Solid Phase Enzymatic Glycerolysis of Beef Tallow Resulting in a High Yield of Monoglyceride

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A mixture of mono-, di- and triglycerides was obtained when beef tallow was reacted with glycerol using lipase enzyme as a catalyst. The reaction was carried out batchwise in a small vessel with agitation by magnetic stirring. The yield of monoglyceride (MG) was greatly influenced by the reaction temperature—at higher temperatures (48–50°C) a yield of approximately 30% MG was obtained, while at lower temperatures (38–46°C) a yield of approximately 70% MG was obtained. A sharp transition was observed between the high and low yield equilibrium states. The temperature at which this transition occurred is called the critical temperature (T_C) and was found to be 46°C in the case of tallow. During the course of the reaction, when approximately 40% MG had been synthesized, the reaction mixture became solid but the reaction continued until approximately 70% MG had been synthesized.

A yield of 70% MG also was obtained with tallow at 42°C when a glycerol/tallow mole ratio ranging from 1.5 to 2.5 was used. The free fatty acid content at equilibrium depended on the water concentration in the glycerol phase and varied from 0.5% to 11.0% when the water content ranged from 0.6% to 12.5%. Above 8% water content, the yield of MG was reduced. Of the commercially available lipases that were investigated, lipase from *Pseudomonas fluorescens* or *Chromobacterium viscosum* resulted in the highest yield of MG.

KEY WORDS: Beef tallow, glycerolysis, lipase, monoglyceride.

Monoglycerides (MG) are an important group of emulsifying agents widely used in the food industry. At present they are produced commercially by the reaction of natural fats and oils with glycerol (glycerolysis) by using an inorganic catalyst at temperatures greater than 220°C (1,2). A yield of MG of approximately 40% is usually obtained. The use of high temperatures results in the formation of dark-colored by-products with an undesirable flavor. Repeated molecular distillation is used to remove impurities and to concentrate the MG. To obtain a product of higher quality and higher yield, and to minimize energy costs, several attempts have been made to synthesize MG at low temperature using lipase enzymes as a catalyst. Enzymatic synthesis by glycerolysis of triglyceride (TG) oils (3,4) or by reaction of free fatty acid (FFA) with glycerol (5) was achieved using either a simple batch system or a membrane bioreactor system, but the yield of MG was low (less than 25%). A microemulsion system incorporating 1,3 specific lipase was recently used to (i) selectively hydrolyze triglyceride at the 1,3 position resulting in 2-MG (6); or (ii) to transesterify acyl groups from triglyceride to glycerol (glycerolysis reaction) (7). This system requires the use of organic solvents and emulsifying agents and therefore is not practical for large scale synthesis.

In the present paper, a process is described for the synthesis of MG from beef tallow by enzymatic glycerolysis with a yield of approximately 70%. The reaction is effective at moderate temperatures (approximately 40°C) and does not require the use of solvents or emulsifying agents.

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EXPERIMENTAL PROCEDURES

Materials. Lipase activity was determined by the olive oil/surfactant non-addition method as described previously (8). One activity unit is described as the amount of enzyme which liberates 1 micromole of free fatty acid per min. Commercially available lipases (E.C. 3.1.1.3) were used, and were obtained from the following companies: *Pseudomonas fluorescens* crude and pure, from Amano Seiyaku Co. Ltd. (Nagoya, Japan); *Chromobacterium viscosum,* from Toyo Jozo Co. Ltd. (Shizuoka, Japan); *Rhizopus japonicus,* Nagase Biochemical Co., Ltd. (Osaka, Japan); *Mucor miehei,* SP398 and Lipozyme, Novo Nordisk Bioindustry Inc. (Tokyo, Japan); and *Candida cylindracea,* from Meito Sangyo Co. Ltd. (Nagoya, Japan). Refined deodorized beef tallow was provided as a gift by the Agriculture and Food Development Authority (Fermoy, Ireland). Glycerol "Special Grade" was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Glycerolysis. A small amount of distilled water or buffer was dissolved in 2.84 g of glycerol to give a final concentration of 3.6% water in glycerol. Lipase powder (7000 units) was suspended in the glycerol/water solution and 13.07 g of fat was added. This resulted in a mole ratio of glycerol/fat of 2.0. Crude lipase from *Pseudomonas fluorescens* was used in all experiments unless otherwise stated. Agitation by magnetic stirring at 800 rpm and temperature control was achieved using an MS-50 enzyme reactor (Matsumoto Manufacturing Co. Ltd., Osaka, Japan). Flat-bottomed glass reaction vessels of 3 cm internal diameter and 10 cm high were tightly stoppered to prevent moisture absorption.

Analysis. During the course of the reaction, samples of approximately 150 mg were intermittently withdrawn from the reaction vessel. Inactivation of the enzyme and removal of excess glycerol was achieved by extraction of the sample into 9 mL of chloroform according to the method of Yamane *et al.* (3). Analysis of the product mixture was carried out by thin-layer chromatography/flame ionization detection (Iatroscan TH-10, Iatron Laboratories, Tokyo, Japan). Chromarod S III quartz rods were used without modification. One μL of chloroform extract was applied to the rod followed by development in benzene/chloroform/acetic acid (70:30:2) solvent. The rods were dried and scanned under the following conditions: hydrogen, 0.7 Kg/cm^2, airflow, 2000 mL/min and 30 sec/scan. Triglyceride (TG), 1,3-diglyceride (1,3-DG), 1,2-diglyceride (1,2-DG), MG and FFA were effectively separated and peak areas were calculated using an SIC Chromatocorder 12 integrator (System Instruments Co. Ltd., Tokyo, Japan). Results are expressed as %peak areas and may vary slightly from the true weight%, as shown previously (9). Moisture contents were determined using a Karl-Fischer moisture meter (MKS-1, Kyoto Electronics Ltd., Kyoto, Japan).

Initial Rates. Initial rate of production of 1,3-DG, 1,2-DG,

MG and FFA is defined as the change in fractional content of the component per hour. Initial rate of conversion of TG is defined as the change in [1-(fractional content of TG)] per hour. Fractional content is defined as wt%/100.

RESULTS

Effect of temperature. Figure 1 shows the time course for the production of MG during the glycerolysis of tallow in the temperature range 40-50°C. Above 46°C, approximately 30% of MG was obtained at equilibrium. At 46°C or lower, approximately 70% MG was produced. After 3 hr at 46°C or lower, the reaction mixture became solid and further stirring was impossible, but the reaction was allowed to continue.

In Figure 2, the change in composition of the reaction mixture during glycerolysis of tallow at 42°C is shown. Initially, a linear decrease in TG concentration and a linear increase in 1,3-DG, 1,2-DG and MG concentration occurred. After 2-3 hr, the 1,3-DG and 1,2-DG concentrations began to decrease while monoglyceride concentration continued to increase linearly. After 3 hr incubation the reaction mixture became solid, corresponding roughly to the beginning of the decrease in 1,3-DG concentration. At the beginning of the reaction, both 1,3- and 1,2-DG were synthesized in approxi-

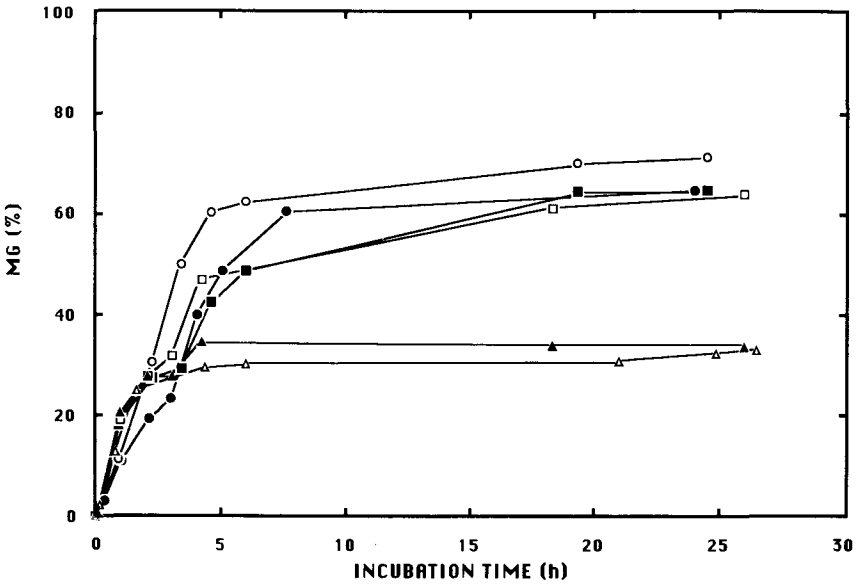

FIG. 1. The effect of temperature on monoglyceride production during enzymatic glycerolysis of beef tallow: 40°C, (●); 42°C, (○); 44°C, (■); 46°C, (□); 48°C, (▲); and 50°C, (△).

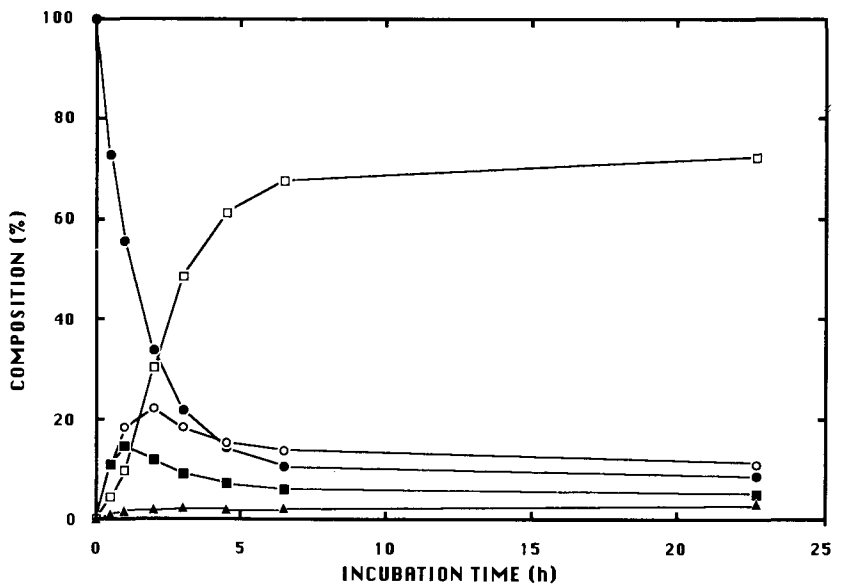

FIG. 2. The composition of the reaction mixture during enzymatic glycerolysis of tallow at 42°C; TG, (●); 1,3-DG, (○); 1,2-DG, (■); MG (□); and FFA, (▲).

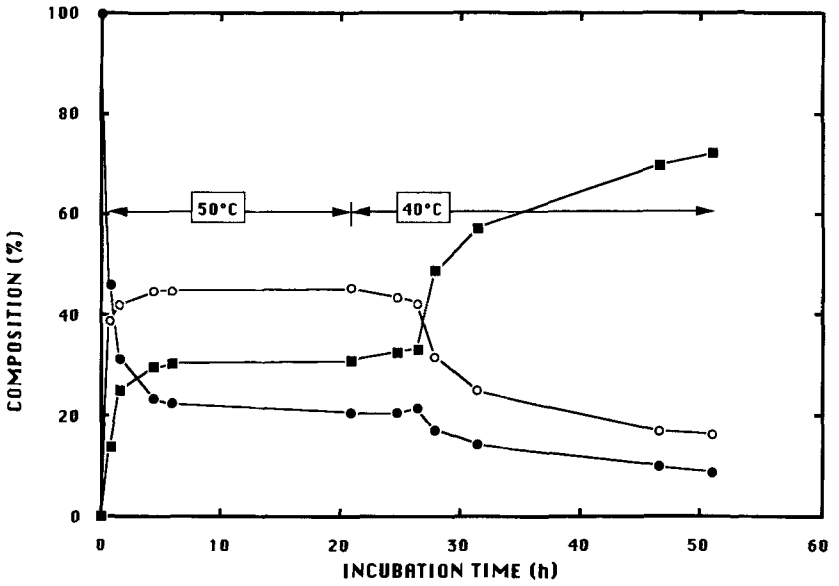

FIG. 3. Enzymatic glycerolysis of beef tallow at 50°C followed by 40°C; composition of the reaction mixture: TG, (●); total DG, (○); and MG, (■).

mately equal amounts, but after 2 hr the main component of the DG fraction was the 1,3-isomer. To check for possible inactivation of lipase at temperatures greater than 46°C (which might account for the low MG yield), the glycerolysis reaction was carried out at 50°C until equilibrium was reached (Fig. 3). When the temperature was reduced to 42°C, a new equilibrium with a high MG yield was attained.

Reproducibility. The glycerolysis of beef tallow was repeated 7 times at 42°C under identical conditions to check the reproducibility of the reaction. The mean and standard deviation (SD) was calculated from the composition of the reaction mixture after 28 hr incubation. The mean (wt%) and SD for MG was 71.3% ± 0.72. For other components of the reaction mixture the mean and CV was: TG, 9.3% ± 0.67; 1,3-DG, 11.9% ± 0.99; 1,2-DG, 5.1% ± 0.19; and FFA, 2.4% ± 0.41.

Effect of water content. Figure 4 shows the initial rates of formation of DG, MG and FFA, as well as the initial rate of conversion of TG during glycerolysis of tallow at 42°C with a water content in the glycerol phase ranging from 0.6% to 12.5%. The initial rate of conversion of TG was roughly proportional to the water content in the range 0.6% to 4.0%. From 4% to 12.5%, the increase in rate was considerably lower. The rate of synthesis of DG was also proportional to water content between 0.6% and 3.5%. Above 3.5%, the initial rate of DG synthesis was constant. The initial rate of MG formation increased with increasing water content from 0.6% to 2.0%, but above 2.0% the water content had little effect on the rate of MG formation. In the case of FFA synthesis, initial rates increased steadily from 3.5% to 12.5%. However, even at the lowest water content (0.6%), a small amount of FFA was synthesized. In Figure 5, the time course for MG production in tallow between 4.5% and 12.5% water in the glycerol phase is shown. Although the initial rate of MG synthesis was high, the rate became very slow at the 8.5%, 10.5% and 12.5% water levels when approximately 30% MG had been synthesized. This resulted in a reduced yield of MG above 8.5% water content. Below the 8.5% level, the yield of MG was unaffected by water content except at the 0.6% level, where a low yield was obtained due to the very low rate of synthesis.

Effect of enzyme concentration. Figure 6 shows the initial

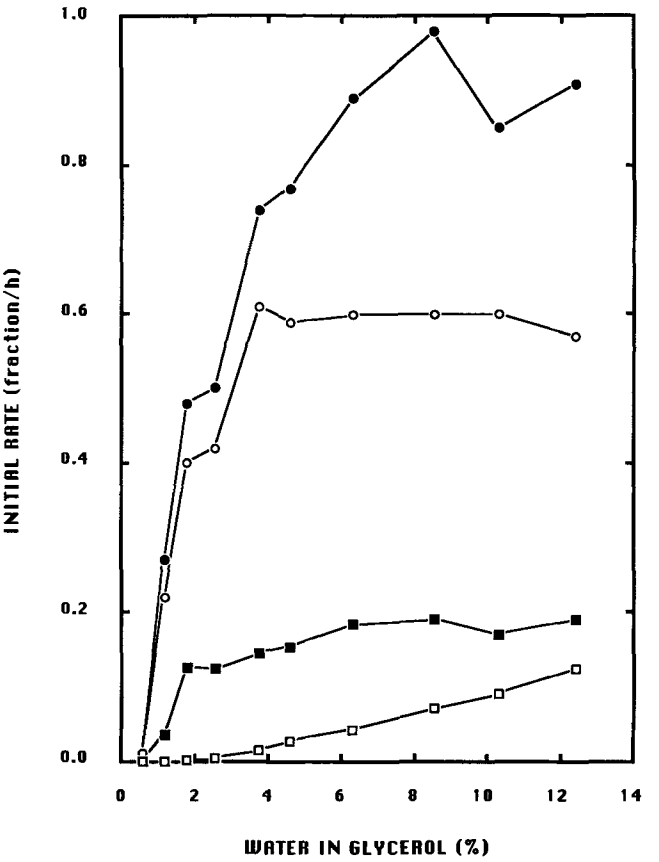

FIG. 4. The effect of water content on the initial rate of conversion of TG and the initial rate of formations of MG, DG and FFA during enzymatic glycerolysis of tallow at 42°C: TG, (●); total DG, (○); MG, (■); and FFA, (□).

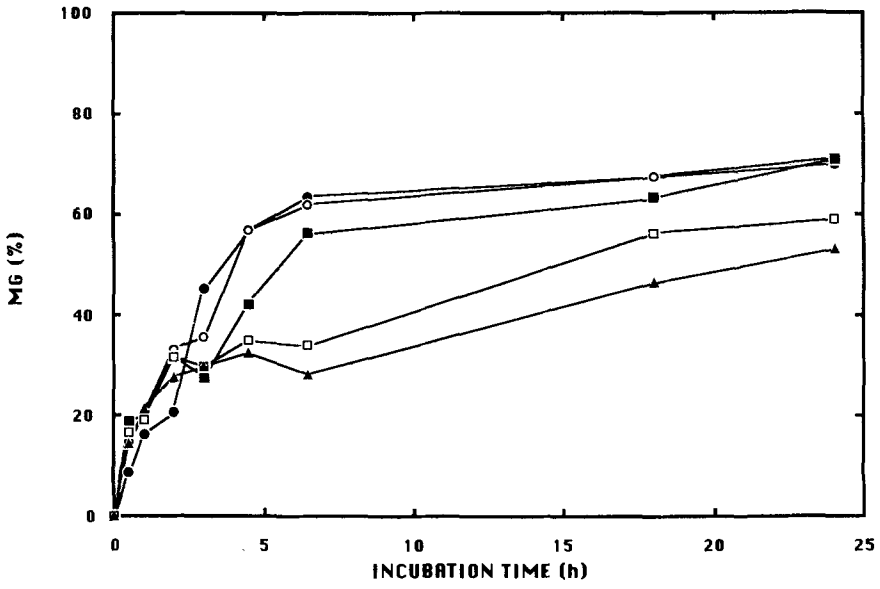

FIG. 5. The effect of water content on the synthesis of MG during enzymatic glycerolysis of tallow at 42°C. Water content in the glycerol: 4.5%, (●); 6.5% (○); 8.5%, (■); 10.5%, (□); and 12.5%, (▲).

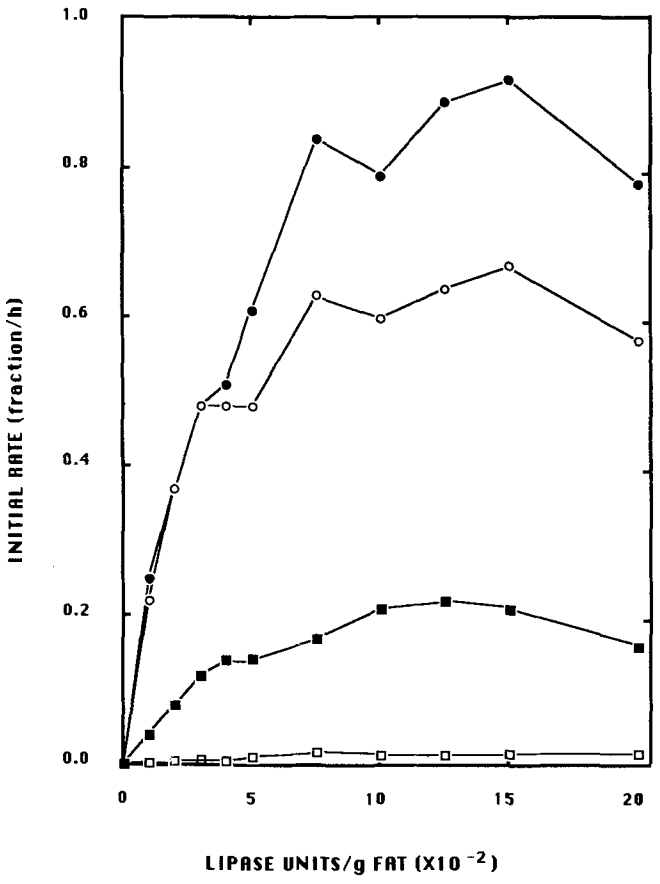

FIG. 6. The effect of lipase concentration on the initial rate of conversion of TG and the initial rate of formations of DG, MG and FFA during glycerolysis at 42°C: TG, (●); total DG, (○); MG, (■); and FFA, (□).

rate of MG and DG production and conversion of TG during glycerolysis of tallow at 42°C in the range 100-2000 units/g fat. For MG and DG, the rate increased proportionally from 100-500 units/g fat, after which only a small increase in rate was observed. The initial rate of conversion of TG followed a similar pattern. The equilibrium composition was unaffected by the enzyme concentration (data not shown).

Glycerolysis activity of various kinds of enzyme. Table 1 shows the equilibrium composition of the reaction mixture after glycerolysis of tallow at 42°C using 8 different lipase preparations. The highest yield of MG was obtained with *Chromobacterium viscosum* and *Pseudomonas fluorescens* lipases. *Mucor miehei* and SP398 lipases were partly effective, while *Candida cylindracea* lipase was inactive. Relatively more 1,2-DG as compared to 1,3-DG was produced by the positionally specific lipases: *M. miehei,* SP398, *R. japonicus* and Lipozyme. During glycerolysis using *M. miehei* enzyme, the relative content of 1,2-DG compared to 1,3-DG gradually decreased as the reaction proceeded.

TABLE 1

Equilibrium Composition of the Reaction Mixture After Glycerolysis of Tallow at 42°C Using Eight Different Lipase Preparations

Lipase preparation	Composition (%)				
	TG	1,3-DG	1,2-DG	MG	FFA
Chromobacterium viscosum	8	11	5	72	3
Pseudomonas fluorescens (crude)	8	11	4	72	3
Pseudomonas fluorescens (pure)	8	13	5	70	3
Candida cylindracea	100	0	0	0	0
Mucor miehei	18	15	11	53	4
SP398	21	12	17	45	5
Rhizopus japonicus	66	7	15	11	2
Lipozyme[a]	45	10	16	25	4

[a]Immobilized enzyme particles of *Mucor miehei* lipase.

DISCUSSION

In this paper it has been shown that a yield of approximately 70% MG can be obtained by glycerolysis of tallow using lipase enzyme as a catalyst without the use of organic solvents or emulsifiers. The procedure is highly reproducible with respect to MG production. Previous attempts to achieve enzyme-catalyzed glycerolysis resulted in low yields of MG (3-5) and required the use of organic solvents and emulsifiers (6,7), which are costly and difficult to separate from the product. The high MG equilibrium state described here is achieved by careful control of the reaction temperature. Below a certain temperature (critical temperature, T_C) a yield of approximately 70% MG can be obtained. Above the T_C only approximately 30% MG can be synthesized. The actual value of T_C for tallow (46°C) is close to the melting point of the fat (46.2°C). As shown in Figure 2, both MG and DG are produced during the early stages of the reaction, presumably by reaction of 1 mole of glycerol with 1 mole of TG. The subsequent decrease in DG concentration is due to reaction of 1 mole of DG with 1 mole of glycerol to give 2 moles of MG.

Monoglycerides have a higher melting point than the corresponding triglycerides (10). This fact, coupled with the observation that the reaction mixture becomes solid during glycerolysis below the T_C, indicates that the MG exceeds its solubility limit and begins to precipitate from solution. This presumably shifts the equilibrium towards synthesis of more MG until a high MG equilibrium is reached. This reaction is analogous to the chemical process of directed interesterification described by Eckey (11), in which the lower solubility of trisaturated triglycerides in the reaction mixture is exploited to alter the equilibrium composition during interesterification of fats and oils. The use of simultaneous fractional crystallization to improve yield of product during the reaction, as described here, may be applicable to other enzyme-catalyzed reactions. This could justify the re-examination of existing procedures and should be considered in the design of new enzyme-catalyzed reactions.

Because of the complexity of the enzyme-catalyzed glycerolysis reaction, the kinetics could not be clarified using the data obtained in the experiments described here. Future research should include measurement of interfacial area between the glycerol and the fat which, due to the synthesis of surface active MG, may change during the course of the reaction.

In a previous study (3) it was found that among 13 commercially available lipases, those from *P. fluorescens* and *C. viscosum* catalyzed the glycerolysis reaction most effectively. This observation has been confirmed in the present study, although SP398 lipase and lipase from *M. miehei* gave moderately high yields of MG and deserve further investigation. Both the highly purified lipase and the crude preparation from *P. fluorescens* were identical with respect to glycerolysis activity, confirming that the glycerolysis activity of crude preparations is due to the presence of lipase. As expected, 1,3-positionally specific lipases produced relatively more 1,2-DG as compared with 1,3-DG. However, the synthesis of a relatively high concentration of 1,3-DG, especially in the case of *M. miehei* lipase, indicates that these enzymes do not possess absolute specificity. The lack of absolute positional specificity for lipases has been previously suggested (12). The low concentration of the 1,2-DG present during catalysis by nonspecific lipases is presumably due to acyl transfer from the 2 position to the 3 position catalyzed by the lipase. Acyl migration from the 2 to the 3 position catalyzed by inorganic catalyst was previously reported (13).

To activate the lipase catalyst, it is essential to dissolve a trace amount of water in the glycerol phase. A similar requirement has been previously reported for other lipase catalyzed reactions where a low water concentration is desired (14-16). However, the moisture content of the glycerol phase must be maintained at low levels to avoid excessive production of FFA. As much as 12% FFA is produced when greater than 8% water is dissolved in the glycerol phase. Moreover, the yield of MG is considerably reduced when 12% water is used. The MG yield is independent of enzyme concentration in the range examined (100-2000 units/g fat), but the reaction rate is proportional to the enzyme concentration between 100 and 1000 units/g fat. The yield of MG is also independent of the glycerol fat mole ratio at 1:5 or greater. At a mole ratio of 1:5, the enzyme catalyzed reaction is very efficient, utilizing essentially all of the glycerol provided. In the commercial chemical process, a large molar excess of glycerol must be used in order to obtain reasonable MG yields (2). The ease of production of a high yield of MG under mild conditions when lipase is used as catalyst provides a possible alternative to the chemical process.

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REFERENCES

1. Sonntag, N.O.V., *J. Am. Oil Chem. Soc. 59*:795A (1982).
2. Sonntag, N.O.V., in *Bailey's Industrial Oil and Fat Products,* Vol. 2, Fourth edn., edited by D. Swern, John Wiley and Sons, 1982, p. 134.
3. Yamane, T., M.M. Hoq, S. Itoh and S. Shimizu, *J. Jpn. Oil Chem. Soc. 35*:625 (1986).
4. Yamane, T., M.M. Hoq, S. Itoh and S. Shimizu, *Ibid. 35*:632 (1986).
5. Hoq, M.M., T. Yamane and S. Shimizu, *J. Am. Oil Chem. Soc. 61*:776 (1984).
6. Holmberg, K., and E. Osterberg, *Ibid. 65*:1544 (1988).
7. Holmberg, K., B. Lassen and M. Stark, *Ibid. 66*:1796 (1989).
8. Yamane, T., *J. Jpn. Oil Chem. Soc. 36*:638 (1987).
9. Tatara, T., T. Fujii, T. Kawase and M. Minagawa, *Lipids 18*:732 (1983).
10. Dawson, R.M.C., D.C. Elliott, W.H. Elliott and K.M. Jones, (eds.) *Data for Biochemical Research,* Clarendon Press, Oxford, 1986, p. 167.
11. Eckey, E.W., *Industrial and Engineering Chemistry 40*:1183 (1948).
12. Machida, H., *Yukagaku 33*:691 (1984).
13. Mattson F.H., and R.A. Volpenheim, *J. Lipid Res. 3*:281 (1962).
14. Macrae, A.R., *J. Am. Oil Chem. Soc. 60*:291 (1983).
15. Kawamoto, R., K. Sonomoto and A. Tanaka, *Biocatalysis 1*:137 (1987).
16. Yamane, T., *Ibid. 2*:1 (1988).

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