Parameters Affecting Diacylglycerol Formation During the Production of Specific-Structured Lipids by Lipase-Catalyzed Interesterification

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ABSTRACT: Diacylglycerols (DAG) are important intermediates in lipase-catalyzed interesterification, but a high DAG concentration in the reaction mixture results in a high DAG content in the final product. We have previously shown that a high DAG concentration in the reaction mixture increases the degree of acyl migration, thus adding to the formation of by-products. In the present study we examined the influence of water content, reaction temperature, enzyme load, substrate molar ratio (oil/capric acid), and reaction time on the formation of DAG in batch reactors. We used response surface methodology (RSM) to minimize the numbers of experiments. The DAG content of the product was dependent on all parameters examined except reaction time. DAG formation increased with increasing water content, enzyme load, reaction temperature, and substrate ratio. The content of *sn*-1,3-DAG was higher than that of *sn*-1,2-DAG under all conditions tested, and the ratio between the contents of the former compounds and the latter increased with increasing temperature and reaction time. The water content, enzyme load, and substrate ratio had no significant effect on this ratio. The DAG content was positively correlated with both the incorporation of acyl donors and the degree of acyl migration.

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KEY WORDS: Acidolysis, acyl migration, batch, diacylglycerols, lipase-catalyzed interesterification, rapeseed oil, response surface methodology, *Rhizomucor miehei*, specific-structured lipids.

The applications of lipases in the oil and fat industry are associated with several attractive features, including (i) efficacy of lipases under mild reaction conditions; (ii) catalysis of specific reactions; (iii) utility in "natural" reaction systems and products; (iv) reduced environmental pollution; (v) availability of lipases from a wide range of sources; (vi) ability to improve lipases by genetic engineering; and in special situations (vii) the use of lipases is the optimal method for the production of particular biomolecules. For these reasons, many nutritional and functional lipids have been produced enzymatically and a great many reports of these studies published over the past 20 yr. A few reviews have been published recently (1–3). One of the exciting applications of lipases is in the production of cocoa butter-like fats, a process currently performed on plant scale (4–6). Another promising application is the production of milk fat substitutes for infant milk formulas, such as Betapol, produced by Unilever (7). Specific-structured lipids (SSL) are triacylglycerols containing both long-chain fatty acids (mostly essential fatty acids), which are located specifically at the *sn*-2 position, and medium-chain fatty acids (capric acid in this paper), which are located specifically at the *sn*-1,3 positions of the glycerol backbone. The nutritional applications of SSL have recently attracted attention (8–10), resulting in increased interest in the production of these fats by lipase-catalyzed interesterification (11–13).

Lipase-catalyzed interesterification (acidolysis) is a two-step reaction involving hydrolysis and esterification (11), as depicted in Figure 1. The diacylglycerols (DAG) produced in the first step are reactants in the second step. The amount of DAG in the reaction mixture therefore affects the overall reaction rate. However, DAG also cause acyl migration or the formation of by-products (13), and as a consequence, the formation of DAG decreases the yield and purity of SSL.

For the reaction between olive oil and myristic acid in hexane saturated with water, a DAG content of 6–7% in the final products has been reported (14–15). The content of DAG was a function of the water content (16–18), reaction time (18), and other reaction parameters (19) in solvent or solvent-free media. In previous publications there has been a general agreement that a higher water content in the reaction mixture would result in more DAG, but for the other parameters, the conclusions have been either contradictory or unclear.

In this study, the contents of DAG and the ratio between *sn*-1,3- DAG and *sn*-1,2(2,3)-DAG (DAG ratio) were determined for the reaction between rapeseed oil and capric acid using a solvent-free medium. The parameters examined for their effect on DAG content were water content (W_c), reaction temperature (T_e), enzyme load (E_l), reaction time (T_r), and substrate molar ratio (oil/capric acid) (S_r). The effects of DAG on the final incorporation of capric acid into rapeseed oil and the degree of acyl migration are also discussed.

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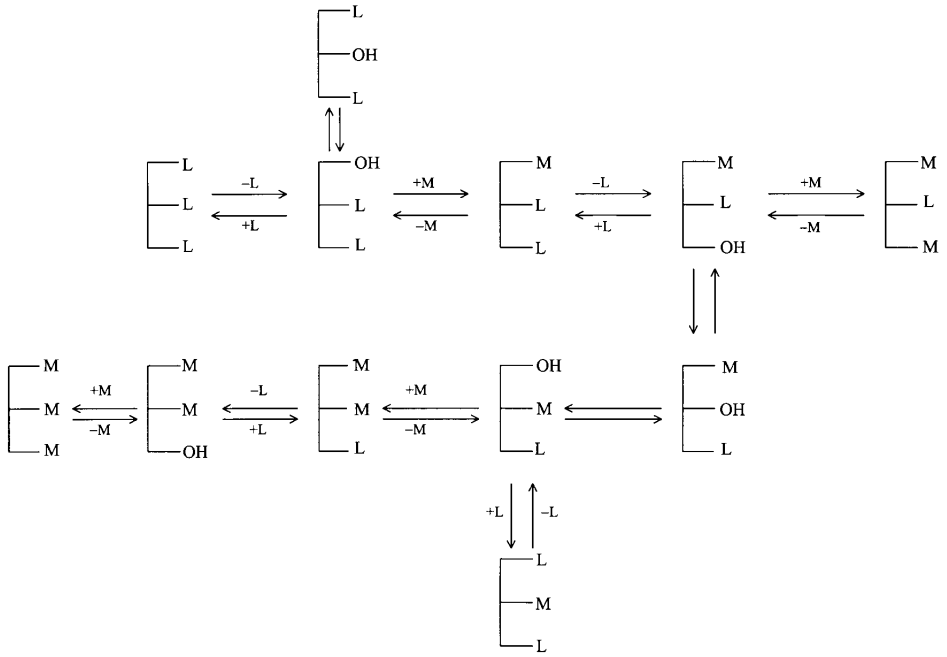

FIG. 1. Diagram of reaction and principle of the lipase-catalyzed interesterification (acidolysis) and side reactions for the production of specific-structured lipids. L, long-chain fatty acids; M, medium-chain fatty acids.

MATERIALS AND METHODS

Materials. Refined rapeseed oil was from Aarhus Oliefabrik A/S (Aarhus, Denmark). The fatty acid composition of the rapeseed oil (mol%) was the following: $C_{16:0}$, 6.0; $C_{16:1}$, 0.2; $C_{18:0}$, 1.6; $C_{18:1n-9}$, 58.8; $C_{18:2n-6}$, 21.9; $C_{18:3n-3}$, 10.0; and $C_{20:1n-9}$, 0.6. Capric acid was purchased from Henkel Kimianika Sdn. Bhd., Selangor, Malaysia (purity 99.6 mol%). *sn*-1,2-Diolein was from Sigma (St. Louis, MO). Lipozyme IM, a commercially immobilized 1,3-specific lipase from the strain *Rhizomucor miehei* (water content 2.3 wt%, measured by the Karl Fischer titration method in our laboratory), was donated from Novo Nordisk A/S (Bagsvaerd, Denmark). All solvents and reagents for analyses were chromatographic or analytical grade.

Experimental design. A fractional factorial design based on the principle of response surface methodology (RSM) was used in this work (20). Using five factors, 30 experimental settings were generated. The five factors were W_c (based on Lipozyme IM), E_l (based on whole substrates), S_r (molar ratio oil/capric acid), T_r, and T_e. The levels of each variable were chosen from previous work (11) as well as recommendations from the manufacturer of the lipase (see *Use of Immobilized Lipases for Interesterification Reactions and Ester Synthesis*, Application sheet, Enzyme business, Novo Nordisk, 1992). The water content of the enzyme and substrates was recalculated as the weight percentages of water in the Lipozyme IM. The ranges of settings for the factors were the following: W_c, 3.25–10.25 wt%; T_e, 30–70°C; E_l, 1–17%; T_r, 10–30 h; and S_r, 0.125–0.292 mol/mol. The average values of the factor settings were 6.75%, 50°C, 9%, 20 h, and 0.208 mol/mol, respectively.

Interesterification 1. The interesterification (acidolysis) between rapeseed oil and capric acid by Lipozyme IM was carried out in a system previously described (13). The enzyme was prepared for the experiments by adding water to the required content and conditioning at 5°C for 12 h. Substrates were preheated at 5°C above the experimental temperature. Preheated substrates (20 g total) were added to the enzyme. The reaction was started after a glass bead was added and the flask tightly closed and stirred by shaking (200 rpm) on a water bath with constant temperature. Following the reaction, the enzyme was removed by filtering and the sample was stored at –40°C.

Interesterification 2. This series of experiments involved lipase-catalyzed reaction (acidolysis) between rapeseed oil and capric acid in a batch reactor. The Lipozyme IM was used directly without further adjustment. The W_c was 3.0 wt%, including the water in the substrates. The other reaction parameters were T_e, 60°C; stirring, 230 rpm; S_r, 1:6 (rapeseed oil/capric acid, mol/mol); and E_l, 5 wt% calculation based on the total substrate (both rapeseed oil and capric acid). One kilogram of the substrates (total) was used for the experiment.

DAG analysis by high-pressure liquid chromatograph (HPLC). An HPLC (Jasco Corporation, Tokyo, Japan) with mini-bore silica column (l = 10 cm, i.d. = 2.1 mm, particle size = 5 μm, Hewlett Packard, Wilmington, DE) was used in the determination of DAG content. The instrument was equipped with two PU-980 pumps, an HG-980-30 solvent mixing module, and an AS-950 autosampler. A Sedex 55 evaporative light-scattering detector (Sedere, Alfortville, France) was set at 40°C. A binary solvent system of heptane and heptane/tetrahydrofuran/acetic acid (80:20:1, vol/vol/vol)

was used at a flow rate of 0.5–1.0 mL/min. Peak areas were calibrated by standard samples of *sn*-1,3- and *sn*-1,2-dipalmitoyl-glycerols. The content of DAG was initially measured as the weight percentage (DAG_t) based on the total mixtures after reaction and recalculated into the weight percentages of acylglycerols in the mixtures according to the substrate ratios and the incorporation of capric acid. The first step was to calculate the average molecular weight of the structured acylglycerols (MW_{SL}) based on the total incorporation of acyl donors (I_f) into the oil (the molecular weights of acyl donors and the oil are defined as MW_{FFA} and MW_{OIL}), that is:

$$MW_{SL} = 3 \times \{I_f \times MW_{FFA} + (100 - I_f) \times MW_{OIL} - 38)/3\} + 38 \quad [1]$$

The second step was to calculate the weight substrate ratios (S_w) according to the molar substrate ratios (S_r), that is:

$$S_w = S_r \times WM_{OIL}/WM_{FFA} \quad [2]$$

The contents of DAG based on the structured acylglycerols (DAG) were then calculated by the following equation:

$$DAG\ (\%) = (DAG_t/S_w)/(MW_{SL}/MW_{OIL}) \quad [3]$$

Grignard degradation, methylation, and gas chromatography. The methods and procedures were the same as those in our previous work (13).

Statistical analysis. The data were analyzed by means of RSM with commercial software (Modde 4.0, Umetri, Umeå, Sweden). Second-order coefficients were generated by regression analysis with backward elimination. Responses were fitted with the factors by multiple regression, and the fit of the model was evaluated by reference to the coefficient of determination (R^2) and analysis of variance. For process factors, the main effect plot displays the predicted change in the response when the factor varies from its low to its high level, all other factors in the design being set on their average.

RESULTS AND DISCUSSION

Main effects of parameters on DAG content. Several reports have included data on the influence of water content on the production of DAG during interesterification. Macrae (16) used a continuous enzyme bed reactor with light petroleum as the solvent and found that an increase in W_c of the feedstream from 0 to 100% saturation increased the DAG content in the product from less than 1.0–3.7%. The interesterification activity was increased from 0.1 to 3.4 g TG/h/g catalyst. Shimada *et al.* (17) used a batch reactor without solvent for the production of structured lipids and found that an increase in W_c from 0 to 100% (w/w) based on the immobilized lipase increased the DAG content from 6 to 25%. The incorporation of caprylic acid increased from 0.4 to 28.9%. Luck and Bauer (18) investigated the influences of residence time on the content of DAG in an enzyme bed reactor with a solvent-free medium. The content of DAG first increased and then reached equilibrium after about 1 d. Bloomer *et al.* (19) observed a similar phenomenon with changes in W_c and T_r in a reaction between triolein and palmitic acid. The content of DAG in the product increased with increasing T_e and decreased with larger E_l and smaller S_r. Our present findings on the effects of W_c, T_e, and S_r are in agreement with the conclusions made in the studies cited above. However, our results on the effects of E_l and T_r are not consistent with these conclusions. Figure 2A shows that W_c and E_l had the most significant effects on the DAG content and were positively correlated. Since the enzyme used was adjusted with water, the use of more enzyme involved the addition of more water to the system and accelerated the hydrolysis step. Increase in T_e and S_r also increased DAG formation, but to a smaller extent. The effects of W_c, E_l, T_e, and S_r on DAG content are further explained by the plots of main effects (Fig. 3). T_r was the least significant parameter, implying that the content of DAG varied little if T_r in the batch reactor was increased. This conclusion was given further weight by a separate experiment, in which DAG content was shown to be relatively stable during the time course (Fig. 4). The equilibrium between hydrolysis and esterification can be reached in a very short time because the esterification step

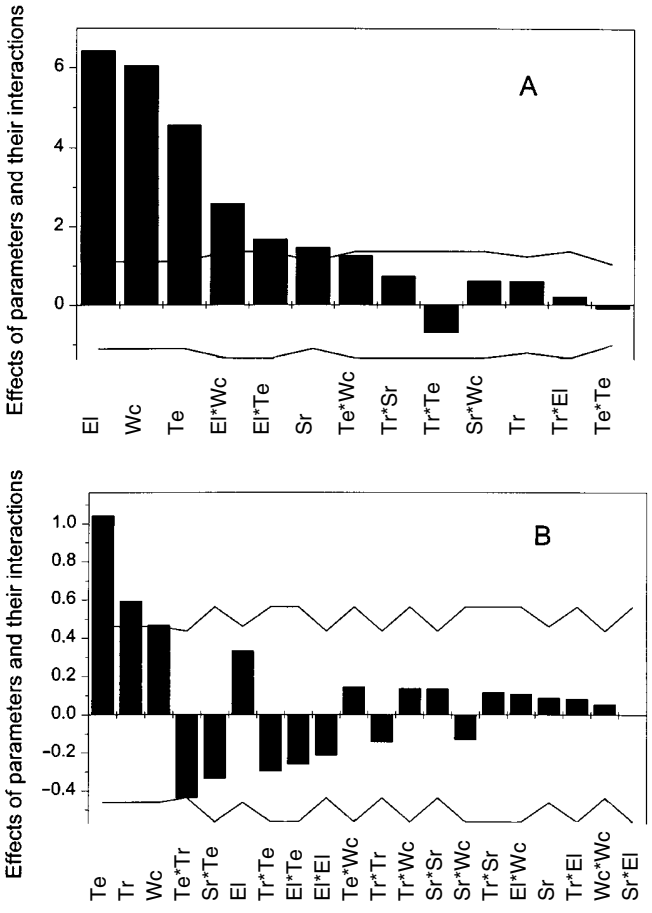

FIG. 2. Effects of parameters and their interactions for (A) DAG content and (B) DAG ratio [the ratio between *sn*-1,3-DAG and *sn*-1,2(2,3)-DAG)]. Abbreviations: DAG, diacylglycerols; T_r = reaction time; S_r = substrate ratio; E_l = enzyme load; T_e = reaction temperature; W_c = water content.

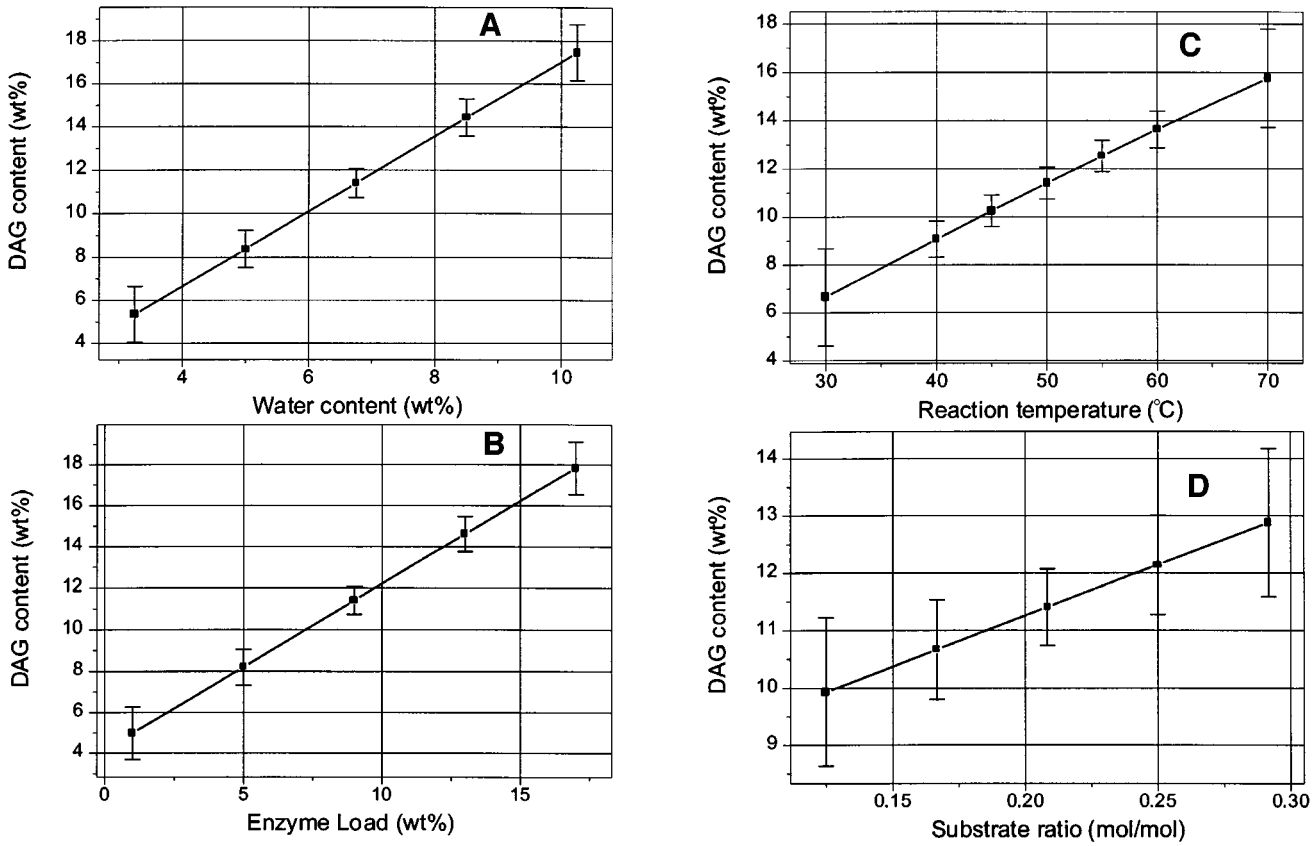

FIG. 3. Main effects of significant parameters for DAG content. (A) Water content; (B) enzyme load; (C) temperature; (D) substrate ratio. When examining each factor as the main effect, all other factors were set to their average values. The average values for the five factors were W_c, 6.75 wt%; T_e, 50°C; E_l, 9%; T_r, 20 h; and S_r, 0.208 mol/mol. For abbreviation see Figure 2.

is much faster than the hydrolysis step (12). Therefore, DAG will be quickly converted to new triacylglycerols, allowing the content of DAG to remain constant.

Main effects of parameters on the ratio between sn-*1,3-DAG and* sn-*1,2(2,3)-DAG.* Only one report has so far addressed the effects of varying reaction parameters on the ratio between *sn*-1,3-DAG and *sn*-1,2(2,3)-DAG (DAG ratio) during lipase-catalyzed interesterification (18). Luck and Bauer (18) described the effect of residence time on the levels of two DAG isomers. The content of *sn*-1,3-DAG increased with higher T_r and stabilized after about 24 h. The content of *sn*-1,2(2,3)-DAG increased during the first hour and decreased thereafter. The DAG ratio at equilibrium was 4. The transition from *sn*-1,2(2,3)-DAG to *sn*-1,3-DAG was closely related to the degree of acyl migration (13). The DAG ratio is therefore useful when choosing reaction parameters to minimize acyl migration.

The parameter that most significantly affected the DAG ratio in the present study was T_e (Fig. 2B). The second most important parameter was T_r, the results being consistent with those in a previous report (18). The main effects of T_e and T_r on the DAG ratio can be seen in Figure 5. The effect of W_c on the DAG ratio was only marginally greater than the error range (Fig. 2B), and therefore not very significant despite the crucial role of water in the process (21–23). The effects of E_l and S_r on the DAG ratio are not significant (Fig. 2B).

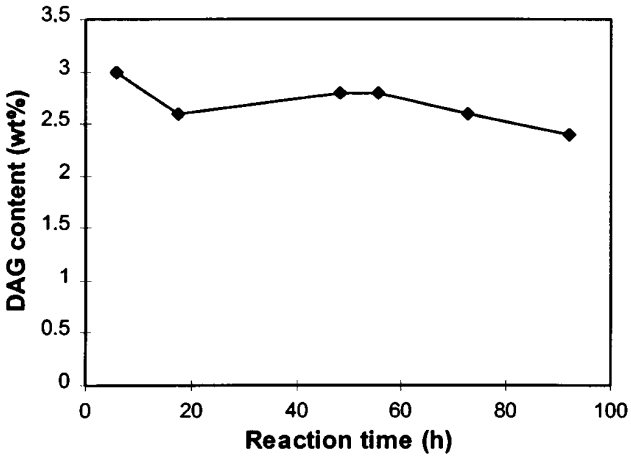

FIG. 4. The time course of DAG content in the lipase-catalyzed interesterification between rapeseed oil and capric acid. For conditions see Interesterification 2 in the Materials and Methods section. For abbreviation see Figure 2.

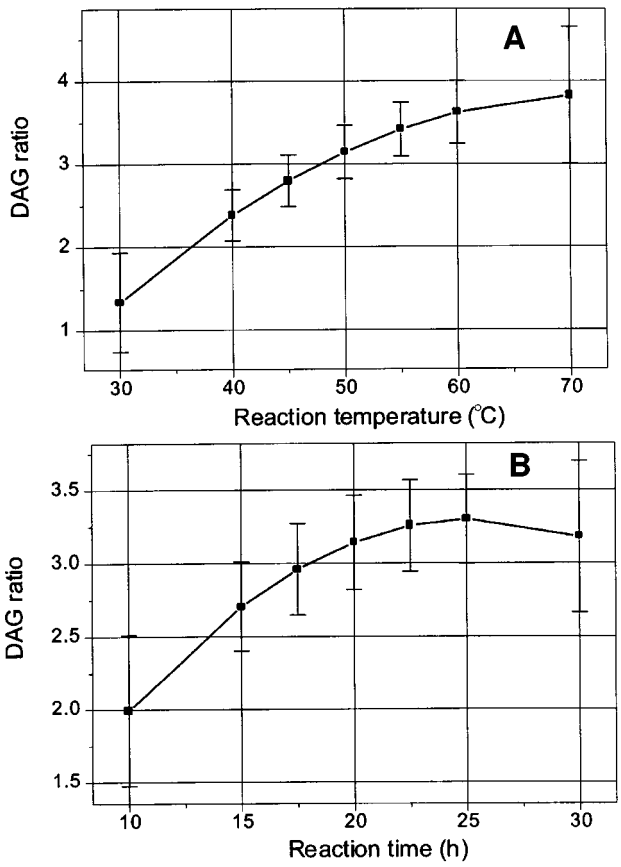

FIG. 5. Main effects of significant parameters for DAG ratio [the ratio between *sn*-1,3-DAG and *sn*-1,2(2,3)-DAG]. (A) Temperature; (B) reaction time. All fixed factors in each situation were set on their average as given in Figure 3. For abbreviation see Figure 2.

Relationship between DAG and incorporation. The increase in DAG during lipase-catalyzed interesterification correlates with the increase in interesterification activity and incorporation of acyl donors (16,17,19). We examined the effects of DAG by adding 3 wt% of *sn*-1,2-diolein to the reaction mixture at the start of the interesterification at similar conditions to those described in Interesterification 2 (see Materials and Methods section; 5 g of the substrates was used in this experiment instead of 1 kg, and a down-scaled reactor was used). The initial interesterification activity (calculated as the incorporation rates of capric acid over the first half hour) increased from 1.9 to 4.4 mol%. This is consistent with DAG being the intermediates between the hydrolysis and the esterification reactions (Fig. 1) and the esterification step being faster than the hydrolysis step in the system used (11). When evaluating the main effects of the varied parameters on both incorporation (24) and DAG formation, we found that the influences of increasing W_c, E_l, and T_e on both the incorporation of acyl donors into *sn*-1,3 positions and on DAG formation were similar. For the other two reaction parameters, T_r and S_r, however, the influences were to the contrary. Longer T_r resulted in higher incorporation (24), whereas the change of the DAG content was not significant (Fig. 2A). The

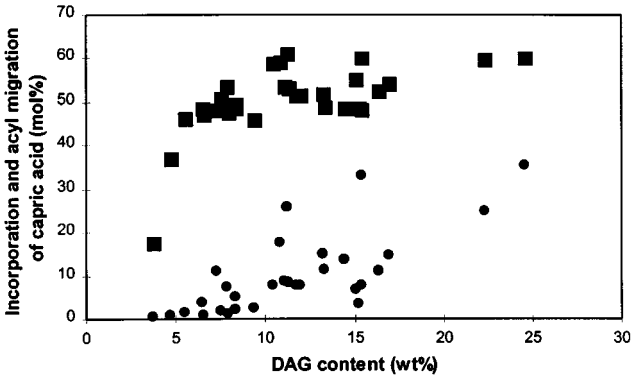

FIG. 6. Correlation between DAG content and incorporation (■) and acyl migration (●) in varying parameters. For abbreviation see Figure 2.

incorporation of acyl donors needs a longer time to reach equilibrium than does the hydrolysis to produce DAG. A lower S_r (more free fatty acids in the substrates) raised the equilibrium level of the reaction, giving higher incorporation, and inhibited the hydrolysis and accelerated the esterification, giving a lower DAG formation (Fig. 3D).

As depicted in Figure 1, reaction equilibria exist not only between substrates and products but also between DAG and byproducts, DAG and substrates, and DAG and products. The reaction equilibria are controlled by both kinetics and thermodynamics. These dynamic equilibria relate to different process parameters, such as W_c, T_e, E_l, T_r, and S_r. To reduce the DAG content in the final products and still retain a high reaction rate and yield, a small increase in water content is necessary at the start of the reaction in addition to the water bound in the lipase. However, the content of water should be reduced gradually when the equilibrium of the reaction is reached. The correlation between DAG content and total incorporation and acyl migration of capric acid was set up with varying parameters (Fig. 6). Total incorporation initially increased and stabilized thereafter. This phenomenon probably depended on the function of water in the present system. If thermodynamic water activity, rather than W_c, was examined, a different relationship might have been observed. Acyl migration correlated with the formation of DAG

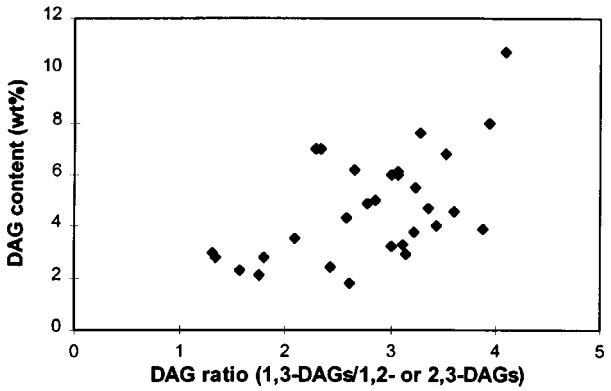

FIG. 7. Correlation between DAG content and DAG ratio (the ratio between *sn*-1,3-DAG and *sn*-1,2(2,3)-DAG) in varying parameters. For abbreviation see Figure 2.

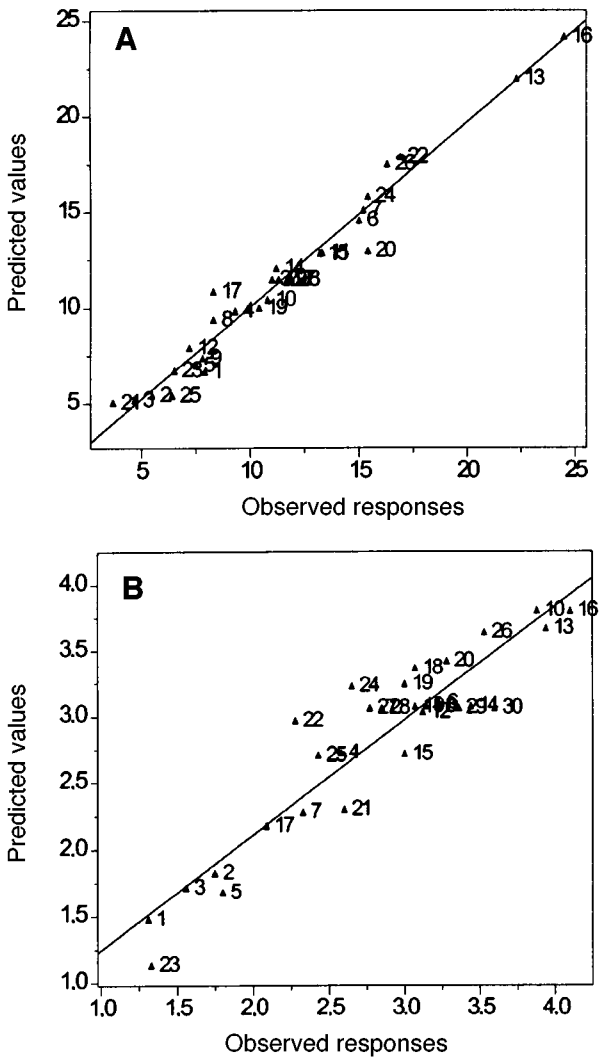

FIG. 8. The linear relationships between the observed responses and those predicted for (A) DAG content and (B) DAG ratio (the ratio between *sn*-1,3-DAG and *sn*-1,2(2,3)-DAG). For abbreviation see Figure 2.

(Fig. 6) as discussed previously (13). The content of DAG in the final products was also correlated with the DAG ratio (Fig. 7), because *sn*-1,3-DAG were not further esterified by Lipozyme IM (since it is an *sn*-1,3-specific lipase). Therefore, the temperature should be as low as possible in order to reduce the DAG ratio (Fig. 5A) if no other responses are considered.

Model fitting. It is not important to model DAG formation and the DAG ratio because of the contradictory roles of DAG during the lipase-catalyzed interesterification. It is difficult to determine whether more or less DAG will be favorable during the reaction before the final optimization of the product yield is made. However, it is necessary to know the goodness-of-fit of all the results obtained in the experiments. The quadratic models were fitted with the responses by multiple regression. The coefficients of determination (R^2) of the models are 0.972 and 0.864 for the two responses, i.e., DAG content and DAG ratio. Predicted results for the DAG formation were well correlated in linearity with the observed results (R^2 = .986), and a linear relationship between the predicted and the observed (R^2 = .929) was also obtained for DAG ratio (Fig. 8). This indicates that the models generally represent the real relationships between the responses and the reaction parameters.

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