Continual Conversion of Free Fatty Acid in Rice Bran Oil to Triacylglycerol by Immobilized Lipase[1]

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Rice bran oil containing 30–50% free fatty acid was continually converted to an oil containing more than 75% of triacylglycerol (TG) by means of immobilized lipase. The reaction was carried out at 60°C for 24 h with dehydration and reactant mixing by dry nitrogen flow under a positive nitrogen atmosphere. Enzymatic TG synthesis with evaporation by heating was not suitable because of the increasing peroxide value of the oil.

KEY WORDS: Continual esterification, dehydration, free fatty acid, immobilized lipase, integration, lipase, peroxide value, rice bran oil, triacylglycerol.

Rice bran oil has attracted much attention as a health food because it was found to lower serum cholesterol (1). Rice bran oil contains oryzanol, tocopherol and sterol, which are thought to have biochemical activities (2). Rice bran itself contains lipase. The bran must be stabilized by heating immediately after milling to eliminate the formation of free fatty acid (FFA) in the bran. However, this task is difficult, because the bran is produced from widely dispersed hullers. Thus, the rice bran oil available in most Asian countries contains 40–50% FFA. The large quantities of soaps obtained after neutralization of FFA induce the formation of stable emulsions, leading to significant loss of oil. World paddy production is around 400 million tons/year, 6% of which is bran—and bran contains 15% oil. However, rice bran oil is not listed in 13 vegetable oils in the world oil and fat production (3). Refining of this high FFA rice bran oil would provide a potentially large and convenient new food resource for Asia (4).

Triacylglycerol (TG) has been synthesized by an enzyme reaction from diacylglycerol (DG) and FFA in palm oil (5). TG has also been synthesized from FFA in high FFA oil (6,7) or from oleic acid (8–10) and glycerol in an enzymatic one-batch reaction. In the present study, we describe a method for continual or repeated batch conversion of FFA, monoacylglycerol (MG) and DG in rice bran oil to TG by means of immobilized lipase.

EXPERIMENTAL PROCEDURES

Materials. Lipase from *Rhizomucor miehei*, immobilized on Duolite weak anionic exchange resin (Lipozyme IM 60 or Lipozyme IM 20), was donated by Novo Nordisk (Chiba, Japan). Molecular sieves, 5Å, were obtained from Wako Pure Chemicals, Ltd. (Osaka, Japan).

Assay methods. Acid value (AV), saponification value, unsaponifiable matter, peroxide value (POV) and fatty acid composition were measured by the standard oil and fat assay methods of the Japan Oil Chemists' Society (11). Glycerol was assayed as previously described (12). The FFA, MG, DG and TG contents of the rice bran oil were determined by high-performance liquid chromatography (GL Science, Tokyo, Japan) equipped with a refractive index detector (Shodex RI SE-61; Showa Denko, Tokyo, Japan) and a chromatointegrator (Hitachi D-2500; Hitachi, Tokyo, Japan). Three columns (Shodex GPC KF-802; Showa Denko) connected in series were used, and the mobile phase was tetrahydrofuran (THF) at a flow rate of 1.0 mL/min. Column temperature was 40°C, and pressure was about 40 kg/cm^2. The sample concentration was 0.5–1%, dissolved in THF, and the injection volume was 50 μL. Calibration curves were made with triolein (Merck, Darmstadt, Germany), diolein and oleic acid (Sigma Chemical Co., St. Louis, MO), and 1-monoolein (Serdary Research Laboratories, London, Ontario, Canada). The heat quantity difference to fuse or to crystallize the waxy substance was measured with a differential scanning calorimeter (Shimadzu DSC-50; Shimadzu, Kyoto, Japan). The water content was measured with a coulometric moisturemeter (Mitsubishi Kasei CA-03; Mitsubishi Kasei, Tokyo, Japan).

Preparation of substrate. Crude, high FFA rice bran oil was heated to 80°C. Then, water (4 w/w%) was added to precipitate gummy substances, which were removed by centrifugation after 15 min. The degummed oil was heated again to 80°C and allowed to cool to room temperature. Hexane was added to extract neutral lipid and FFA to provide dewaxed oil. Dewaxing was confirmed by differential scanning calorimetry (DSC). The symmetrical transition on the DSC chart for waxy substance was around 56–70°C for the crude oil. This transition was not found in dewaxed oil. The AVs of the dewaxed oil and crude rice bran oil were measured. No difference between these AVs was observed, which confirms that there was no loss of FFA during wax crystallization. The dewaxing process was not necessary for rice bran oil that was extracted at low temperature (about 10°C) because waxy substance does not extract at that temperature.

High FFA rice bran oil. The high FFA rice bran oil, used in a reactor after evaporating water by heating, contained 43.4% (w/w) TG, 14.9% DG, 0.2% MG and 41.4% FFA. The unsaponifiable matter content was 4.6%. AV was 91.0, and POV was 12 meq/kg. The glycerol content was 0.023%, and the moisture content was 620 ppm. The average molecular weight was calculated as the sum of products from the fatty acid content and their molecular weights, as shown in Table 1. The average molecular weights of fatty acid, MG, DG and TG were 276, 351, 610 and 869, respectively. The molar amount of DG in 1 g of the high FFA rice bran oil was calculated by:

$$1 \times (1 - 0.046) \times 0.149/610 = 2.33 \times 10^{-4} \text{ (mol)} \quad [1]$$

where (1 − 0.046) was the saponifiable matter content, 0.149 was the DG content and 610 was the average molecular weight of DG. Other components were calculated similarly. The stoichiometric amount of glycerol (G in the following equations) necessary for converting all

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TABLE 1

Average Molecular Weight of Fatty Acid in Rice Bran Oil

Fatty acid	Molecular weight (M)	Concentration[a] (C%)	MC/100
16:0	256.4	18.434	47.265
16:1	254.4	0.294	0.748
18:0	284.4	2.019	5.742
18:1	282.5	41.822	118.147
18:2	280.4	36.101	101.227
18:3	278.4	1.310	3.647
Average molecular weight of fatty acid			275.776

[a]High free fatty acid rice bran oil was methylated, and the fatty acid composition was analyzed by gas chromatography.

FFA into TG is (molar amount of FFA)/3, because of the following formula:

$$FFA + G/3 = TG/3 + H_2O \quad [2]$$

Stoichiometric amounts of glycerol produced by converting MG and DG into TG are 2(molar amount of MG)/3 and (molar amount of DG)/3, respectively, because:

$$MG = TG/3 + 2G/3 \quad [3]$$

$$DG = 2TG/3 + G/3 \quad [4]$$

Thus, the stoichiometric amount of glycerol necessary for converting all FFA, MG and DG into TG in 1 g of the high FFA rice bran oil was 0.036 g as calculated from the following equation:

$$\begin{aligned}\text{weight of glycerol required (g)} = 92 \times (1/3)\\ \{(\text{the molar amount of FFA}) - 2\,(\text{the molar amount of MG})\\ - (\text{the molar amount of DG})\}\\ - \text{glycerol in 1 g of the high FFA rice bran oil}\end{aligned} \quad [5]$$

where 92 is glycerol's molecular weight. If TG is synthesized from the main component of the FFA, the weight of glycerol necessary for synthesizing TG from 1 g of the high FFA rice bran oil was estimated as 0.05 g, as calculated by following the equation derived from the definition of AV:

$$\text{glycerol (g)} = (92/3) \times (\text{AV}/1000) \times (1/56.11) \quad [6]$$

where 92 is glycerol's molecular weight and 56.11 is the molecular weight of potassium hydroxide. The lot used in the continual reactor with dry nitrogen contained 58.2% TG, 13.2% DG, 0.6% MG and 27.6% FFA. The unsaponifiable matter content was 4.5%, and the moisture content 406 ppm.

Continual or repeated batch loop reactor with evaporation of water by heating. The fixed-bed reactor and ethylene glycol thermostated bath shown in Figure 1 were maintained at 58–81°C. The reaction mixture, containing 25 g high FFA rice bran oil and 0.9 g glycerol, was circulated at 5 mL/min in the loop. The reaction mixture was changed every 24–48 h. Product components, POVs and moisture contents of the products were measured as shown in Table 2.

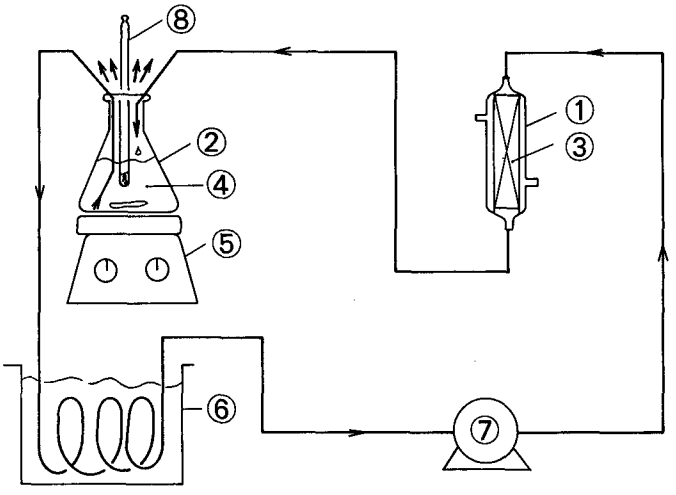

FIG. 1. Reactor with heating for dehydration. 1, Fixed-bed reactor; 2, dehydrator; 3, 1.25 g of Lipozyme IM 60 (Novo Nordisk, Chiba, Japan); 4, reaction mixture; 5, hot stirrer; 6, thermostat; 7, liquid pump and 8, thermometer. The solid line represents the path of the reactant.

Continual or repeated batch loop reactor with dry nitrogen. The system in Figure 2 was operated under a positive nitrogen atmosphere. The reaction mixture, containing 25 g high FFA rice bran oil and 0.55 g glycerol, was circulated at a flow rate of 6 mL/min. A plate was placed in the middle of the dehydrator to break foams and disperse the reactants, which entered the dehydrator between the plate and the bottom of the dehydrator. Dry nitrogen entered the dehydrator near the bottom, passed through the reactant and was collected from the top of the dehydrator. Dried reactant was collected from the bottom of the dehydrator. The reaction mixture was changed every 24–144 h. The reaction products were measured as shown in Table 3. The molecular sieves were regenerated every 5–10 d by heating at 500°C. The weight difference of used molecular sieves and the regenerated sieves was 8.2–21.8 g.

RESULTS AND DISCUSSION

Continual or repeated batch TG synthesis reactor. Table 2 shows that the increase in the heating temperature of the dehydrator in Figure 1 from 58 to 73°C raised the dehydration efficiency and the TG yield. However, at heating temperatures of more than 80°C, the color of the reactant changed from brown to yellow, and the TG yield decreased. The TG yield did not recover, even when the heating temperature was reduced to 60°C. The POVs of the products were fairly high as compared with the substrate high FFA rice bran oil POV of 12–16 meq/kg.

Table 3 shows the results from the continual reactor, shown in Figure 2, with dry nitrogen flow under positive nitrogen atmosphere. The product contained 73–75% of TG in a batch reacted for 24 h. This productivity was maintained for 37 d. POVs of the products were lower than 20. The mean molecular sieve weight increase was 1.93 g/d. There was no decrease of TG formation or increase in POV for more than one month. In an early experiment in the continual reactor, the nitrogen cylinder was turned off after 5 min because the reactor loop was saturated

TABLE 2

Continual TG Synthesis with Heat Evaporation[a]

Total[b] (d)	Batch[c] (h)	Temperature[d] (°C)	Moisture[e] (ppm)	POV[f] meq/kg	Product component[g] (w/w%)			
					TG	DG	MG	FFA
1	24	60	739		49.8	26.2	1.4	22.6
2	24	58	764	33.8	49.4	26.4	1.2	22.9
5	24	59	569	128.0	54.8	27.6	0.8	16.8
7	48	62	393		64.2	23.1	0.6	12.1
9	24	72	454	42.7	59.8	25.1	0.5	14.5
10	24	72	444	140.7	59.3	26.1	0.5	14.0
11	24	72	422	137.7	60.7	26.1	0.5	12.8
12	24	73	388		61.4	26.4	0.4	11.8
14	48	73	306		69.5	21.1	0.2	9.1
16	24	81	288	58.1	58.4	27.4	0.4	13.9
18	24	81	349	81.3	48.7	32.7	0.7	17.9
24	26	60		48.3	40.0	25.0	4.1	30.6
26	24	60	412	38.4	39.1	28.4	3.7	28.8
28	48	60	704	46.0	39.7	36.2	1.7	22.4

[a]Triacylglycerol (TG) synthesis was carried out in the reactor, as shown in Figure 1.
[b]Total reaction time.
[c]Reaction time for one batch.
[d]Heating temperature of dehydrator.
[e]Water content of product.
[f]Peroxide value (POV) of product.
[g]DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acid.

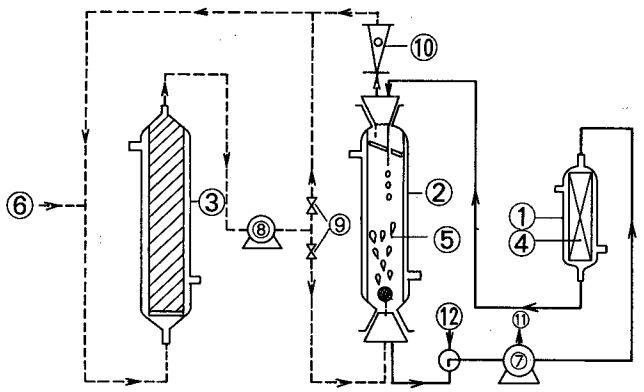

FIG. 2. Reactor with dry-nitrogen dehydrator. 1, Fixed-bed reactor at 60°C; 2, dehydrator at 60°C; 3, 250 g of molecular sieves, 5Å; 4, 1.25 g of Lipozyme IM 60; 5, reaction mixture; 6, nitrogen cylinder; 7, liquid pump; 8, air pump; 9, needle valve; 10, flow meter; 11, outlet for product and 12, inlet for substrate. The solid line represents the path of the reactant and the dotted line indicates the path of the nitrogen gas. See Figure 1 for Lipozyme's source and address.

with nitrogen. After 22 d of continual operation, the TG yield was decreased from 85 to 62%. The yield did not recover by hydration of the substrate, or by washing the immobilized column with hexane or glycine buffer at pH 10.5 containing 6.7% mannitol or 6.7% vitamin C or 175,000 units of superoxide dismutase. Oxygen contamination in the loop caused inactivation of lipase because the POVs of several products were 60–133.7 meq/kg (data not shown). This phenomenon was prevented by maintaining a positive pressure from the nitrogen cylinder after bubbling nitrogen.

Integration of dehydration and esterification in an immobilized lipase reactor. Conversion of FFA, MG and DG to TG by immobilized lipase seems to be driven by dehydration of the water that formed by condensation or the reverse reaction of lipolysis. The dehydration rate was faster than the deacidification rate in this conversion (13). The water removal process was an important part for the process integration of esterification and dehydration. High FFA rice bran oil and dry glycerol can be continuously reacted with Lipozyme IM 20 in a countercurrent fluidized-bed reactor, as reported earlier (14). Although the reaction mixture contained an excess amount of glycerol, the deacidification ability was not significant (15). The typical product contained 39.9% TG, 21.3% DG, 3.1% MG and 35.7% FFA. Dehydration and esterification should be carried out at the same time.

Enzymatic TG synthesis with evaporation of water by heating the reaction mixture is a simple method to study kinetics (9,10). However, TG synthesis with free evaporation is not suitable for industrial production of edible oil, because evaporation by heating often causes increased POV of the oil. Ergan *et al.* (8) have suggested that there is no reason to use molecular sieves or dry air bubbling because these methods are complicated, expensive and no more efficient than free evaporation. However, this assessment ignored lipid oxidation. Our experiment showed that evaporation by heating caused increased POV. Heating at more than 80°C caused the oil to become yellow, and it reduced the TG content. The thermal stability limit of Lipozyme is 60–80°C (unpublished data, Eigtved, Hansen and Sakaguchi). This suggests that secondary lipid oxidation products cause the inactivation of lipase.

Enzymatic FFA, MG and DG conversion to TG with a water trap and a vacuum pump to evaporate water under reduced pressure at 5–10 Torr is useful for small-scale TG synthesis. Although an ordinary liquid pump cannot cycle reactant in a vacuum, a modified pump, which can send the liquid into a vacuum atmosphere, is industrially utilized. This process will be applicable if such a modified pump is utilized. The product from stoichiometric substrate drying by vacuum evaporation after 24 h with

TABLE 3

Continual TG Synthesis with Dry Nitrogen Dehydration[a]

Total[b] (d)	Batch[c] (h)	Moisture[d] (ppm)	POV[e] meq/kg	Product component (w/w %)			
				TG	DG	MG	FFA
1	24	77	21.3	73.4	16.7	0.2	9.7
4	24	40	20.2	76.2	14.5	0.1	9.1
8	72	29	10.7	86.2	5.5	0.0	8.6
10	27	50	18.3	75.8	15.7	0.4	8.0
12	48	37	14.9	81.1	10.8	0.1	8.1
14	29	43	12.9	76.2	15.3	0.2	8.3
16	24	57		74.3	16.2	0.;0	9.5
20	48	63		77.1	13.5	0.0	9.5
26	72	27		81.8	12.3	0.0	9.5
30	24	31		73.0	21.4	0.0	5.8
36	144	39		81.2	11.7	0.0	7.2
37	24	33		73.0	20.0	0.0	7.0

[a]TG synthesis was carried out in the reactor shown in Figure 2. Abbreviations as in Table 2.
[b]Total reaction time.
[c]Reaction time for one batch.
[d]Water content of product.
[e]Peroxide value of product.

Lipozyme IM 20 at 60°C without a water trap was 55.4% TG, 41.3% DG, 1.3% MG and 2.1% FFA. The product obtained by vacuum evaporation with a water trap was 66.8% TG, 25.7% DG, 0.1% MG and 7.4% FFA. The water trap used with the reaction vessel and vacuum pump contained silica gel or molecular sieves. The trap was necessary for the vacuum evaporation to achieve adequate dehydration.

We have found that the best method for the enzymatic conversion of FFA, MG and DG conversion to TG includes the use of nitrogen. The dry nitrogen is easily regenerated by passing the gas through a molecular sieve column, which can be regenerated by heating. Lipid oxidation could easily be prevented by maintaining positive nitrogen pressure. The amount of water (ΔW) produced in the TG synthesis can be calculated by the following equation, because water is formed by the condensation reaction of FFA (13):

$$\Delta W(g) = 18 \times \text{substrate amount } (\Delta[FFA] + \Delta[H_2O]) \quad [7]$$

where Δ[FFA] and Δ[H_2O] are differences of concentration (mol/g) from product to substrate. The calculated value was 0.316 g/d. In contrast, the observed weight increase of molecular sieves was 1.93 g/d. The calculated value was less than the observed weight increase of the molecular sieves, 5Å, which suggests that the molecular sieves not only trap water, but also volatile materials, in the reaction mixture.

High FFA rice bran oil containing 30–50% FFA could be converted to oil containing more than 75% TG by means of immobilized lipase. This process can be called "enzymatic refining." Continual enzymatic refining is the first step to reduce the enzyme cost, which is a major factor in industrializing the technology.

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