Optimization of Reaction Conditions for the Production of DAG Using Immobilized 1,3-Regiospecific Lipase Lipozyme RM IM

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ABSTRACT: Oils with a high DAG (1,3-DAG) content have attracted considerable attention as a healthful food oil component. In this study, we report on the synthesis of 1,3-DAG from a mixture of FA, constituted largely of oleic and linoleic acids, using an immobilized 1,3-regioselective lipase from *Rhizomucor miehei* in a solvent-free system. The kinetics of 1,3-DAG production from FA and glycerol were investigated on the basis of a simplified model, taking into consideration the acyl migration reaction, the removal of water, and glycerol dissolution in the oil phase in addition to the esterification reactions. Both the yield of 1,3-DAG and the purity of DAG were evaluated under a variety of experimental conditions, including reaction temperature, pressure, and amount of enzyme present. When either the reaction temperature or the amount of enzyme used was increased, the 1,3-DAG production rate increased, but yield remained relatively constant. The 1,3-DAG yield as well as the purity of DAG gradually decreased because of the enhancement of acyl migration at later stages of the reaction after the 1,3-DAG concentration reached a maximum. Vacuum was important for attaining high yields of 1,3-DAG. Under conditions of a high vacuum (1 mm Hg) at 50°C, 1.09 M 1,3-DAG was produced from 1.29 M glycerol and 2.59 M FA in an 84% yield and in 90% purity.

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KEY WORDS: Acyl migration, diacylglycerol, esterification, immobilized lipase, kinetics, 1,3-regioselective lipase, water removal.

DAG occurs naturally as a minor component of edible fats and oils from various sources (1). DAG has been utilized as a cocoa butter blooming agent (2) and as an intermediate in the synthesis of structural lipids (3,4). Recent studies on the nutritional properties and dietary effects of DAG (5–8) have revealed that DAG, of which 1,3-DAG is a major component, in contrast to TAG, had the ability to reduce serum TAG concentrations (5) and, as a result, to decrease both body weight and visceral fat mass (6).

DAG structures can have two configurations, namely, 1,3-DAG and 1,2(2,3)-DAG. In natural oil, 1,3-DAG constitutes approximately 70% of the total DAG. Similarly, in commercial oils with a high DAG content, 1,3-DAG reaches approximately 70% of total DAG as a result of the equilibration during the refining process and storage following the synthesis process.

1,3-DAG can be synthesized chemically from fats and oils using glycerol *via* a chemical glycerolysis reaction at temperatures in excess of 200°C using an alkaline catalyst (9). However, the yield and purity are low in this method, making it unsuitable for industrial production. Numerous investigators have studied the production of 1,3-DAG *via* a biochemical reaction using a lipase. A method for producing 1,3-DAG by the hydrolysis of triolein using lipase was reported (10), but the yield was only 43% since a large amount of MAG was formed by a side reaction. In producing 1,3-DAG by the enzymatic glycerolysis of beef tallow, Yamane *et al*. (11), achieved a yield of around 90% by lowering the reaction temperature from 60°C (where tallow is a liquid) to 48°C (where tallow is a solid). However, their method is too time-consuming for application to the industrial production of 1,3-DAG. Berger *et al*. (12) reported on the production of 1,3-DAG by esterification using FA and glycerol in an organic solvent containing a trace amount of water, under which conditions the equilibrium of the esterification reaction is shifted in favor of the formation of 1,3-DAG. However, an esterification reaction in an organic solvent also is unsuitable for industrial production since the recovery of organic solvent is costly and time-consuming. Furthermore, in the method of Berger *et al*., glycerol must be adsorbed on silica gel before starting the reaction so as to achieve high yield and good rates, which is also unsuitable for large-scale industrial production.

As an alternative to using an organic solvent, Rosu *et al*. (13) synthesized 1,3-DAG by esterification of FA and glycerol in a solvent-free system using 1,3-regioselective lipases with the simultaneous removal of water. They achieved a 1,3-DAG yield of 84.6% with a purity of 96% when caprylic acid was used as the source of FA. Thus, this method has some potential for the production of high-purity 1,3-DAG on an industrial scale. However, the reaction conditions were not optimized in their study.

The goal of this study was to determine suitable conditions for the efficient production of 1,3-DAG from FA prepared from natural edible soybean oil and rapeseed oil and glycerol in a solvent-free system using an immobilized 1,3-regioselec-

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tive lipase. The effects of various reaction conditions on the yield of 1,3-DAG and the purity of DAG were examined. Analyses also were performed on the basis of a simplified kinetic model, to simulate the course of 1,3-DAG synthesis with the aim of its use in industrial production.

EXPERIMENTAL PROCEDURES

Materials. Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on macroporous anion exchange resins) was supplied by Novozymes Industry (Bagsvaerd, Denmark). Glycerol, *n*-hexane, and silylation reagents were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan), and oleic acid and linoleic acid were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Monoolein, diolein, and triolein were purchased from Sigma Chemical Co. (St. Louis, MO). To prepare a FA substrate, soybean oil and rapeseed oil were first subjected to countercurrent hydrolysis with steam at 250°C and 50 mm Hg. Then the hydrolysate was gradually cooled to crystallize saturated FA, and the uncrystallized liquid fraction was collected as FA. The resulting FA preparation was mainly composed of oleic acid (18:1) and linoleic acid (18:2) with a small amount of TAG (1.2%), DAG (3.7%), and MAG (2.3%) as shown in Table 1.

Esterification reaction. The esterification of FA with glycerol was performed in a 1-L four-neck round-bottomed flask. FA was added to the immobilized enzyme in the flask and gently agitated at 125 rpm for 10 min. Then, the agitation rate was increased to 450 rpm and the glycerol was added quickly. The standard reaction mixture consisted of 434 g of FA, 66 g of glycerol (molar ratio of FA to glycerol = 2.0), and 25 g of Lipozyme RM IM (5% on a dry weight basis). During the reaction the mixture was agitated at 450 rpm by an impeller at 50°C, and water removal was performed by maintaining the pressure at 3 mm Hg by means of a vacuum pump with a vacuum controller. At appropriate time intervals a 2-mL aliquot was withdrawn, and the immobilized enzyme resins in the sample were quickly removed by centrifugation at 1,000 × *g* for 5 min. The concentrations of glyceride, FA, and water in the sample mixture were analyzed by using the methods described below. The esterification of FA with MAG in *n*-hexane also was performed in a magnetically stirred screw-capped 100-mL Erlenmeyer flask at 50°C to determine the substrate concentration dependency on the initial rate. MAG was used instead of glycerol since glycerol is nearly insoluble in *n*-hexane. The reaction was initiated by adding 2 g of Lipozyme RM IM to 50 mL of *n*-hexane containing FA and monoolein at different concentrations. The initial reaction rate was calculated based on the amount of FA consumed during the reaction.

TABLE 1
FA Composition of FA Substrate Used in This Study

FA component	Content (wt%)	FA component	Content (wt%)
Palmitic acid (16:0)	3.1	Arachidic acid (20:0)	0.7
Stearic acid (18:0)	1.3	Gadoleic acid (20:1)	0.6
Oleic acid (18:1)	38.2	Behenic acid (22:0)	0.1
Linoleic acid (18:2)	7.5	Erucic acid (22:1)	0.2
Linolenic acid (18:3)	7.5		

Measurement of equilibrium constants. Twenty grams of the mixture of the substrates (2.70 g of FA, 0.10 g of glycerol, 2.70 g of 1-MAG, 0.05 g of 2-MAG, 4.50 g of 1,2(2,3)-DAG, 9.50 g of 1,3-DAG, 0.40 g of TAG) and 1 g of immobilized enzyme resins were placed in a screw-capped 50-mL Erlenmeyer flask. The flask was shaken at 150 rpm at different temperatures (40–60°C) for 7 d. At appropriate time intervals, an aliquot of about 0.5 mL of the reaction mixture was withdrawn, and the immobilized enzyme resins in the sample were removed as described above. The concentrations of all types of FA and glycerides, as well as the water content, were then analyzed by the methods described below. After 7 d of reaction, the concentration of each component was found to be nearly constant, and thus equilibrium appeared to be attained at this stage. The equilibrium constants were calculated as follows:

$$K_1 = k_1/k_2 = ([H_2O][1\text{-MAG}])/([FA][\text{glycerol}]_{OIL}) \quad [1]$$

$$K_2 = k_3/k_4 = ([H_2O][1,3\text{-DAG}])/([FA][1\text{-MAG}]) \quad [2]$$

$$K_3 = k_5/k_6 = [1,2(2,3)\text{-DAG}]/[1,3\text{-DAG}] \quad [3]$$

$$K_4 = k_7/k_8 = ([H_2O][TAG])/([FA][1,2(2,3)\text{-DAG}]) \quad [4]$$

$$K_5 = k_{11}/k_{12} = [2\text{-MAG}]/[1\text{-DAG}] \quad [5]$$

where K_1–K_5 are the equilibrium constants, k_1–k_{12} the rate constants in the reaction scheme shown in Scheme 1, and [ ] denotes the respective concentrations.

Analytical methods. FA concentrations were determined by the AOCS official method (14). Namely, an aliquot (about 10 mg) of the oil sample containing 1-MAG, 2-MAG, 1,2(2,3)-DAG, 1,3-DAG, TAG, and glycerol was silylated by the addition of 0.5 mL of silylation reagent according to the AOCS official method (15). This mixture was allowed to stand for 15 min at 70°C, and then 1.5 mL distilled water was added. The resulting mixture was extracted into 1.5 mL of *n*-hexane. The silylated sample (1 µL), dissolved in *n*-hexane, was analyzed using a gas chromatograph equipped with an FID detector and a DB-1HT capillary column (7 m, 0.25 mm I.D., film thickness 0.1 µm; J&W Scientific, Folsom, CA). Helium was used as a carrier gas at a flow rate of 2.45 mL/min. Both the injector and detector temperatures were 350°C. The temperature program was as follows: initial temperature of 80°C and then heating to 340°C at 10°C/min. The final temperature was 340°C and was held for 15 min. Monoolein, diolein, and triolein were used as standards for MAG, DAG, and TAG, respectively.

The water content was measured by the Karl-Fischer titration method using an AQ-7 aquacounter (Hiranuma Co. Ltd., Mito, Japan).

Purity of DAG. The oil produced in the esterification reaction contains unreacted glycerol, FA, MAG, DAG, and TAG. To increase the DAG content in the product, it is necessary to reduce the contents of glycerol, FA, MAG, and TAG. Al-

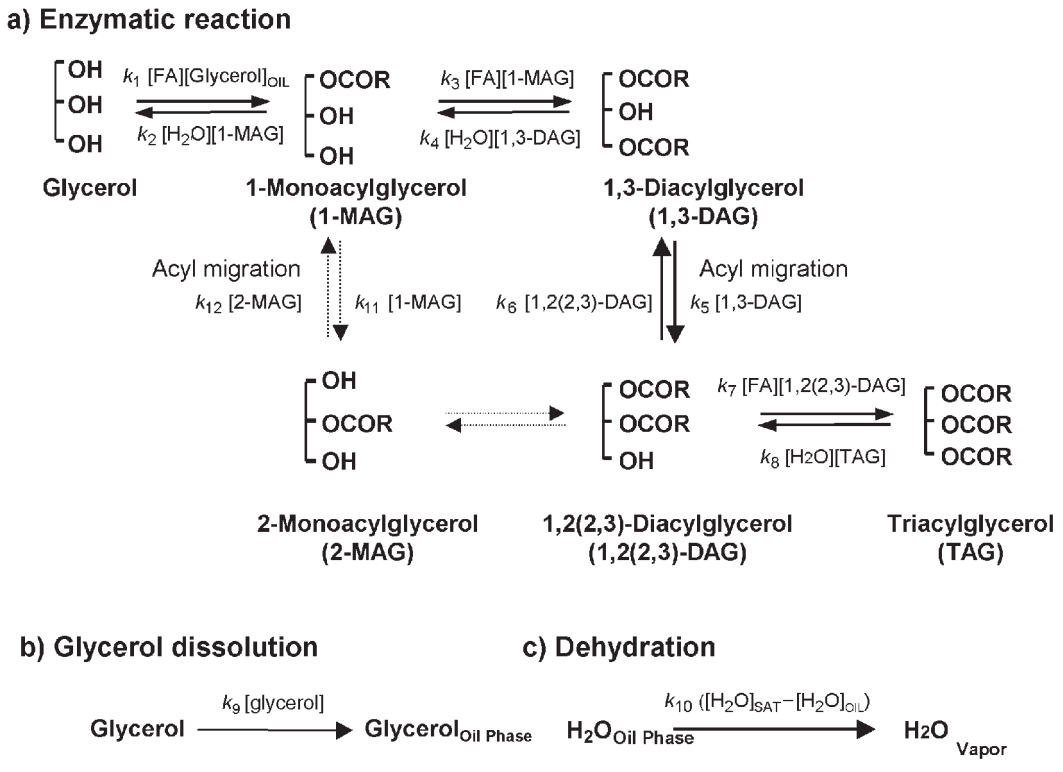
though glycerol, FA, and MAG could easily be separated from the product oil by distillation, the separation of TAG from the product oil is difficult because of the small vapor pressure difference between TAG and DAG. Therefore, TAG produced during the esterification reaction is considered to remain in the final product oil even after distillation. Thus, the DAG purity was defined as Equation 6 in this study, taking into consideration the DAG content after distillation:

$$\text{DAG purity (\%)} = \text{DAG (\%)}/[\text{DAG (\%)} + \text{TAG (\%)}] \times 100 \quad [6]$$

RESULTS AND DISCUSSION

For modeling the kinetic behaviors of 1,3-DAG production by immobilized lipase in a solvent-free system, we adopted the synthetic scheme shown in Scheme 1 on the basis of the model for triolein synthesis proposed by Lortie *et al*. (16). In this scheme, the rate of glycerol dissolution into the oil phase and rate of water removal from the oil phase, in addition to the rates of the enzymatic reactions, were taken into consideration. Some assumptions were made in the formulation of the rate equations. First, the rate of synthesis of 2-MAG from FA and glycerol was assumed to be negligible (as indicated by the dotted arrows for this reaction) since an enzyme with a high 1,3-selectivity was used. In fact, 2-MAG produced during the esterification was found experimentally to be very small. Acyl migration from 1-MAG to 2-MAG was not considered since the equilibrium constant for 1-MAG/2-MAG was five times lower than those for the rest of the reactions. Generally, it is considered that the lipase reaction occurs at the oil/water interface. In the reaction using the immobilized enzyme, the esterification of FA with glycerol would occur not in the glycerol phase but in the oil (FA) phase. That is, the esterification reaction did not take place when the immobilized enzyme resins first came into contact with glycerol followed by FA. In this case, aggregation of the immobilized enzyme resins was observed, which might be partly responsible for the failure of the reaction. On the other hand, when the immobilized enzyme resins initially came into contact with FA followed by glycerol, a reaction occurred. At an early stage of the esterification reaction, the substrate solution separated into two phases. With the progress of the reaction, glycerol gradually dissolved in the oil phase and the substrate solution became homogeneous. Because of this, we took into consideration the dissolution rate of glycerol into the oil phase, as shown in Scheme 1.

The esterification of FA with glycerol includes various reaction steps with several substrates, as shown in Scheme 1, and thus the derivation of kinetic equations that consider the overall reaction would be complicated. To approximate the esterification reaction rates using the simplified equations, the substrate concentration dependencies of the initial reaction rates were measured in *n*-hexane. The initial reaction was proportional to the MAG concentration in the concentration range of 0.125 to 0.5 M for a constant 1 M FA concentration. It was also proportional to the FA concentration in the concentration range of 0.125 to 1 M at a constant 0.5 M MAG concentration. The FA substrate used for the reaction was composed of several types of FA, mainly oleic acid (18:1) and



SCHEME 1

linoleic acid (18:2) as shown in Table 1. The initial reaction rates measured in *n*-hexane using oleic acid, linoleic acid, and the FA substrate did not differ by more than 5% (data not shown). Thus the FA substrate was treated as a single component in the kinetic study. On the basis of these findings, we assumed that the esterification reaction rates using the immobilized lipase would follow first-order kinetics with respect to the concentration of each substrate involved in the respective reaction step as shown in Equations 7–10 (see below).

The esterification reaction, accompanied by water removal, was carried out to increase the yield of 1,3-DAG. Thus, the rate of water removal from the oil phase was considered, as shown in Equation 12, in which the water removal rate was expressed as being proportional to the difference between the saturated and actual water contents in the oil phase. Equation 11 shows the rate of dissolution of glycerol into the oil phase as pointed out previously:

$$d[\text{1-MAG}]/dt = k_1[\text{FA}][\text{glycerol}]_{\text{OIL}} - k_2[\text{H}_2\text{O}][\text{1-MAG}] \quad [7]$$

$$d[\text{1,3-DAG}]/dt = k_3[\text{FA}][\text{1-MAG}] - k_4[\text{H}_2\text{O}][\text{1-MAG}] \quad [8]$$

$$d[\text{1,2(2,3)-DAG}]/dt = k_5[\text{1,3-DAG}] - k_6[\text{1,2(2,3)-DAG}] \quad [9]$$

$$d[\text{TAG}]/dt = k_7[\text{FA}][\text{1,2(2,3)-DAG}] - k_8[\text{HO}]\,[\text{TAG}] \quad [10]$$

$$d[\text{glycerol}]_{\text{OIL}}/dt = k_9[\text{glycerol}] \quad [11]$$

$$d[\text{H}_2\text{O}]_{\text{OIL}}/dt = k_{10}([\text{H}_2\text{O}]_{\text{SAT}} - [\text{H}_2\text{O}]_{\text{OIL}}) \quad [12]$$

To solve Equations 7–12, the rate constants k_2, k_4, k_6, and k_8 were replaced by k_1/K_1, k_3/K_2, k_5/K_3, and k_7/K_4, respectively, which include the equilibrium constants. The equilibrium constants were determined in separate experiments, as described above. The reaction rate constants were determined so as to obtain the best fit between the experimental data and results calculated by Equations 7–12 by the simplex method using the commercially available computer software Batch CAD (GSE System Inc., Columbia, MD), in which the Runge–Kutta method is used for solving the differential equations. The values of [1-MAG], [1,3-DAG], [1,2(2,3)-DAG], [TAG], and $[\text{H}_2\text{O}]_{\text{OIL}}$ at the initial reaction time were set to each concentration in the FA substrate. The values of $[\text{glycerol}]_{\text{OIL}}$ at the initial reaction time were set to zero. The reaction rate constants were determined using experimental data at different temperatures at 3 mm Hg.

Figure 1 shows typical experimental time courses for 1,3-DAG synthesis using the immobilized enzyme resins in a solvent-free system, in which the initial concentrations of FA and glycerol were 2.59 and 1.29 M, respectively. The saturated water content, which was used for calculation, was measured experimentally. The rate constants determined at 50°C are summarized in Table 2. The experimental and calculated results are in agreement. FA is consumed gradually at the early stage of the reaction, as shown in Figure 1 and 1,3-DAG simultaneously increases, reaching a maximum value of 1.05 M with a yield of 81% after 4 h of reaction. The purity of 1,3-DAG was calculated to be 96% at 2.5 h and then decreased gradually to 92% at 4 h. Rosu *et al*. (13) reported a yield of 74% with a 97% purity from linoleic acid and a yield of 61% with a 98% purity from oleic for a 12-h reaction by esterification in a solvent-free system at 25°C. The yield obtained in this study was higher than their results, and the synthetic rate was also much higher. The fact that the yield reported by Rosu *et al*. was lower than that obtained in this study is mainly ascribed to the lower reaction rate since they conducted the reaction at 25°C, much lower than the temperature used in this study. Thus, unreacted oleic acid and linoleic acid remained at 20.6 and 12.2 wt%, respectively, even after 12 h of reaction. The concentration of 2-MAG formed was negligible throughout the reaction as shown in Figure 1, indicating the very high 1,3-regioselectivity of the enzyme used in this study. On the other hand, the 1,2(2,3)-DAG and TAG concentrations begin to increase at a later stage of the reaction, when high concentrations of 1,3-DAG accumulate and then undergo partial acyl migration.

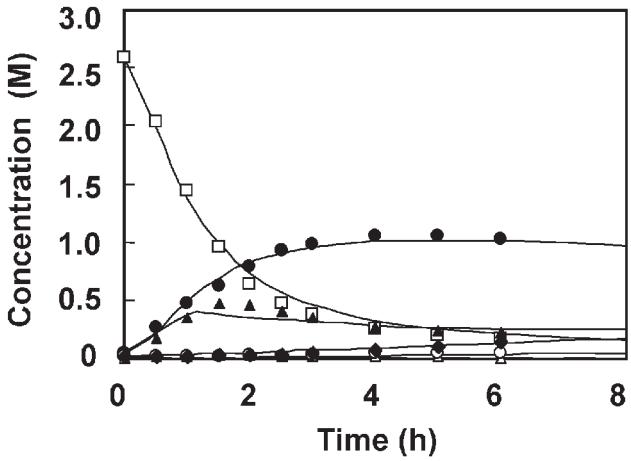


FIG. 1. Time courses of esterification reaction using Lipozyme RM IM starting with 2.59 M FA and 1.29 M glycerol. The reaction was performed at 50°C, the molar ratio of FA to glycerol was 2.0, and an immobilized enzyme resin concentration of 5% (dry weight basis) was used. Water removal was performed under 3 mm Hg vacuum. (□), FA; (▲), 1-MAG; (△), 2-MAG; (●), 1,3-DAG; (○), 1,2(2,3)-DAG; (◆), TAG. Solid lines are results calculated using Equations 7–12.

Figure 2 shows the results for an esterification reaction carried out at various temperatures (30–60°C). The solid lines show the calculated results using Equations 7–12 with the parameters determined by the same method as described previ-

TABLE 2
Values of Parameters Determined at 50°C

Equilibrium constants	$K_1 = k_1/k_2 = 1.87$
	$K_2 = k_3/k_4 = 0.41$
	$K_3 = k_5/k_6 = 0.39$
	$K_4 = k_7/k_8 = 0.64$
	$K_5 = k_{11}/k_{12} = 0.08$
Rate constants	$k_1 = 7.50 \times 10^{-4}$ (L/mol/s)
	$k_3 = 2.91 \times 10^{-4}$ (L/mol/s)
	$k_5 = 7.85 \times 10^{-6}$ (1/s)
	$k_7 = 1.84 \times 10^{-3}$ (L/mol/s)
	$k_9 = 3.59 \times 10^{-4}$ (1/s)
	$k_{10} = 1.20 \times 10^{-2}$ (1/s)

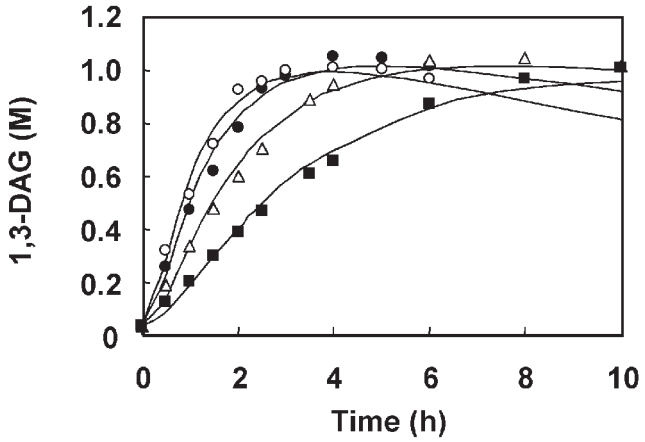


FIG. 2. Effect of temperature on 1,3-DAG synthesis. The reaction was performed at 3 mm Hg vacuum. The molar ratio of FA to glycerol was 2.0, with an immobilized enzyme resin concentration of 5% (dry weight basis). (■), 30°C; (△), 40°C; (●), 50°C; (○), 60°C. Solid lines are results calculated using Equations 7–12.

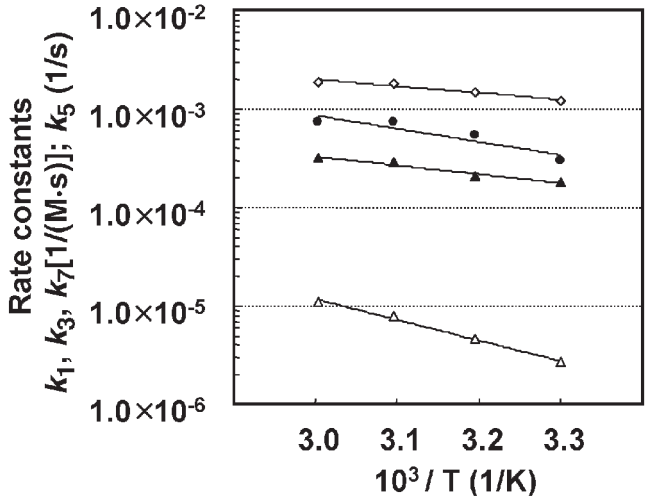


FIG. 3. Arrhenius plots for various reaction rate constants. (●), k_1; (▲), k_3; (△), k_5; (◇), k_7.

ously. The equilibrium constants (K_1, K_2, K_3, and K_4) and the saturated water content at 40 to 60°C were measured experimentally. However, the equilibrium constants at 30°C were extrapolated from those determined at 40, 50, and 60°C. Although the rate of esterification became higher with increasing reaction temperature, the maximal 1,3-DAG concentration was consistently 1 M with a yield of around 80%. The DAG purity at the maximal 1,3-DAG concentration was increased slightly with decreasing temperature, and was 90, 92, 93, and 93% at 60, 50, 40, and 30°C, respectively. With an increase in the reaction temperature, the rate of TAG production was also increased. As a result, the DAG purity began to decrease in the later stages of the reaction, reaching 93, 86, 85, and 81% at 30, 40, 50, and 60°C respectively, at the end of reaction (9 h for 30 and 40°C, 6 h for 50 and 60°C) (data not shown). On the other hand, at 30°C the DAG purity was constant at 93% even toward the end of the reaction.

Figure 3 shows the Arrhenius plots for the reaction. From the Arrhenius plots of the k_1, k_3, k_5, and k_7 values, the apparent activation energies were evaluated to be 25.5, 17.2, 40.2, and 13.0 kJ/mol, respectively. The esterification rate constants k_1 and k_7, determined at 60°C in this study, were both several times higher than the results reported by Lotie *et al.* (16). These differences are believed to be due to the fact that the immobilized lipase used in this study has a higher activity than the enzyme used in their study (Lipozyme IM-20). The temperature dependency for the acyl migration rate constant, k_5, is pronounced in comparison with those for k_1, k_3, and k_7; the reason for this is not known at present. However, the finding that the temperature dependency of the acyl migration rate is stronger than that of the 1,3-DAG synthesis rate would support the fact that the 1,3-DAG purity became higher with decreasing reaction temperatures, as described previously.

Water removal during the reaction is very important (13,17,18) since the equilibrium is shifted in favor of synthesis with a decrease in water content. Several methods for water removal are available, such as the removal of water by a stream of nitrogen, the use of water removal agents, and the use of a vacuum pump. In this study, a vacuum pump was used to remove water, as would be likely for industrial production. Figure 4 shows the effect of vacuum conditions on the esterification reaction. Solid lines show the calculated results using Equations 7–12 with the rate constants determined from the fitting, as described above. The saturated water contents at each vacuum condition were obtained experimentally and were determined to be 0.011% at 1 mm Hg, 0.032% at 3 mm Hg, 0.053% at 6 mm Hg, and 0.10% at 10 mm Hg, respectively. The maximal 1,3-DAG concentration was increased with increasing vacuum, and was 1.09, 1.05, 0.95, and 0.94 M at 1, 3, 6, and 10 mm Hg, respectively. A vacuum lower than 1 mm Hg could not be attained using the apparatus in this study. It is

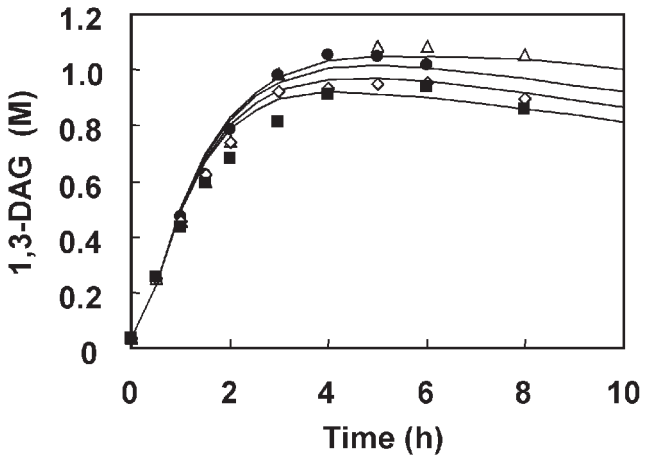


FIG. 4. Effect of vacuum on 1,3-DAG synthesis. The reaction was performed at 50°C, the molar ratio of FA to glycerol was 2.0, and an immobilized enzyme resin concentration of 5% (dry weight basis) was used. Water removal was performed under 1 mm Hg (△), 3 mm Hg (●), 6 mm Hg (◇), 10 mm Hg (■). Solid lines are results calculated using Equations 7–12.

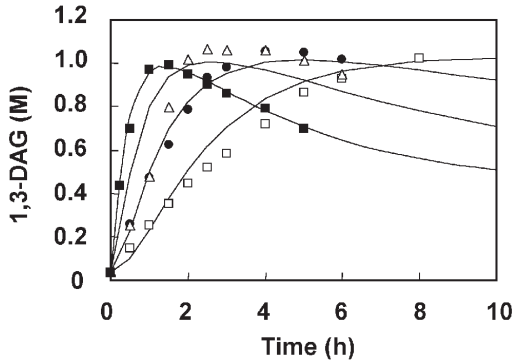


FIG. 5. Effect of enzyme load on 1,3-DAG synthesis. The reaction was performed under 3 mm Hg vacuum at 50°C, and the molar ratio of FA to glycerol was 2.0. Enzyme load: (□), 2.5%; (●), 5%; (△), 10%; (■), 20% (dry weight basis). Solid lines are results calculated using Equations 7–12.

known that a small amount of water is necessary for the lipase reaction, in order to maintain enzyme structure and to achieve catalytic activity (19,20). In the range of vacuum studied in this study, a decrease in reaction rate was not observed (Fig. 4). The water content including that in the immobilized enzyme resins was around 3% at the end of reaction at a vacuum of 3 mm Hg, which was similar to that before reaction. Thus, the amount of water essential for an enzyme reaction can be maintained during the reaction. These findings suggest that the immobilized enzyme could be used repeatedly for esterification reactions under the vacuum condition used here.

Xu *et al*. (21,22) reported that acyl migration becomes appreciable with increasing water content in an interesterification reaction. They examined acyl migration in the range of water contents in the reaction mixture higher than 3%. In this study, the water content in the reaction mixture reached higher levels when the water removal rate was decreased, but it did not exceed 0.5% at any time for any of the vacuum conditions used. The TAG production rate was increased slightly and, accordingly, the DAG purity decreased with a decrease in the water removal rate. The DAG purity was 92% at 3 mm Hg and 90% at 10 mm Hg at 4 h of reaction. The decrease in purity might be caused by acyl migration, but the degree was not large.

Mass transfer at a gas–liquid interface also may affect the water removal rate. Mass transfer might be affected by the agitation rate in a batch reaction. When the agitation was done at 150 rpm, the esterification rate for the reaction decreased in comparison with that at 450 rpm, and the maximal 1,3-DAG concentration was only 0.8 M. The purity decreased to 87%. These results strongly suggest that sufficient agitation, in addition to the low vacuum conditions, is a major factor for attaining high 1,3-DAG yield and DAG purity.

Figure 5 shows the effect of the amount of enzyme used on the esterification reaction. The solid lines show the calculated results obtained by the fitting method, described previously. As shown in Figure 5, the enzyme load barely affected the maximal 1,3-DAG concentration and yield, as expected. With a higher enzyme load, the esterification rate was increased, and the DAG purity at the maximum 1,3-DAG concentration was maintained at 92%. The DAG purity at 4 h of reaction was 95, 92, 87, and 68% for enzyme concentrations of 2.5, 5, 10, and 20%, respectively. With the higher enzyme load, TAG content was increased significantly in the later stage of reaction and, as a result, DAG purity was decreased. Thus, the reaction time should be carefully chosen so as to obtain a higher 1,3-DAG concentration. It should also be noted that the immobilized enzyme resins should be separated from the product oil as quickly as possible after the 1,3-DAG concentration reaches a maximum in order to obtain high DAG purity.

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