High-Yield Diacylglycerol Formation by Solid-Phase Enzymatic Glycerolysis of Hydrogenated Beef Tallow

Tsuneo Yamane[a,*], Sung Tae Kang[b], Katsuyoshi Kawahara[c] and Yoshito Koizumi[c]

[a]Laboratory of Molecular Biotechnology, Department of Applied Biological Sciences, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan, [b]Laboratory of Biochemical Engineering, Department of Food Science and Engineering, Seoul National Polytechnic University, 172 Kongnung-dong, Nowon-gu, Seoul, Korea and [c]Riken Vitamin Co., Ltd., Deguchi 1-1-32, Hirakata City, Osaka 573, Japan

Diglyceride (DG) was prepared by reaction of hydrogenated beef tallow and glycerol in the presence of a *Psesudomonas* lipase. The yield of DG depended strongly on the reaction temperature. After initial incubation at 60°C for 2 h, followed by the first temperature shift down to 55°C for 4 h and then the second shift down to 48°C for up to 3 d, the reaction mixture became solid and a yield of approximately 90% DG was obtained. About 95% of total DG was 1,3-DG. The yield of DG was also dependent on the glycerol (GL) to triglyceride (TG) molar ratio. At the molar ratio of 1:2 (GL/TG), the enzyme-catalyzed reaction was highly efficient and utilized essentially all of the glycerol. The free fatty acid (FFA) content at equilibrium depended on the water concentration in the glycerol phase. The initial rate of FFA formation was low and was hardly affected by the moisture content between 0.5 and 4%, but, at higher water content (4–6.7%), there was a small increase in the rate.

KEY WORDS: Diacylglycerol, diglyceride, glycerol, glycerolysis, hydrogenated beef tallow, triglyceride.

Diglycerides (DG) find some utilization in the fats and oils industry. For example, a Japanese company is now making a special cooking oil that contains about 10% DG together with 1% phosphatidic acids (products of lecithin hydrolyzed by phospholipase D). Addition of DG into triglycerides (TG) makes oil more hydrophilic, which raises attachability of oil to cooked food materials. DG is also utilized in the food industry as estranger oil to separate materials from molds easily and as an adjuster of fat crystals.

Previously, Yamane *et al.* (1–3) reported that a high yield of monoglycerides (MG) (approximately 70%) from tallow in a simple batch solid-phase glycerolysis system was obtained by careful control of the reaction temperature. Since neither organic solvents, emulsifiers nor high temperatures were required, this system provided a practical alternative to chemical glycerolysis. However, MG yield from hydrogenated beef tallow by lipase-catalyzed glycerolysis was as low as 39% (2).

The work reported here describes the application of our enzymatic glycerolysis process to the synthesis of DG from hydrogenated beef tallow under a variety of conditions. Comprehensive experiments were carried out to increase the DG yield. Effects of the initial molar ratio of glycerol (GL)/TG, incubation temperature and water content in the GL phase on the DG yield were primarily investigated.

EXPERIMENTAL PROCEDURES

Materials. Lipase activity was determined by the olive oil/surfactant nonaddition method as described previously (4). One activity unit is the amount of enzyme that liberates one micromole of free fatty acid (FFA) per min at 37°C. A commercially available *Pseudomonas* lipase (EC 3.1.1.3) was obtained from Kurita Water Industries Ltd. (Tokyo, Japan). Hydrogenated beef tallow was provided by Riken Vitamin Co. (Hirakata, Japan). Its fatty acid composition was (%): C_{14}, 2.3; C_{16}, 28.4; C_{17}, 1.2; C_{18}, 66.1; others, 2.0. Reagent-grade glycerol was purchased from Wako Chemicals Co. (Osaka, Japan).

Glycerolysis. A mixture of 0.37 g (unless otherwise stated) GL, a trace amount of water, lipase powder and 6.67 g hydrogenated beef tallow under investigation was prepared in a flat-bottomed glass vessel of 3 cm internal diameter and 7 cm height. This resulted in a molar ratio of GL/TG of *ca.* 0.5. Unless otherwise stated, water concentration in the GL phase was 3.7%. Lipase from *Pseudomonas* sp. (50,000 units/g fat) was suspended in the GL/water solution. An enzyme reactor (model MS-50; Matsumoto Manufacturing Co. Ltd., Osaka, Japan) was used for temperature control and magnetic stirring at 800 rpm. For temperature programming, the reaction mixture was fist incubated at 60°C with magnetic stirring for at least 2 h to melt TG. The mixture was then transferred to 55°C. After incubation of reaction mixture for 4 h at 55°C, the mixture was finally transferred to 48°C. Magnetic stirring was discontinued at this stage because the reaction mixture had solidified.

Analysis. The course of glycerolysis was monitored by intermittent sampling (150 mg), followed by chloroform extraction (5). The extract was analyzed for TG, 1,3-DG, 1,2-DG, MG and FFA by use of a thin-layer chromatograph/flame-ionization detector (TLC/FID) (Iatroscan TH-10; Iatron Laboratories, Tokyo, Japan). Chromarod S III quartz rods coated with silica impregnated with 3% boric acid were used. Chloroform extracts (1 μL) were applied to the rods. A first development in $CHCl_3$ was followed by a second development in $CHCl_3$/methanol/NH_3 (70:0.04:0.01, vol/vol/vol). The rods were dried and scanned as described elsewhere (3). Results are expressed as percentage of peak areas and may differ slightly from the true weight percent as described by Tatara and co-workers (6). Moisture contents were determined with a Karl-Fischer moisture meter (model MKS-1; Kyoto Electronics Ltd., Kyoto, Japan).

Initial rates. Initial rates of appearance of total DG, MG and FFA are defined as follows: initial rate of appearance = fraction of glyceride appearing as a new component/h. Initial rate of disappearance of TG is defined as: initial rate of disappearance = (1-fraction of TG)/h. These rates were calculated from the tangential straight lines passing through the origin of the time-course curves for the components. GL included in the sampled reaction mixture was removed by chloroform extraction so that the percentages add up to 100.

RESULTS

Effect of molar ratio of GL to TG. Figure 1 shows the composition of reaction mixtures at 72 h after glycerolysis of

*To whom correspondence should be addressed.

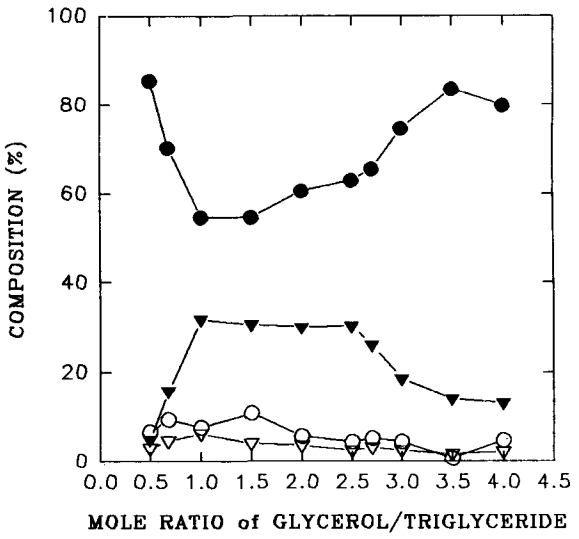

FIG. 1. The effect of glycerol/triglyceride (TG) ratio on diglyceride (DG) production after 72 h enzymatic glycerolysis of hydrogenated beef tallow. TG (○), DG (●), monoglyceride (▼), free fatty acids (▽).

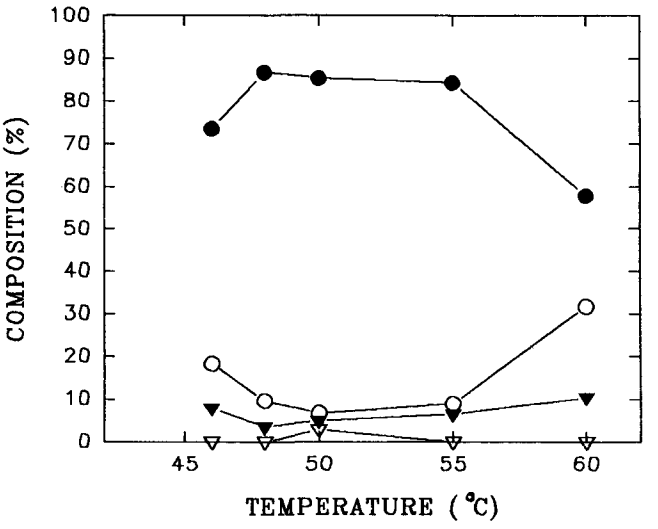

FIG. 2. The effect of temperature on diglyceride production after 72 h enzymatic glycerolysis of hydrogenated beef tallow for the molar ratio glycerol/TG = 0.5. TG (○), DG (●), monoglyceride (▼), free fatty acids (▽). See Figure 1 for abbreviations.

hydrogenated beef tallow with GL/TG molar ratios ranging from 0.5 to 4 at 50°C. More than 95% of TG was converted to other glycerides or FFA in all ranges of GL/TG investigated. The yield of DG showed a sharp decrease with increasing GL/TG ratio from 0.5 and 1. This result follows from the stoichiometric mole ratio GL/TG = 0.5 for complete conversion of TG to DG:

$$2\ TG + GL \rightarrow 3\ DG \qquad [1]$$

At this ratio, 85.5% was obtained by the lipase-catalyzed glycerolysis reaction, but in the range GL/TG 1–2.5 there was only a small increase in DG production. The yield of DG increased again with increasing GL/TG above 2.7. Whereas there was a large increase in the content of MG with increasing GL/TG from 0.5 to 1, above the ratio of 1 the production of MG decreased. The yield of FFA was low (1.6–2.6%) throughout and was hardly affected by GL/TG over the range of 0.5–4.

Effect of temperature. Figure 2 shows the final compositions of reaction mixtures with respect to DG, MG, FFA and TG as a function of incubation temperature during the glycerolysis. The optimum temperature was 48°C with the highest DG yield of 87%. The yield of DG decreased at higher or lower temperatures. There was a large increase in the yield of DG with solidification of the reaction mixture. During the incubation of a reaction mixture at 60°C, it remained liquid throughout glycerolysis with a DG yield of 58%. By controlling the temperature to be lower than 60°C, reaction mixtures solidified. More than 74% of DG was obtained after 72 h reaction. The final yield of MG was only slightly affected by reaction temperature; production of MG was roughly proportional to temperature above 48°C. FFA synthesis was nearly zero and was hardly affected by temperature. The temperature at which high TG conversion was observed was 50°C, with 6.7% remaining. At higher and lower temperatures, there were large increases in TG content.

Effect of water content on final yield and initial rates. Figure 3 depicts the variations in initial rates of formation of DG, MG and FFA and of conversion of TG during glycerolysis of hydrogenated beef tallow at 60°C with water contents in the GL phase ranging from 0.5 to 6.7%. The initial rate of conversion of TG was roughly proportional to water content between 0.5 and 2%, but above 3%, the rate of DG synthesis stabilized. The initial rate of MG formation increased with increasing water content from 0.5 to 6.7%. In the case of FFA synthesis, the initial rate was low and hardly affected by moisture content between between 0.5 and 4%. At higher water content (4–6.7%) there was little increase in rate.

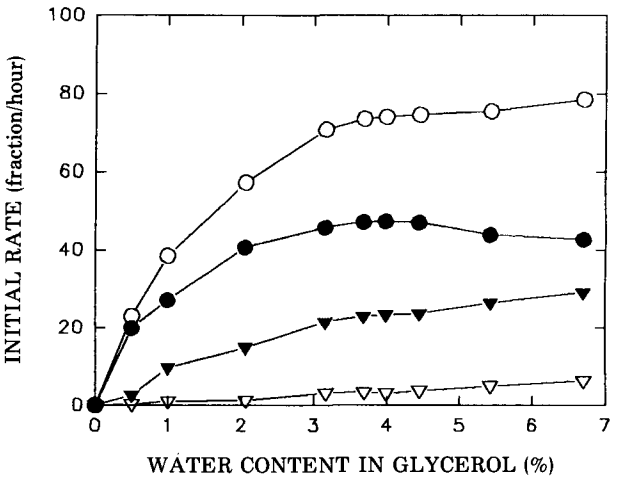

FIG. 3. The effect of initial water content on the initial rate of disappearance of TG and the initial rates of appearance of monoglyceride (MG), DG and free fatty acids (FFA) during enzymatic glycerolysis of hydrogenated beef tallow. TG (○), DG (●), MG (▼), FFA (▽). See Figure 1 for abbreviations.

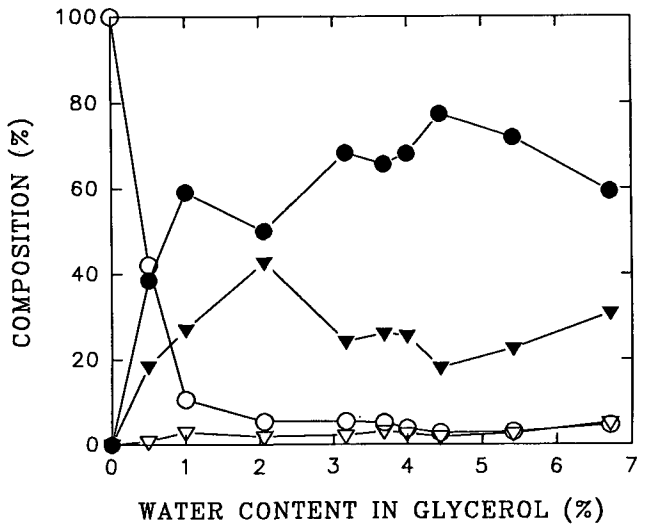

FIG. 4. The effect of initial water content on diglyceride production after 72 h enzymatic glycerolysis of hydrogenated beef tallow for the molar ratio glycerol/TG = 0.5. TG (○), DG (●), MG (▼), FFA (▽). See Figures 1 and 3 for abbreviations.

As shown in Figure 4, the final yield of DG was also affected by water content in the GL phase. The conversion of TG was roughly proportional to water content between 0.5 and 1%, but above 1% the degree of TG conversion was almost constant. Formation of DG increased with increasing water content from 0.5 to 1%. In the case of FFA formation, the yield was low and hardly affected by moisture content between 0.5 and 3.1%. At high water contents (3.4–6.7%), there were only slight increases in FFA yields with increasing water content.

Time course of DG formation from hydrogenated beef tallow. Detailed changes in glyceride composition of a reaction mixure during the course of glycerolysis are given in Figure 5. The reaction mixture was first incubated at 60°C with magnetic stirring for 2 h. It was then incubated at 55°C for 4 h and subsequently at 48°C. Initially, the rate of DG synthesis was high, but after 6 h the rate decreased. There was a large decrease in the content of TG to 29% during the first 6 h. The concentration of MG remained low throughout the reaction with a final yield of 3.5%. Equilibrium was almost reached in 72 h with a DG concentration of 87%. Figure 6 is a TLC/FID chromatogram of the reaction mixture incubated for 72 h. Up to 90.3% of TG was converted to other glycerides. Total DG was composed of 94.5% 1,3-DG and 5.5% 1,2-DG. FFA as not detected by the TLC/FID analyzer. Only 3.5% of 1-MG was produced.

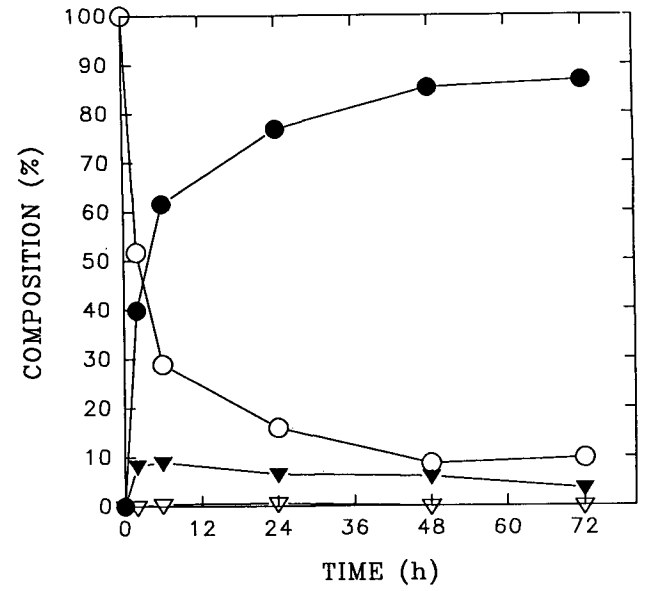

FIG. 5. The composition of the reaction mixture during enzymatic glycerolysis of hydrogenated beef tallow at 48°C for the molar ratio glycerol/TG = 0.5. TG (○), DG (●), MG (▼), FFA (▽). See Figures 1 and 3 for abbreviations.

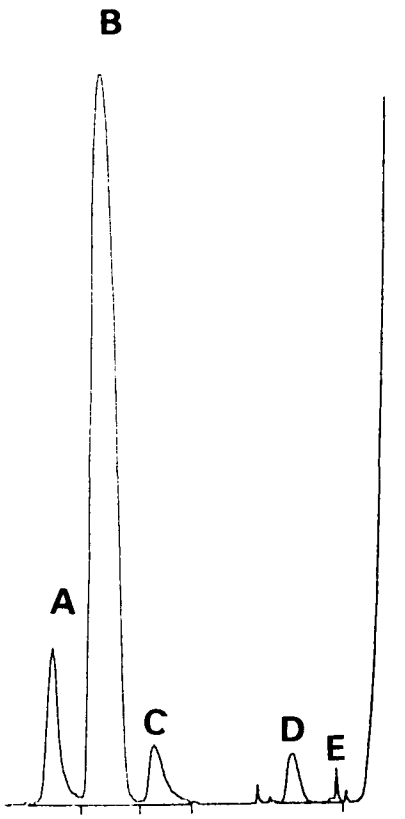

FIG. 6. Chromatogram by thin-layer chromatography/flame-ionization detector analyzer of the reaction mixture for the reaction at 48°C at molar ratio glycerol/TG = 0.5. Sampling was carried out after 72 h incubation. (A, TG; B, 1,3-DG; C, 1,2-DG; D, 1-MG; and E, glycerol). See Figures 1 and 3 for abbreviations.

DISCUSSION

In this paper it is shown that a yield of approximately 90% DG can be obtained by glycerolysis of hydrogenated beef tallow with lipase as a catalyst without use of organic solvents or emulsifiers. The procedure is highly reproducible with respect to DG production. Previous attempts were mainly for the production of MG (1–3).

The high DG equilibrium state described here is achieved by careful control of reaction temperature, water content and the reaction composition. The fats become

solid below a certain temperature during the course of the reaction only when DG is synthesized in high yield. The high DG yield is presumably due to lower solubility of DG in the reaction mixture, which results in shifting the equilibrium toward synthesis of more DG.

To activate the lipase, it is necessary to dissolve a trace amount of water in the GL phase. However, the water content of the GL phase must remain at low levels to avoid excessive production of FFA. As much as 6.4% FFA is produced when 6.7% water is dissolved in the GL phase. Moreover, the yield of DG is considerably reduced when 6.7% water is used.

The yield of DG is also dependent of the GL/fat molar ratio. At a molar ratio of 1:2 (GL/TG), the enzyme-catalyzed reaction is efficient and utilized essentially all of the GL provided. This result means that no GL is present in the product oil. Even though the enzyme-catalyzed reaction is also efficient at GL/TG molar ratios of 3.5–4.0:1, the large amounts of GL remaining after glycerolysis would need to be removed. Because of the lipase-catalyzed glycerolysis reaction, the kinetics could not be clarified by use of the data obtained in this experiment. More detailed research on the interfacial area between the GL and the fat should be carried out with variation of GL/TG during the course of the reaction.

The ease of production of DG in high yield under mild conditions with lipase as catalyst provides a possible alternative to the chemical process.

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