A_0.

Table 3 gives a comparison of the experimentally determined incorporation of FA with the predicted values by solving Equation 2. The standard error (S_e) was determined as the sum of the squares of the differences between the experimental and predicted yields for each run. The estimated error S_b is given by

$$S_b = \sqrt{\frac{S_e^2}{N}} \qquad [3]$$

Student's *t*-test was performed to determine the significance of each FA employed.

$$t\text{-value} = \text{coefficient}/S_b \qquad [4]$$

TABLE 2
Statistical Data Showing *t*-Values and Coefficients from Experimental Yields of the Incorporated FA[a]

	10:0		12:0		14:0		16:0		18:0		18:1		18:2		18:3		20:4		22:0		22:6	
Run no.	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl	Cof	*t*-vl
Constant	1.46	0.58	36.6	30400	21.5	24.2	13.97	3491	1.89	7570	11.6	1118	5.08	2343	1.67	452	1.74	21289	2.68	4.63	1.19	0.78
1	0.37	0.15	0.23	190	0.08	0.09	–0.52	1308	0.24	606	–0.63	60	0.19	89	0.48	129	–0.34	4134	0.17	0.29	0.37	0.24
2	–0.25	–0.1	0.22	183	0.23	0.26	–0.21	521	0.05	216	–0.38	37	0.18	85	0.12	32	0.37	4470	0.42	0.73	–0.31	0.21
3	–0.07	–0.03	–0.13	108	1.66	1.87	–0.62	1550	0.05	190	–0.04	3.9	–0.22	103	0.42	115	0.24	2960	–0.2	0.35	–0.44	0.28
4	–0.003	–0.007	–0.11	89	–1.67	1.89	1.76	4408	–0.07	277	0.48	46	–0.02	7.3	–0.14	13	–0.04	490	–0.35	–0.61	–0.42	0.27
5	0.05	0.02	1.28	1062	–1.71	1.93	0.61	1527	0.14	550	0.48	46	–0.57	261	0.17	45	–0.43	5266	–0.7	1.23	0.2	0.19
6	–0.25	–0.1	–2.46	883	–0.59	0.67	0.29	729	–0.20	790	1.74	167	–0.003	1.2	–0.02	5.8	–0.21	2551	0.5	0.87	0.55	0.36
7	–0.04	–0.14	–0.96	793	0.14	0.16	–0.1	238	–0.4	1596	–0.07	6.9	1.51	694	0.13	36	0.34	4102	–0.53	0.92	0.45	0.3
8	–0.2	–0.08	–1.88	1557	0.03	0.04	0.35	882	0.16	623	1.09	104	–0.19	86	0.75	203	0.23	2837	–0.23	0.4	–0.71	0.46
9	0.31	0.12	0.58	479	–1.11	1.26	–0.68	1704	0.08	337	–0.39	37	0.003	1.2	0.13	34	0.29	3511	–0.21	0.37	0.59	0.38
10	0.15	0.06	–0.72	601	–0.26	0.29	–0.67	1670	–0.98	390	0.13	12.8	–0.69	317	0.04	12.4	0.36	4368	1.95	3.4	0.23	0.15
11	–0.03	–0.01	–1.92	1596	0.098	0.11	0.58	1454	0.2	816	0.38	37	0.27	12.3	–0.5	135	0.17	2020	1.00	1.74	0.86	0.56

[a]Cof = Coefficients, *t*-vl = *t*-value.

TABLE 3
Comparison of Predicted and Experimental Incorporation of FA[a]

Run no.	10:0		12:0		14:0		16:0		18:0		18:1		18:2		18:3		20:4		22:0		22:6	
	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd	Expt	Prd
1	2.1	0	34.3	34.3	26.4	27.2	11.2	11.2	2.4	2.4	9.5	9.4	7.7	7.7	2.8	2.8	2.6	2.6	0.8	0.8	0.3	2.0
2	2.2	7.4	39.1	39.1	19.1	18.2	13.2	13.2	2.4	2.4	9.4	9.4	3.5	3.5	2.9	2.9	2.5	2.5	4.3	4.3	1.0	0
3	1.7	4.4	41.1	41.0	22.3	23.2	11.1	11.1	2.5	2.5	9.1	9.1	2.5	2.5	0.7	0.7	2.7	2.7	5.3	5.3	0.3	2.0
4	1.7	3.6	32.3	32.2	23.2	24.1	14.8	14.8	1.9	1.9	12.9	12.9	3.6	3.6	0.7	0.7	1.1	1.1	7.0	7.0	0.4	2.1
5	1.6	2.7	39.9	39.9	18.8	19.7	17.4	17.4	2.0	2.0	9.3	9.3	7.7	7.7	0.7	0.6	0.9	0.8	0.8	0.8	0.4	2.1
6	0.7	4.4	37.0	37.0	23.9	23.1	12.9	12.9	2.6	2.6	13.3	13.3	3.4	3.4	3.8	3.8	0.5	0.5	0.7	0.7	0.8	0
7	0.8	2.2	36.8	36.7	21.8	20.9	14.0	14.0	0.7	0.7	11.4	11.4	7.5	7.5	1.4	1.4	2.7	2.7	0.1	0.1	2.3	0.6
8	1.2	4.9	37.5	37.5	20.9	20.1	15.8	15.8	1.2	1.2	12.9	12.9	4.2	4.2	2.9	2.9	2.1	2.1	0.7	0.7	0.1	0
9	1.2	0	33.5	33.5	14.6	15.5	19.0	19.0	2.3	2.3	16.3	16.3	3.6	3.6	0.9	0.9	0.7	0.7	0.8	0.8	0.2	1.9
10	2.6	3.5	38.4	38.4	17.6	16.7	11.0	11.0	1.2	1.2	11.3	11.3	5.5	5.5	1.9	1.8	0.7	0.7	3.2	3.2	6.3	4.5
11	0.2	0.8	27.0	27.0	23.8	24.7	13.6	13.6	1.1	1.1	14.5	14.5	6.7	6.7	1.0	1.0	3.2	3.2	7.0	7.0	0.2	1.9
12	1.4	0	42.5	42.5	24.6	23.7	13.1	13.1	1.7	1.7	8.8	8.8	4.6	4.6	0	0	0.7	0.7	0.8	0.8	1.5	0

[a]Expt = experimental yield; Prd = predicted yield.

A *t*-value ($P < 0.001$) of 4.44 corresponded to a confidence level of 99.9%. Values for others were worked out using a *t*-value table (23).

DISCUSSION

The preference of lipases to catalyze interesterification reactions normally depends on the type of FA to be incorporated into the acylglycerol moiety as well as the types of acyl groups already existing on the glycerol backbone. In order to determine the FA preference in acidolysis reactions catalyzed by lipase from *R. miehei*, we employed the PB experimental design. In any given experiment, we selected six externally added FA at a concentration of 0.015 M each. This means that there will be an increase of 7.5% incorporation of any of these particular FA if they are completely incorporated into coconut oil TAG. The maximal incorporation observed with certain FA was found to be in this range, confirming the preference of the lipase in incorporation of these FA into TAG. Thus, the preference referred to in this work indicates a maximal increase of around 7.5% of that specific FA into coconut oil TAG. However, the lower limit of incorporation is again dependent on the preferences of the lipase for the externally added FA to the displaced ones, which already existed on the TAG. Table 4 indicates the level of significance for the FA replaced and FA incorporated in its place. The confidence level obtained for each FA indicates the preference for the incorporation of that FA into coconut oil. Higher confidence levels indicate higher preference for that FA by *R. miehei* lipase for incorporation into coconut oil and vice versa.

In the case of replacement of 10:0 and 22:6, the significance of incorporation of other FA was less than 80%, whereas for the replacement of 14:0, the significance of incorporation of 18:0, 16:0, and 14:0 was above 90%. Similarly, in the case of replacement of 22:0, the significance of incorporation of the same acid was 99.5%, whereas that for 12:0, 16:0, 18:0, 18:1, 18:2, 18:3, and 20:4 was 99.9% (Table 4). In the present study we observed that the saturated FA with longer chain length were incorporated better (Table 4) into coconut oil, with 18:0 being shown maximal preference followed by 16:0, 12:0, and 10:0. However, 22:0 and 14:0 did not follow this trend. The unsaturation in the FA also influenced the rate of incorporation into coconut oil TAG. Higher incorporation was observed for 18-carbon FA that had a lower level of unsaturation (18:0 > 18:1 > 18:3) (Table 4). Linoleic acid (18:2), however, was incorporated to a slightly higher extent than 18:1. Similarly, the preference for 22:0 > 20:4 > 22:6 shows that the lipase preferred a fully saturated FA over a monounsaturated FA, which in turn is preferred over PUFA. This observation is similar to previous reports where lipase from *R. miehei* preferred FA with a single double bond to those containing two or three double bonds (9). Similarly, Haraldsson *et al.* (24) observed that lipase from *R. miehei* has less preference for FA with a double bond close to the carboxylic end. The lower reactivity of long-chain PUFA, such as 20:5 and 22:6, may be caused by an unfavorable orienta-

TABLE 4
Significance of the Order of Replacement of FA by Lipase

FA replaced	FA incorporated	Confidence level (%)
10:0	10:0, 22:0	≪80
12:0	22:6 > 18:3 > 18:0 > 18:1 > 18:2 > 22:0 > 20:4	99.9
14:0	18:0 > 16:0 > 14:0	90.0
16:0	16:0 > 20:4 > 22:0 > 14:0 > 18:0 > 22:6 > 10:0	99.9
18:0	18:2	99.9
18:1	18:1 > 18:3	99.9
18:2	18:2 > 22:0 > 18:3	99.9
18:3	18:3 > 22:6 > 10:0 > 14:0	99.9
20:4	18:0 > 12:0, 22:0 > 10:0 > 18:2 > 20:4 > 14:0 > 18:3 > 18:1 > 22:6	99.9
22:0	22:0	99.5
22:6	All	≪80

tion of the FA chain in the active site of the lipase due to a kink in the chain caused by a series of *cis* double bonds, more so if nearer to the carboxyl end (25).

It is interesting to note that the lipase showed lesser preference for 12:0, which is the major FA found in coconut oil. However, the lowest 12:0 content observed was 27%. A number of FA efficiently replaced 12:0 from coconut oil TAG. The lipase from *R. miehei* is known to hydrolyze the FA on the *sn*-1 and -3 positions of TAG. Hence, during hydrolysis, the enzyme removed 12:0 and 14:0, which occur at these positions (22). However, since the enzyme showed low affinity for 12:0, the lipase preferred to replace 12:0 with almost all the long-chain FA in the TAG (Table 4).

Although 18:0 is present at a very low concentration (<2%) in coconut oil, the lipase showed highest preference for this FA. Since 18:0 has been known to enhance the activity of lipase from *Rhizopus japonicus*, it is possible that such an activation may have taken place for lipase from *R. miehei* too (26). TAG of coconut oil contained about 87% saturated FA and no FA beyond 18:2 in length. However, the enzyme was capable of incorporating longer-chain saturated, monounsaturated, and polyunsaturated FA into the TAG. Our study indicated that the overall preference of lipase for the incorporation of various FA into coconut oil TAG as inferred from the significance levels is in the order of 18:0 > 18:2, 22:0 > 18:1, 18:3, 14:0, 20:4, 22:6 > 16:0 > 12:0 >> 10:0 (Table 4).

The PB design employed here helped us to arrive at the preference of *R. miehei* lipase for different FA in the incorporation into coconut oil TAG. The design helped us choose the appropriate FA to use for modifying coconut oil for specific requirements, using the least number of experiments

ACKNOWLEDGMENTS

The authors acknowledge the encouragement of Dr. Vishweshwariah Prakash, Director, CFTRI; Dr. Santhoor Bhat, head of the Department of Biochemistry and Nutrition, CFTRI; and Dr. Ganesh Karanth, head of the Department of Fermentation Technology and Bioengineering, CFTRI, for their keen interest during the course of this investigation. Reena Rao acknowledges the Senior Research Fellowship from the Council of Scientific and Industrial Research, New Delhi. We also thank Drs. Rashesh Doshi and Ak Kutty, Arun & Co., Mumbai, India, for donating Lipozyme IM 60 from Novo Nordisk.

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[Received October 29, 2001; accepted April 2, 2002]