Control of Lipase-Mediated Glycerolysis Reactions with Butteroil in Dual Liquid Phase Media Devoid of Organic Solvent

Baokang Yang,[†,‡,§] W. James Harper,[∥] and Kirk L. Parkin*[,‡]

Center for Dairy Research and Department of Food Science, University of Wisconsin, 1605 Linden Drive, Madison, Wisconsin 53706

Factors affecting the progress of glycerolysis reactions with butteroil mediated by two lipase preparations (from *Pseudomonas* sp.) in dual liquid phase mixtures devoid of solvent were evaluated. Within the range of parameters evaluated, the conditions best supporting the formation of monoacylglycerols (MAG) were 35 °C, 2.5–4.8% water in glycerol, and 0.33–0.44 g of glycerol/g of butteroil (equivalent to a molar ratio of acyl groups to glycerol of 0.66–0.85). Under these conditions, percent yields of MAG formation from butteroil were 50–55% (mass fraction) relative to the other acylglycerol species and fatty acids accumulated. Little interaction was observed between these critical parameters. At temperatures greater than 35 °C, up to a 50% reduction in yield of MAG was noted. Temperature shift experiments indicated that MAG and other acylglycerol components were interconvertible. Incubation of reactive mixtures at 35 °C after a prior incubation at 52 °C restored the yield of MAG from about 25% to the near-maximal levels of 50–55%. The effect of this temperature shift was manifest as a net and reversible interconversion of MAG and 1,3-DAG, and changes in other acylglycerol components did not take place. Temperature control of reaction equilibria was not attributable to changes in availability of water.

INTRODUCTION

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) have been studied for the purpose of producing monoacylglycerols (MAG) and diacylglycerols (DAG) from different fats and oils via hydrolysis (Holmberg and Osterberg, 1988), esterification (Tsujisaka et al., 1977; Ergan et al., 1990; Akoh et al., 1992), and acyl-transfer or alcoholysis reactions (Schuch and Mukherjee, 1989; Holmberg et al., 1989; McNeill et al., 1990). Reaction mixtures have been configured as reverse micelles with anhydrous solvents as the continuous phase (Holmberg et al., 1989), with enzyme suspended in organic solvents (Pecnik and Knez, 1992; Akoh et al., 1992), or with enzyme simply suspended in substrate without added solvent (Tsujisaka et al., 1977; Schuch and Mukherjee, 1989; McNeill et al., 1990, 1991, 1992; Weiss, 1990; Ergan et al., 1990; McNeill and Yamane, 1991). Reaction parameters have been studied for each configuration in attempts to optimize MAG production. While optimization of each reaction configuration is important to ultimately judge its technical feasibility, what appears to be lacking is a proper comparison of reaction configurations that would allow evaluations of relative process feasibility. Furthermore, reaction parameters have been studied often in terms of their singular effects, with little regard for interaction between these parameters.

We set out to evaluate the reaction parameters that control lipase-mediated glycerolysis reactions with butteroil in reaction configurations containing, and devoid of, solvent (2-methyl-2-propanol). Two lipases from *Pseudomonas* sp. were used since they are equally effective in both reaction configurations (Yang et al., 1993a). Our results are presented in this and the preceding companion paper (Yang et al., 1993b).

* Author to whom correspondence should be addressed [telephone (608) 263-2011].

† Center for Dairy Research.

‡ Department of Food Science.

§ Present address: Department of Food Science and Animal Industries, Alabama A&M University, P.O. Box 264, Normal, AL 35762.

∥ Present address: Department of Food Science and Technology, The Ohio State University, 2121 Fyffe Road, Columbus, OH 43210.

EXPERIMENTAL PROCEDURES

Materials. The same lots of lipase preparations from *Pseudomonas* sp., classified as type AK and PS-30, that were used in the preceding paper (Yang et al., 1993b) were also used here (obtained from Amano International Enzyme Co., Inc., Troy, VA). These preparations were used at the same level of addition in the reactive mixtures studied and without any further purification or modification unless otherwise stated. Hexane, acetone, chloroform, and 2-methyl-1-propanol were of high-performance liquid chromatography (HPLC) grade (Aldrich Chemical Co., Milwaukee, WI). Glycerol and glycerol esters of dodecanoic (lauric) acid were obtained from Sigma Chemical Co. (St. Louis, MO). Anhydrous butteroil (<0.15% moisture, Level Valley Dairy, West Bend, WI) was stored at 2–4 °C prior to use. When needed, the butteroil was melted at 50 °C. All water used was distilled and deionized. Other reagents used were of reagent grade or the best grade commercially available.

To evaluate the effect of pH on enzyme activity (only for lipase PS-30), samples and controls were prepared as described in the preceding paper (Yang et al., 1993b). The water content of the glycerol reagent (about 0.1%) and lipase preparations was determined by the Karl Fischer method.

Reaction Mixtures and Analysis for Acylglycerol Components. Unless otherwise stated, the reaction mixtures contained 4.5 g of butteroil, 2.0 g of glycerol, and 40 μL of water in a closed 50-mL flask, and the contents were incubated at 35 °C in a rotary shaker at 300 rpm. Reactivity was initiated by the addition of 250 mg of lipase powder. Sample handling and analysis involved the same procedures described in the preceding paper (Yang et al., 1993b). Triacylglycerol (TAG), 1,3- and 1,2-(2,3)-diacylglycerol (DAG), monoacylglycerol (MAG), and FA component contents are reported as mass fractions. Each experiment was done four times; the coefficient of variation averaged about 4% and was always less than 10% for each component analysis.

RESULTS AND DISCUSSION

Time Course of Glycerolysis. The progress of glycerolysis for lipases AK (Figure 1A) and PS-30 (Figure 2A)

0021-8561/93/1441-1905$04.00/0

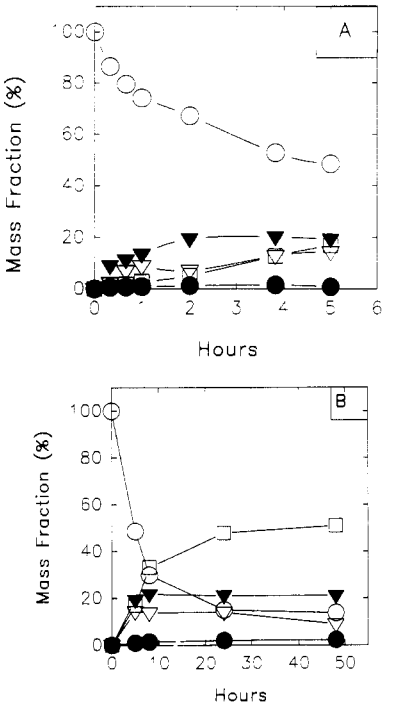

Figure 1. Time course of glycerolysis mediated by lipase AK after short- (A) or long-term (B) incubation. Reactive mixtures contained 4.5 g of butteroil, 2.0 g of glycerol (2.1% water), and 250 mg of lipase AK and were incubated at 35 °C. Acylglycerol species are designated TAG (○), MAG (□), 1,2-DAG (▼), 1,3-DAG (▽), and FA (●).

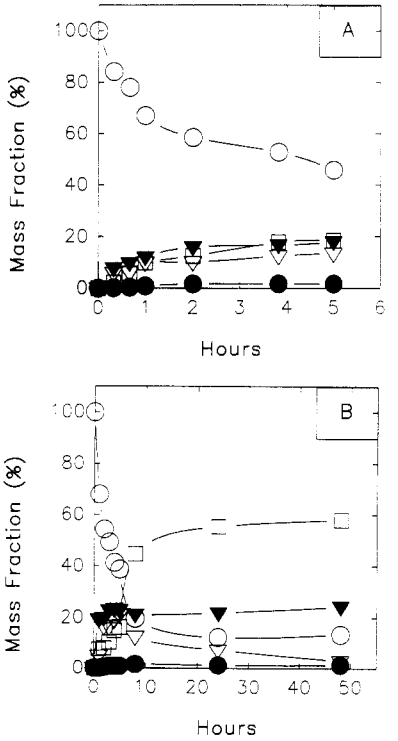

Figure 2. Time course of glycerolysis mediated by lipase PS-30 after short- (A) or long-term (B) incubation. Reactive mixtures contained 4.5 g of butteroil, 2.0 g of glycerol (2.1% water), and 250 mg of lipase PS-30 and were incubated at 35 °C. Symbols are the same as for Figure 1.

indicated that after the first 5 h of reaction, only about 50% transformation of TAG took place for both enzymes. The products of the reaction during the initial 5-h period were almost evenly distributed (mass basis) between MAG, 1,2(2,3)-DAG, and 1,3-DAG, with only trace quantities of

Table I. Interconversion of Glycerol Esters of Dodecanoic Acid[a]

initial reactants	hours at 52 °C	mass fraction (%) of acylglycerol components before and after 24 h of incubation at 52 °C		
		TAG	DAG	MAG
MAG	0	0	0	100
	24	30	33	37
MAG + DAG	0	0	48	52
	24	26	45	27
MAG + TAG	0	56	0	44
	24	37	40	23

[a] Reaction mixtures contained 100 mg of each acylglycerol as indicated (MAG, *rac*-dodecanoylglycerol; DAG, *rac*-didodecanoylglycerol; TAG, tridodecanoylglycerol) and 25 mg of lipase PS-30. Mixtures without added enzyme showed no change in distribution of acylglycerol species, and ≤2% FA accumulation was observed in mixtures with or without enzyme.

FA accumulating. This is in sharp contrast to the reaction taking place in the presence of solvent in which very little 1,3-DAG accumulated at any point during the progress of reaction (Yang et al., 1993b).

In view of the reported (Berger and Schneider, 1991) hydrolytic regioselectivity (*sn*-1,3) of the lipases used, production of 1,3-DAG can arise from acyl migration of the 1,2(2,3)-DAG species or acylation at the *sn*-1(3) position of an *sn*-1(3)-MAG. The latter possibility assumes reversibility of the reaction steps involved in glycerolysis. The reactions involved in MAG production were reversible (Table I). Simple reaction mixtures containing individual or selected combinations of glycerol esters of dodecanoic acid yielded the full spectrum of acylglycerol species (MAG, DAG, and TAG) after 24 h of incubation.

During more extended periods of incubation up to 24–48 h, about 85% transformation of TAG took place as MAG accumulated to a level of about 50–60% of the total products in the mixture (Figures 1B and 2B). The secondary reaction product was 1,2(2,3)-DAG (20–25%), whereas 1,3-DAG levels were limited to 10% (lipase AK) or less (lipase PS-30), and FA accumulation remained below 5% of the product mixture with both lipases.

The time frame of lipase-mediated glycerolysis observed here is similar to those previously reported for reaction mixtures devoid of organic solvents (McNeill et al., 1990, 1991, 1992; Ergan et al., 1990; McNeill and Yamane, 1991; Yang et al., 1993a). Reaction progress was substantially faster in the presence of solvent (Yang et al., 1993b) than in its absence (this paper). Thus, a single liquid reaction phase appears to offer some kinetic advantage over the biphasic liquid reaction mixture. However, steady-state acylglycerol product profiles and yield of MAG were similar for both reaction configurations.

In the presence of solvent, lipase AK was more active than lipase PS-30 (Yang et al., 1993b), whereas in the absence of solvent the reverse was true (this study). This difference may be related to the structural differences between the enzymes and resultant relative efficiencies of the enzymes in single or dual phase liquid media. The relative activity of many lipases on different substrates varies with the nature of the substrate and whether the reaction medium is homogeneous or heterogeneous (Vorderwülbecke et al., 1992).

Effect of pH. For lipase PS-30, both initial reaction velocities and yield of MAG after 24 h were maximal at pH 7.5 and declined markedly at pH 6 and 9 (Figure 3). The pH of an enzyme solution was about 7.8, and after lyophilization, the enzyme had activity slightly less than that observed after adjustment to optimum pH (7.5). The enzyme reagent used without any modification provided

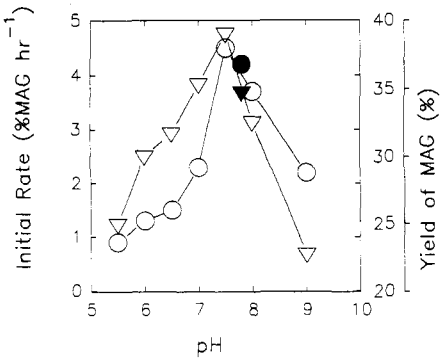

Figure 3. Effect of pH on rate and extent of glycerolysis for lipase PS-30. Reactive mixtures contained 4.5 g of butteroil, 2.0 g of glycerol (2.1% water), and 250 mg of lipase PS-30 and were incubated at 35 °C. Solid symbols represent enzyme dissolved in water and lyophilized without pH adjustment. Open symbols represent pH-adjusted enzyme. Circles indicate percent yield MAG after 24 h, and triangles indicate initial rates of MAG formation.

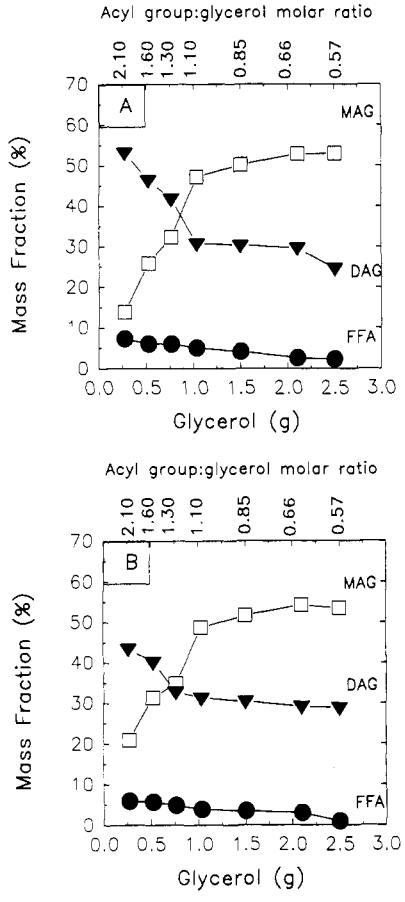

Figure 4. Effect of glycerol content on extent of glycerolysis. Reactive mixtures contained 4.5 g of butteroil, 250 mg of lipase AK (A) or PS-30 (B), and various levels of glycerol (2.1% water) and were incubated for 24 h at 35 °C.

for an initial rate of 6.3% MAG/h and a yield of MAG of 54% after 24 h of incubation. Thus, there was no advantage in modifying the pH of the enzyme reagent as supplied by the manufacturer. A similar behavior of the enzyme was noted for the solvent-based reaction mixtures (Yang et al., 1993b).

Effect of Glycerol Concentration. As glycerol was increased to 100 mg/mL, the yield of MAG after 24 h progressively increased to about 50% for both lipases (Figure 4). The yield of MAG increased slightly at greater glycerol levels. Steady-state DAG levels decreased to 25–30% as glycerol was increased to 75–100 mg/mL, whereas FA levels declined with increasing glycerol content throughout the range evaluated.

Maximum percent yields of MAG were observed when the fatty acyl groups/glycerol molar ratio was reduced to 0.66–0.85, and further reduction in this ratio had little effect. The optimum range of fatty acyl groups/glycerol molar ratio was similar to that found (0.61–0.76) for reaction mixtures in the presence of 2-methyl-2-propanol (Yang et al., 1993b). Other studies of lipase-mediated synthesis of MAG used fatty acyl groups/glycerol ratios of 0.77–1.0 (Schuch and Mukherjee, 1989; McNeill et al., 1990; Ergan et al., 1990), although only one (Ergan et al., 1990) evaluated various glycerol levels as a reaction parameter.

These results for reaction mixtures devoid of solvent are in marked contrast to those observed for the solvent-based reaction mixtures with lipase PS-30. In the latter case, elevated levels of glycerol were inhibitory, and this was attributed to the 2-methyl-2-propanol/glycerol mixture's stripping essential water from the lipase (Yang et al., 1993b). For reaction mixtures devoid of solvent, glycerol was not inhibitory, and this is consistent with other studies on biphasic reaction mixtures designed for MAG production (Ergan et al., 1990; Holmberg et al., 1989). In the biphasic solvent-free system, any water-sorbing effect of glycerol may be ameliorated by the aqueous glycerol phase remaining proximal to the enzyme. Thus, the net dilution effect of glycerol in the biphasic (solvent-free) system is probably less extensive than in the solvent-based system in which 2-methyl-2-propanol also will desorb water from the enzyme.

Effect of Added Water Content. For lipase AK at least 40 μL [2% (w/w) of glycerol phase] of added water was necessary to obtain maximal yield of MAG (Figure 5A), whereas for lipase PS-30 40–80 μL (2–4% of glycerol phase) of added water was necessary to obtain maximal yield of MAG (Figure 5B). Above these levels of added water, FA accumulation progressively increased with increased addition of water. The slight difference in optimum water contents between the enzymes is probably because the lipase AK preparation had a greater initial moisture content (6.5%) than did the PS-30 preparation (2.4%). Other lipase-mediated processes designed for MAG production have optimal water contents in the range of 0.5% for reverse micelles (Holmberg et al., 1989) and 2–4% (McNeill et al., 1990, 1991; McNeill and Yamane, 1991) to <10% (Weiss, 1990) in solvent-free media.

Initial rates of MAG formation were also dependent on added water content (data not shown) and paralleled the trends in Figure 5. Maximal initial rates for MAG formation of 2.1–2.8% and 4.1–5.7% MAG/h were observed at 40–160 μL of added water for lipases AK and PS-30, respectively, whereas at 0 μL of added water the initial rates of MAG formation were 0.3–0.5% MAG/h. Other studies found maximal initial rates of MAG formation at 2–6% of added water (McNeill et al., 1990, 1991), and FA tend to accumulate at greater rates and to greater extents when water is provided in slight excess (Holmberg et al., 1989; McNeill et al., 1990, 1991). Caution must be exercised when optimum water contents are compared between studies of reactions in microaqueous organic media because all sources of water are important. Accounting for all sources of water, including enzyme, glycerol, and butteroil, optimal water content in our reaction mixtures was 50–97 μL [2.5–4.8% (w/w) of the glycerol phase, or 0.8–1.5% (v/v) of the total reaction mixture] for both lipases.

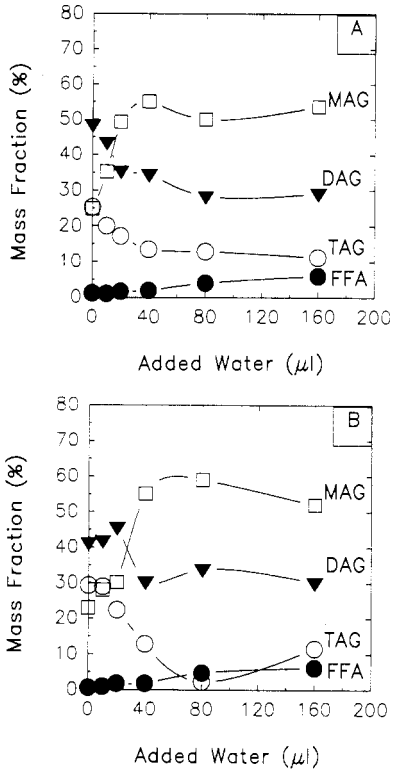

Figure 5. Effect of added water on extent of glycerolysis. Reactive mixtures contained 4.5 g of butteroil, 2.0 g of glycerol (0.1% water), and 250 mg of lipase AK (A) or PS-30 (B), with various amounts of water added. Reactive mixtures were incubated at 35 °C, and mass fractions of reactive components were determined after 24 h of incubation.

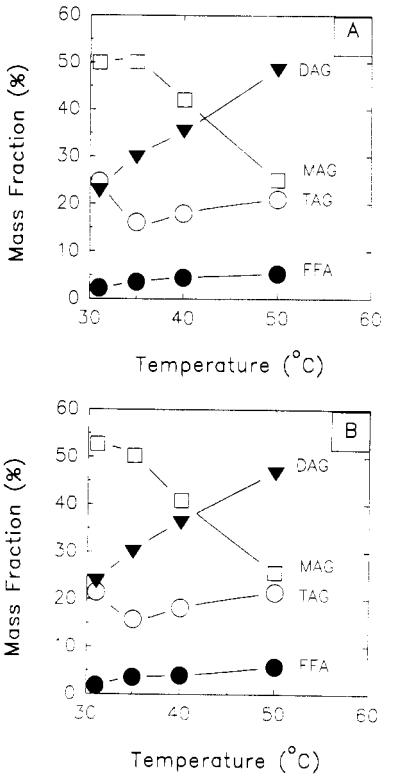

Figure 6. Effect of temperature on extent of glycerolysis. Reactive mixtures contained 4.5 g of butteroil, 2.0 g of glycerol (2.1% water), and 250 mg of lipase AK (A) or PS-30 (B). Mass fractions of reactive components were determined after 24 h of incubation.

Effect of Temperature. For both lipases, as reaction temperature was increased from 35 to 50 °C, a progressive decrease in percent yield of MAG after 24 h was noted (Figure 6). This corresponded to a reciprocal increase in DAG, whereas TAG and FA levels remained relatively constant. The decreased yield of MAG was not because of thermal inactivation of the enzyme. Reaction mixtures containing either enzyme preparation initially incubated at 50 °C yielded 25% MAG after 24 h, and after a decrease in temperature to 40 °C and subsequent 24-h incubation, the resultant yield of MAG increased to about 50% (data not shown). Others have noted a similar dependence of percent yield MAG on temperature in reaction mixtures void of solvent (McNeill et al., 1990, 1991, 1992; Weiss, 1990). Recent work indicates that progressive solidification of some of the newly formed MAG may effectively direct the reaction toward the formation of MAG (McNeill et al., 1992).

The effect of temperature was reversible over an extended period of time (Figure 7). The net and reciprocal change between MAG and DAG took place specifically for the 1,3-DAG species, whereas the levels of 1,2(2,3)-DAG species remained relatively constant. Increasing temperature from 30 to 50 °C also reduced the yield of MAG in reaction mixtures containing 2-methyl-2-propanol (Yang et al., 1993b). In this latter case, water availability appeared to be the critical factor controlling acylglycerol transformation since supplementation of reaction mixtures incubated at 55 °C with additional water restored yields of MAG to 50–60%. Increasing the added water in the solvent-free reaction mixtures from 40 to 160 μL increased the initial rate of MAG production (about 3-fold) at 50 °C but did not affect the steady-state yield of MAG, which remained at about 25%. Thus, any restriction in water availability at elevated temperatures had little net effect on reaction equilibria that affect the steady-state yield of MAG.

Conclusions. Conditions optimum for MAG production from butteroil in a dual-liquid phase medium in the presence of glycerol and in the absence of organic solvent by lipases from *Pseudomonas* sp. were established. Critical factors include (1) water content of the enzyme and glycerol, (2) molar ratio of fatty acyl groups to glycerol, and (3) temperature. Only slight interaction between these parameters was observed, in contrast to the substantial interaction between these parameters for similar reaction mixtures dispersed as a single liquid phase in 2-methyl-2-propanol (Yang et al., 1993b).

The most important differences between reaction mixtures containing 2-methyl-2-propanol and those void of solvent were the effects of glycerol and temperature. Elevated glycerol content was not inhibitory in the absence of solvent, whereas it was in the presence of solvent for lipase PS-30 (Yang et al., 1993b). This inhibition was relieved by the addition of water. Elevating the temperature from 35 to over 50 °C reduced the yield of MAG by half in both reaction configurations. In the presence of 2-methyl-2-propanol, this inhibition was relieved by the addition of small amounts of water (Yang et al., 1993b), whereas in the reaction mixtures without added solvent addition of water had no effect on steady-state levels of acylglycerol products. Thus, reaction equilibria as controlled by temperature were dependent on different factors in reaction mixtures containing or devoid of solvent.

A similarity in reaction control for both reaction configurations was that glycerol levels (*viz.* fatty acyl groups/glycerol molar ratios) could be manipulated to obtain various ratios of MAG to DAG in the product mixture.

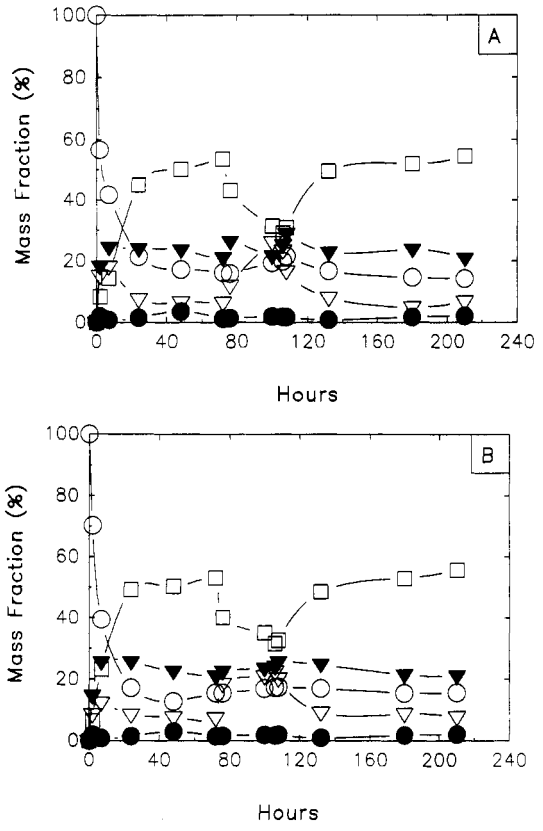

Figure 7. Effect of temperature shifts on progress of glycerolysis. Reactive mixtures contained 4.5 g of butteroil, 2.0 g of glycerol (2.1% water), and 250 mg of lipase AK (A) or PS-30 (B). Mixtures were incubated at 35 °C for 0–72 h, at 52 °C for 72–108 h, and at 35 °C for 108–210 h.

ABBREVIATIONS USED

MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; FA, fatty acid.

ACKNOWLEDGMENT

This work was supported by the National Dairy Promotion and Research Board, the Wisconsin Milk Marketing Board, and the University of Wisconsin—Madison Center for Dairy Research and the College of Agricultural and Life Sciences. We thank Amano International Enzyme Co. for the gift of the lipase preparations.

LITERATURE CITED

Akoh, C. C.; Cooper, C.; Nwosu, C. V. Lipase G-catalyzed synthesis of monoglycerides in organic solvent and analysis by HPLC. *J. Am. Oil Chem. Soc.* **1992**, *69*, 257–260.

Berger, M.; Schneider, M. P. Regioselectivity of lipases in organic solvents. *Biotechnol. Lett.* **1991**, *13*, 333–338.

Christie, W. W. The composition and structure of milk lipids. In *Developments in Dairy Chemistry*—2; Fox, P. F., Ed.; Applied Science: New York, 1983; pp 1–35.

Ergan, F.; Trani, M.; André, G. Production of glycerides from glycerol and fatty acid by immobilized lipases in non-aqueous media. *Biotechnol. Bioeng.* **1990**, *35*, 195–200.

Holmberg, K.; Osterberg, E. Enzymatic preparation of monoglycerides in microemulsion. *J. Am. Oil Chem. Soc.* **1988**, *65*, 1544–1548.

Holmberg, K.; Lassen, B.; Stark, M-B. Enzymatic glycerolysis of a triglyceride in aqueous and nonaqueous microemulsions. *J. Am. Oil Chem. Soc.* **1989**, *66*, 1796–1800.

Krog, N. J. Food emulsifiers and their chemical and physical properties. In *Food Emulsions*; Larsson, K., Friberg, S. E., Eds.; Dekker: New York, 1990; pp 127–180.

McNeill, G. P.; Yamane, T. Further improvement in the yield of monoglycerides during enzymatic glycerolysis of fats and oils. *J. Am. Oil Chem. Soc.* **1991**, *68*, 6–10.

McNeill, G. P.; Shimizu, S.; Yamane, T. Solid phase enzymatic glycerolysis of beef tallow resulting in a high yield of monoglyceride. *J. Am. Oil Chem. Soc.* **1990**, *67*, 779–783.

McNeill, G. P.; Shoichi, S.; Yamane, T. High-yield enzymatic glycerolysis of fats and oils. *J. Am. Oil Chem. Soc.* **1991**, *68*, 1–5.

McNeill, G. P.; Borowitz, D.; Berger, R. G. Selective distribution of saturated fatty acids into the monoglyceride fraction during enzymatic glycerolysis. *J. Am. Oil Chem. Soc.* **1992**, *69*, 1098–1103.

Pecnik, S.; Knez, Z. Enzymatic fatty ester synthesis. *J. Am. Oil Chem. Soc.* **1992**, 69, 261–265.

Schuch, R.; Mukherjee, K. D. Lipase-catalyzed reactions of fatty acids with glycerol and acylglycerols. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 322–336.

Sonntag, N. O. V. Glycerolysis of fats and methyl esters—status, reviews and critique. *J. Am. Oil Chem. Soc.* **1982**, *59*, 795A–802A.

Tsujisaka, Y.; Okumura, S.; Iwai, M. Glyceride synthesis by four kinds of microbial lipases. *Biochim. Biophys. Acta* **1977**, *489*, 415–422.

Vorderwülbecke, T.; Kieslich, K.; Erdmann, H. Comparison of lipases by different assays. *Enzyme Microb. Technol.* **1992**, *14*, 631–639.

Weiss, V. A. Enzymatische herstellung von festen fettsäuremonoglyceriden. *Fat Sci. Technol.* **1990**, *92*, 392–396.

Yang, B.; Chen, J. Analysis of neutral lipids and glycerolysis products from olive oil by liquid chromatography. *J. Am. Oil Chem. Soc.* **1991**, *68*, 980–982.

Yang, B.; Harper, W. J.; Parkin, K. L.; Chen, J. Screening of commercial lipases for production of mono- and diacylglycerols from butteroil by enzymic glycerolysis. *Int. Dairy J.* **1993a**, in press.

Yang, B.; Harper, W. J.; Parkin, K. L. Control of lipase-mediated glycerolysis reactions with 2-methyl-2-propanol. *J. Agric. Food Chem.* **1993b**, preceding paper in this issue.

Received for review April 9, 1993. Revised manuscript received August 10, 1993. Accepted September 7, 1993.®

® Abstract published in *Advance ACS Abstracts*, October 15, 1993.