Further Improvements in the Yield of Monoglycerides During Enzymatic Glycerolysis of Fats and Oils

Gerald P. McNeill and **Tsuneo Yamane***
Laboratory of Bioreaction Engineering, Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

Three approaches were used in an effort to increase the yield of monoglycerides (MG) during the lipase catalyzed reaction of glycerol with triglyceride fats and oils: i) various commercially available lipases were screened for ability to catalyze MG synthesis; ii) mixtures of lipases were compared with single lipases; and iii) two-step temperature programming was applied during the reaction. Of these, temperature programming was found to be the most effective. With an initial temperature of 42°C for 8–16 hr followed by incubation at 5°C for up to 4 days, a yield of approximately 90 wt% MG was obtained from beef tallow, palm oil and palm stearin. When the second incubation temperature was greater than 5°C, the yield of MG was progressively lower with increasing temperature. In the case of screening of newly available commercial lipase preparations, lipases from *Pseudomonas* sp. were found to be most effective, giving a yield of approximately 70 wt% MG at 42°C from tallow. Lipases from *Geotrichum candidum, Penicillium camembertii* (lipase G) and *Candida rugosa* were inactive. A mixture of lipases from *Penicillium camembertii* and *Humicola lanuginosa* was found to be more effective than either enzyme alone, giving a yield of approximately 70 wt% MG using beef tallow or palm oil. A mixture of *Penicillium camembertii* lipase with either *Pseudomonas fluorescens* lipase or *Mucor miehei* lipase was not more effective than *Pseudomonas fluorescens* or *Mucor miehei* lipase alone.

KEY WORDS: Fats and oils, glycerolysis, high yield of monoglyceride, lipase mixtures, lipases, monoglycerides, temperature programming.

Due to the worldwide importance of monoglycerides (MG) and their derivatives as surface active additives in a wide range of foods (1), considerable attention recently has been paid to improvements in the method of MG synthesis. The current method of manufacture by the glycerolysis reaction in which natural fats and oils undergo ester exchange with glycerol in the presence of an inorganic catalyst (2,3) suffers from several drawbacks. A molar excess of glycerol is used and because the reaction temperature is greater than 220°C, dark-colored byproducts with an undesirable flavor are formed, necessitating purification by molecular distillation. Moreover, the yield of MG is rather low (30–40%). In an attempt to overcome these problems, several investigations have been made using lipase enzyme (E.C. 3.1.3.3) as a catalyst (4–9) for MG synthesis by glycerolysis or partial hydrolysis of triglyceride and by esterification of fatty acid with glycerol. The use of lipase is potentially advantageous because it is an efficient and selective catalyst at ambient temperatures. In most cases, however, a low yield of MG was obtained. In a new approach recently reported by the authors (10–12), a high yield of MG (60–75 wt%) could be obtained from commonly used fats and oils using a batch glycerolysis system by carefully controlling the reaction temperature. Although this system provides a possible alternative to chemical synthesis because of the mild conditions and high yields of MG, further investigations have been carried out in an attempt to obtain a higher yield of MG and to broaden the range of useful lipase preparations. The results presented here show that it is possible to obtain nearly theoretical yields of MG (approximately 90 wt%) using *Pseudomonas* sp. lipase and a yield of approximately 80 wt% using *Mucor miehei* lipase under appropriate conditions.

*To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Glycerolysis. A mixture of 2.84 g glycerol, a trace amount of water, lipase powder and 13.07 g of the triglyceride fat under investigation was prepared in a flat-bottomed glass vessel of 3 cm internal diameter and 10 cm high, as previously described (11). Screening of individual lipases and lipase mixtures for glycerolysis activity was performed with a water concentration in the glycerol phase of 3.6% in order to accurately compare their results with previous results (in references 11 and 12, a water concentration of 3.6% was applied). For experiments at lower temperatures a slightly higher water concentration in glycerol, 4.6%, was used to increase the reaction rates. A water concentration of 4.6% results in higher reaction rates without excessive production of free fatty acid. Unless otherwise stated, 500 units of lipase/g fat was used and a mole ratio of glycerol/fat of 2 was chosen. 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 first incubated at 42°C with magnetic stirring for at least 6 hr. The mixture was then transferred directly to a water bath at the second incubation temperature. Magnetic stirring was not necessary 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. The extract was analyzed for triglyceride (TG), 1,3 diglyceride (1,3 DG), 1,2 diglyceride (1,2 DG), monoglyceride (MG) and free fatty acid (FFA) using a thin-layer chromatography (TLC) flame ionization detector. Details of the extraction and analysis are described elsewhere (11). Results are expressed as percent peak areas and may differ slightly from the true weight percent as described by Tatara and co-workers (13).

Materials. Lipase activity was determined by the olive oil/surfactant non-addition method as described previously (14). One activity unit is described as the amount of enzyme which liberates 1 micromole of free fatty acid per min at 37°C. Commercially available lipases (E.C. 3.1.1.3) in dry powder form were used and were obtained

TABLE 1

Glyceride Composition of the Reaction Mixture Following Glycerolysis of Tallow Using Newly Available Lipases as Catalyst (500 Units/g Fat, 42°C, 800 rpm) (Incubation Time is the Minimum Time Required to Reach the Equilibrium Composition)

Lipase (Manufacturer's name and code)	Composition (%)					
	TG	1,3 DG	1,2 DG	MG[b]	FFA	Incubation time (hr)
Pseudomonas sp. (Amano P)[a]	8.2	11.4	5.4	72.6	2.4	25
Pseudomonas sp. (Nagase P)	52.8	13.0	8.0	25.2	1.1	100
Pseudomonas sp. (Amano AK)	6.4	11.1	4.8	76.4	1.3	45
Pseudomonas sp. (Amano CES)	7.3	12.1	5.1	74.3	1.3	29
Candida rugosa (Amano AY)	99.8	0.2	0	0	0	54
Penicillium camembertii (Amano G)	99.4	0.3	0.3	0	0	45
Geotrichum candidum (Amano GC)	99.6	0.3	0.1	0	0	46

[a]Taken from ref. 11. [b]Mostly 1-MG.

from the following companies: *Pseudomonas* sp. (Amano P, lipase AK and lipase CES), *Candida rugosa* (lipase AY), *Penicillium camembertii* (lipase G), and *Geotrichum candidum* (lipase GC), from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). *Mucor miehei* powder and *Humicola lanuginosa* (SP398), from Novo Nordisk Bioindustry Inc. (Tokyo, Japan). *Pseudomonas* sp. (Nagase P) was from Nagase Biochemical Industry Co. Ltd. (Fukuchiyama, Kyoto-fu, Japan).

Refined fats and oils were provided free of charge from the following companies—Palm Oil Research Institute of Malaysia, palm stearin; Fuji Oil Co. Ltd., Osaka, Japan, palm oil; and Agriculture and Food Development Authority, Fermoy, Co., Cork, Republic of Ireland, beef tallow.

RESULTS

Screening of commercial lipases for glycerolysis activity. The glyceride composition of the reaction mixture after glycerolysis of beef tallow at 42°C using several enzyme preparations is shown in Table 1. The reaction temperature (42°C) was below the critical temperature for tallow and was expected to give an optimum yield of monoglyceride (MG) (11). The highest MG yield was obtained when *Pseudomonas* sp. lipase AK or CES was used. Lipase CES was more active as the maximum MG concentration was reached in half the time required by lipase AK. The previous result obtained with Amano P lipase (11) is shown for comparison and was similar to that for lipase CES. Use of *Pseudomonas* sp. Nagase P lipase resulted in a yield of 25% MG after 100 hours. The low yield may reflect the low activity of this enzyme and a longer incubation time may result in a higher yield. Lipase from *Geotrichum candidum* (lipase GC), *Candida rugosa* (lipase AY) and *Penicillium camembertii* (lipase G) were inactive under the conditions applied here.

Glycerolysis using lipase mixtures. Lipase G *(Penicillium camembertii)* was mixed individually with three

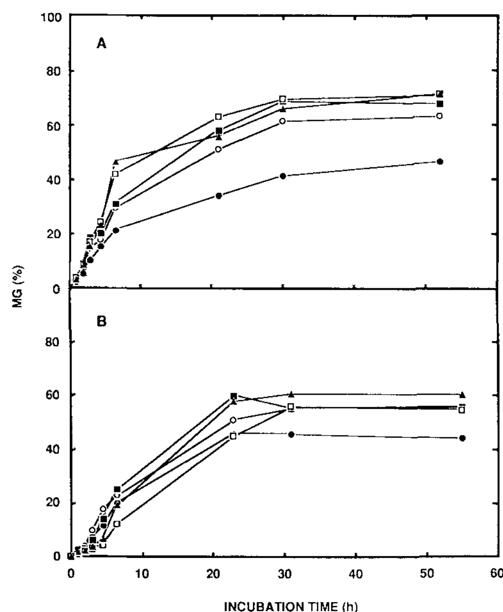

FIG. 1. The effect of mixing lipase G with SP398 lipase on monoglyceride production during enzymatic glycerolysis of tallow and palm oil. A, tallow: 500 units SP398/g fat mixed with lipase G; 0 units (●), 50 units/g fat (○), 100 units/g fat (■), 200 units/g fat (□), 300 units/g fat (▲). B, palm oil: 500 units SP398/g fat mixed with lipase G; 0 units (●), 50 units/g fat (○), 100 units/g fat (■), 200 units/g fat (□), 300 units/g fat (▲).

other lipase preparations in order to improve the rate of glycerolysis or the yield of MG. In Figure 1A, the production of MG during the course of glycerolysis of tallow is shown for mixtures of SP398 lipase (500 units/g fat) with lipase G (50–300 units/g fat). In all cases the final equilibrium yield of MG is higher when a mixture is used as compared to SP398 alone. In general, higher lipase G concentrations resulted in a higher yield and higher

TABLE 2

The Ratio of 1,3 DG to 1,2 DG in the Reaction Mixture During Enzymatic Glycerolysis of Beef Tallow at 42°C (*M. miehei* and SP398 lipases were used at 500 units/g fat and lipase G at 300 units/g fat)

Incubation time (hr)	*M. miehei*	*M. miehei* + lipase G	SP398	SP398 + lipase G
3	0.44	0.67	0.40	0.56
5	0.47	0.75	0.34	0.65
8	0.72	1.08	0.29	0.81
22	1.58	2.13	0.33	2.27
30	2.18	2.75	0.39	2.52

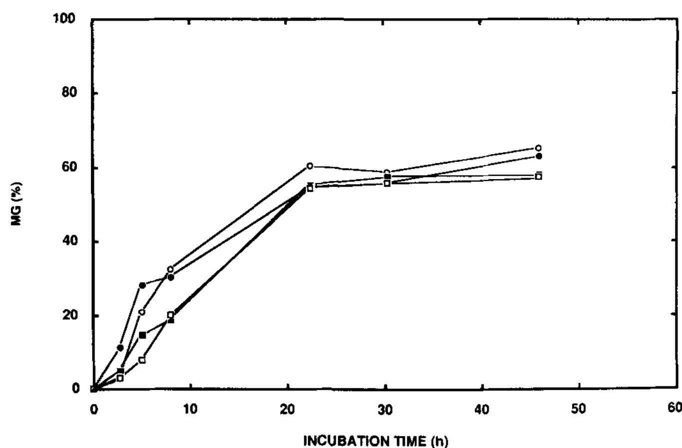

FIG. 2. The effect of mixing lipase G with *Mucor miehei* lipase on monoglyceride production during enzymatic glycerolysis of tallow and palm oil. Tallow: *Mucor miehei* lipase (500 units/g fat) mixed with lipase G; 0 units (●), 200 units/g fat (○). Palm oil: *Mucor miehei* lipase (500 units/g fat) mixed with lipase G; 0 units (■), 200 units/g fat (□).

reaction rate. During the reaction, the major diglyceride (DG) detected in the reaction mixture using SP398 only was 1,2 DG (Table 2). When lipase G was included, the major DG was found to be the 1,3 isomer after 8 hr incubation. Figure 1B shows the effect of mixing SP398 lipase with lipase G on MG production from palm oil. As in the case of tallow, a higher yield of MG was obtained from lipase mixtures as compared to SP398 alone. In this case, however, the equilibrium MG yield was independent of lipase G concentration. The pattern of DG formation was found to be the same as that observed with tallow.

Mixing *Mucor miehei* lipase with lipase G resulted in only a slightly higher yield of MG for tallow and no difference in yield for palm oil (Fig. 2). As shown in Table 2, during the early stage of the reaction mainly 1,2 DG was formed when *Mucor miehei* lipase was used alone, but after 22 hr, the 1,3 isomer became the major DG. In the mixed system the major DG was the 1,3 isomer after 8 hr.

Addition of lipase G to *Pseudomonas* sp. lipase (Amano P) did not result in an increased rate of synthesis or increased equilibrium concentration of MG from tallow (Fig. 3). The highest level of lipase G (300 units/g fat) caused a reduction in the rate of MG synthesis.

Temperature programming. Tallow, palm oil and palm stearin were reacted with glycerol using either *Pseudomonas* sp. (Amano P) lipase, *Mucor miehei* lipase or

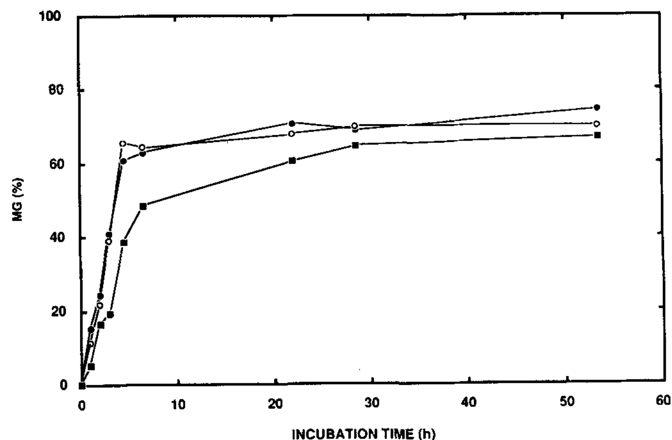

FIG. 3. The effect of mixing lipase G with *Pseudomonas* sp. lipase (Amano P) on monoglyceride production during enzymatic glycerolysis of tallow. *Pseudomonas* sp. lipase (500 units/g fat) mixed with lipase G; 0 units (●), 100 units/g fat (○), 300 units/g fat (■).

TABLE 3

Glyceride Composition in the Reaction Mixture After Enzymatic Glycerolysis of Fats at 42°C (8 hr) Followed by 5°C (4 Days) Using Three Different Enzyme Preparations [Activity of *Pseudomonas* sp. (Amano P) and *M. miehei* lipase powders was 500 units/g fat and lipase G activity was 200 units/g fat]

Fat	Lipase	Composition (wt%) TG	1,3 DG	1,2 DG	MG[a]	FFA
Tallow	Amano P	2.9	4.1	1.7	89.9	1.4
Palm oil	Amano P	2.2	4.7	1.0	90.9	1.3
Palm stearin	Amano P	1.1	2.7	0.9	94.0	1.4
Tallow	*M. miehei*	7.5	5.1	3.6	82.0	1.7
Tallow	*M. miehei*+G	7.7	6.1	3.2	80.5	2.5
Palm oil	*M. miehei*+G	7.9	5.6	3.1	81.3	2.0

[a]Mostly 1-MG.

Mucor miehei + lipase G as catalyst at 42°C for 8 hr and were then transferred immediately to a waterbath at 5°C for a further 4 days. It was decided to try to increase the MG yield using *Mucor miehei* lipase in preference to SP398 lipase, as the former has been commercially available for a long period and is already approved for use in the food industry. The glyceride composition at the end of the reaction is shown in Table 3. In the case of *Pseudomonas* sp. (Amano P) lipase, approximately 90% MG was detected for all three fats examined. In the case of *Mucor miehei* or *Mucor miehei* + lipase G the final MG yield was only approximately 80%. In all cases the free fatty acid content was less than 2.6%. Figure 4 shows the effect of the second temperature on the production of MG during glycerolysis of tallow. It can be seen clearly that as the second temperature was reduced the yield of MG increased. The lowest yield was obtained for the control (73%), which was kept at 42°C throughout the reaction. The rate of MG production during glycerolysis at 5°C was lower than the rates at 10°C to 30°C, although the final MG yield was higher.

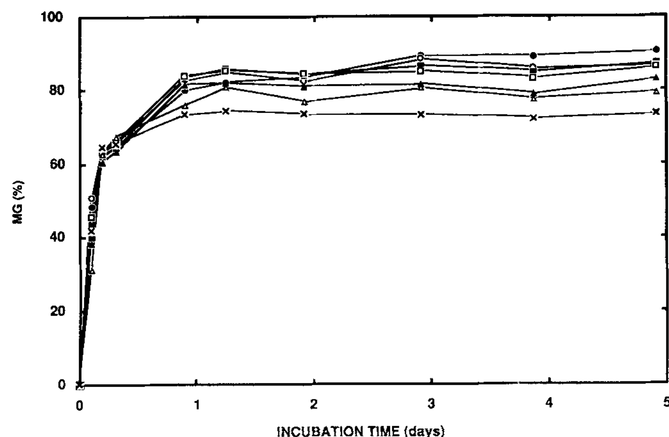

FIG. 4. The effect of temperature programming on MG production during enzymatic glycerolysis of tallow with Amano P lipase. Initial temperature, 42°C for 7 hr followed by incubation at: 5°C (●), 10°C (○), 15°C (■), 20°C (□), 30°C (▲), 35°C (△), and 42°C (×).

DISCUSSION

Previous research in our laboratory established a procedure for efficient production of MG by enzymatic glycerolysis of commonly occurring fats and oils (10–12). In the case of high melting point fats such as tallow and palm oil, a yield of 65–75 wt% MG was obtained at 42°C without the use of organic solvents or emulsifying agents. However, this yield is lower than the MG concentration of "distilled monoglyceride" (>90%), which is manufactured by a chemical process and is a popular, commercially available grade of MG (1). In order to avoid an unnecessary concentration step to upgrade enzyme synthesized MG, it was decided to further investigate the reaction with a view to obtaining an improved MG yield.

Lipases which were not previously available were investigated, but a higher yield of MG than the reported value could not be obtained. As previously found, lipases from *Pseudomonas* sp. were highly active. Surprisingly, the rate of MG production varied considerably among the enzymes even though they were produced by organisms of the same genus. These lipases are reported to differ in their temperature and pH optima (15), characteristics which may be of importance during scale-up of this process. Lipase from *Geotrichum candidum* was inactive and this observation may be partly explained by the known preference for unsaturated fatty acids which this lipase possesses. The lipase from *Penicillium camembertii* (lipase G) exhibits a strong preference for DG and MG as substrate and the observed lack of glycerolysis activity may be due to the virtual absence of DG and MG in refined beef tallow. Lack of glycerolysis activity with *Candida rugosa* lipase was reported previously using an enzyme from a different manufacturer (12).

To provide lipase G with DG substrate, lipase G was mixed with lipases which are active in the glycerolysis system. When mixed with SP398 lipase, the lipase G became active, as shown by an increase in 1,3 DG concentration as compared to the 1,2 DG concentration (Table 2). SP398 is a 1,3 specific lipase and resulted in predominantly 1,2 DG, whereas lipase G is nonspecific and could generate 1,3 DG. More importantly, the MG yield was higher when the enzymes were used in combination as compared to SP398 alone. A combination of lipase G and *Mucor miehei* lipases was only slightly more effective than *Mucor miehei* lipase alone. This may be due to the weak 1,3 specificity of this lipase as shown by DG analysis during the reaction (Table 2). In a related reaction, Park *et al.* (16) showed that a mixture of lipase G with 1,3 specific enzymes from *Rhizopus* sp. efficiently catalyzed hydrolysis of soybean oil compared to the use of single enzymes only.

Although the above results allow the use of a wider range of enzymes in this glycerolysis procedure than previously reported, the yield of MG is not greater than that previously obtained using *Pseudomonas* sp. (Amano P) lipase (11). This indicates that optimum conditions for enzyme activity have been reached and that the final MG concentration is under thermodynamic control.

According to the theory proposed previously (11,12), below a critical temperature (Tc) high melting point MG precipitates from the reaction mixture promoting further synthesis of MG. For high melting point fats, Tc is 35–45°C, but for liquid oils Tc is 5–10°C. During glycerolysis of tallow the less soluble MGs are presumably esters of saturated fatty acids. These will continue to precipitate from the reaction mixture until the tallow is completely depleted of saturated fatty acids. The fatty acid composition of the remaining mixture of glycerides may be calculated by eliminating the saturated fatty acids from the fatty acid composition of tallow. The calculated composition is very similar to that of olive oil. As the Tc for olive oil is 10°C, a reduction in the temperature of the partly reacted tallow should bring about further MG precipitation with a corresponding increase in the MG yield. This was observed to be the case for tallow, palm oil and palm stearin from which a MG yield of approximately 90% was obtained using Amano P lipase as catalyst. It is noted here that MG yield was 90% when olive oil was subjected to glycerolysis at 10°C (12). With *Mucor miehei* lipase the same effect was observed, but a lower final MG concentration was obtained (approximately 80%). Nevertheless, this represents a considerable improvement over the yield of 57% MG previously reported for the single temperature reaction with *Mucor miehei* lipase (12).

The improvements in the Tc method of enzymatic glycerolysis of fats which are reported here result in an extremely high yield of MG and allow a broad range of lipase preparations to be used effectively. In particular, a yield of 80% MG can be obtained using *Mucor miehei* lipase. Using temperature programming with lipase from *Pseudomonas* sp., a yield of 90% MG can be obtained. As this is close to the value found in commercial distilled MG preparations, the MG product produced by the enzymic method may be used without any molecular distillation. The method described here is expected to provide a high quality, inexpensive alternative to the currently used chemical method. However, method(s) of removing or recovering the enzyme included in the solid mixture after the reaction completion must be established in order to realize the lipase-catalyzed solid phase glycerolysis process industrially.

ACKNOWLEDGMENTS

This work was supported in part by the Nakano Foundation and the Commission of the European Communities (DG XII). We thank

Amano Pharmaceutical Co., Ltd., Novo Nordisk Bioindustry Inc., and Nagase Biochemical Co. Ltd., for the lipase preparations.

REFERENCES

1. Swern, D., *Bailey's Industrial Oil and Fat Products Vol. 1,* John Wiley and Sons, 1979, p. 134.
2. Lauridsen, J.B., *J. Am. Oil Chem. Soc. 53*:400 (1976).
3. Sonntag, N.O.V., *Ibid. 59*:795A (1982).
4. Hoq, M.M., T. Yamane and S. Shimizu, *Ibid. 61*:776 (1984).
5. Yamane, T., M.M. Hoq, S. Itoh and S. Shimizu, *J. Jpn. Oil Chem. Soc. 35*:625 (1986).
6. Yamane, T., M.M. Hoq, S. Itoh and S. Shimizu, *Ibid. 35*:632 (1986).
7. Fletcher, P.D.I., R. Freedman, B. Robinson, G. Rees and R. Schomacker, *Biochim. Biophys. Acta 912*:278 (1987).
8. Holmberg, K., and E. Österberg, *J. Am. Oil Chem. Soc. 65*:1544 (1988).
9. Holmberg, K., B. Lassen and M. Stark, *Ibid. 66*:1796 (1989).
10. McNeill, G.P., S. Shimizu and T. Yamane, *Inforam 1*:304 (1990).
11. McNeill, G.P., S. Shimizu and T. Yamane, *J. Am. Oil Chem. Soc. 67*:779 (1990).
12. McNeill, G.P., and T. Yamane, *Ibid.,* in press (1990).
13. Tatara, T., T. Fujii, T. Kawase and M. Minagawa, *Lipids 18*:732 (1983).
14. Yamane, T., *J. Jpn. Oil Chem. Soc. 36*:638 (1987).
15. Lipase Catalog, Amano Pharmaceutical Co. Ltd., 1990.
16. Park, Y.K., G.M. Pastore and M.M. de Almeida, *J. Am. Oil Chem. Soc. 65*:252 (1988).

[Received June 28, 1990; accepted November 23, 1990]