Control of Lipase-Mediated Glycerolysis Reactions with Butteroil in Single Liquid Phase Media with 2-Methyl-2-propanol

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Factors affecting the progress of glycerolysis reactions with butteroil mediated by two lipase preparations (from *Pseudomonas* sp.) in single liquid phase mixtures containing 2-methyl-2-propanol were evaluated. Within the range of parameters evaluated, the conditions best supporting the formation of monoacylglycerols (MAG) were 25–40% butteroil substrate, 10–20 mg of enzyme/mL of substrate mixture, 35 °C, and 100–150 mg of glycerol/mL of substrate mixture (equivalent to a molar ratio of fatty acyl groups to glycerol of 0.61–0.76). Under these conditions, the percent yield of MAG formation from butteroil was 50–60% (mass fraction) relative to the other acylglycerol species and fatty acids accumulated. These conditions were somewhat dependent on the source of enzyme and probably related to the water content of the enzyme reagents. At temperatures greater than 35 °C, and at the greatest levels of glycerol (150 mg/mL) used, a severe restriction of activity was noted, particularly for the lipase preparation with the lesser water content. However, activity and percent yields of MAG could be restored to near-optimal levels by the simple addition of water of up to 1% of the reaction volume. The ability of the chosen lipases to mediate glycerolysis reactions with butteroil, and other oils, appears to be principally controlled by water availability in the reaction mixture.

INTRODUCTION

Mixtures of monoacylglycerols (MAG) and diacylglycerols (DAG) are widely used as emulsifiers in the food and pharmaceutical industries (Krog, 1990). Typically, these emulsifiers are prepared from triacylglycerol (TAG)-rich oils and glycerol using alkaline catalysts and temperatures above 200 °C (Krog, 1990; Sonntag, 1982). Limitations of this process include maximum product yields of about 40–60% and the formation of undesirable byproducts.

Enzymic methods have been evaluated for the preparation of mixtures of MAG and DAG from TAG-rich lipids. These processes use lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) in reaction systems configured as reverse micelles with organic solvents as the continuous phase (Holmberg et al., 1989) or with the enzyme simply suspended in these solvents (Pecnik and Knez, 1992). Using this approach, mixtures of MAG and DAG are formed by acyl-transfer (alcoholysis) reactions between the native lipid substrate and added glycerol. MAG and DAG can also be produced from TAG-rich oils through hydrolytic processes (Holmberg and Osterberg, 1988), but this approach is subject to limits in yields of these products because much of the original substrate is liberated as fatty acids. Perhaps the most promising area of these most recent efforts is the approach in which lipase is simply combined with glycerol and the oil to be modified in the absence of organic solvent (Schuch and Mukherjee, 1989; McNeill et al., 1990, 1991, 1992; McNeill and Yamane, 1991). Proper selection of temperature provides for yields of MAG approaching 90% (mass basis) for several food lipids evaluated (NcNeill et al., 1991, 1992; McNeill and Yamane, 1991). Different sources of lipase display different degrees of reactivity, and organic solvents are not required to facilitate reaction.

Our initial efforts to prepare mixtures of MAG and DAG from butteroil focused on the screening of several lipases for their ability to promote glycerolysis reactions with butteroil (Yang et al., 1993a). Reactivity was dependent on the source of lipase and whether reaction mixtures contained an organic solvent as the principal component of the continuous phase. The primary physical effect of organic solvent (specifically, 2-methyl-2-propanol) in supporting glycerolysis reactions is to promote a single liquid phase, whereas in the absence of added solvent two immiscible liquid phases exist. This difference in physical properties between reaction mixtures with and without added solvent prompted our current efforts to develop an understanding of the factors that influence glycerolysis activity and how these factors interact in reaction systems containing, or void of, organic solvent. Butteroil was chosen as substrate because of its regional economic importance, as well as its heterogeneous nature. The wide range of fatty acyl constituents in butteroil (Christie, 1983) implies that our observations with butteroil are likely to be applicable to other TAG-rich oils. Our findings are reported in this and the companion paper (Yang et al., 1993b).

MATERIALS AND METHODS

Materials. Commercial lipase preparations were obtained from *Pseudomonas* sp. and classified as types AK (lot 7070TY, 6.5% moisture, 22.0 units/mg) and PS-30 (lot LPSA009517, 2.4% moisture, 33.8 units/mg) and supplied by Amano International Enzyme Co., Inc. The units of activity were stipulated by the manufacturer as the amount required to liberate 1 μmol of fatty acid/min from an olive oil emulsion at 37 °C and pH 7.7.

Hexane, acetone, chloroform, and 2-methyl-2-propanol were of high-performance liquid chromatography (HPLC) grade (Aldrich Chemical Co., Milwaukee, WI). Anhydrous butteroil

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(<0.15% moisture; Level Valley Dairy, West Bend, WI) was stored at 2–4 °C prior to use. Molecular sieves, glycerol, and other oils were obtained from Sigma Chemical Co. (St. Louis, MO). When needed, the oils were dissolved in 2-methyl-2-propanol at 30 °C as a 25% (w/w) solution, and about 5% (w/v) molecular sieves (3 Å, Sigma) were added 24 h prior to use to remove any residual moisture. All water used was distilled and deionized. Other reagents used were of reagent grade or the best grade commercially available.

Reaction Mixtures. Unless otherwise stated, reaction mixtures contained 10 mL of 25% (w/w) butteroil in 2-methyl-2-propanol and 1.0 g of glycerol (PS-30 lipase) or 1.5 g of glycerol (AK lipase) 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 200 mg of lipase powder. At selected intervals, 0.25 mL of the reaction mixture was removed and added to 0.5 mL of chloroform, and the mixtures were passed through a 0.45-μm membrane to remove the lipase powder. Prior studies indicated that this procedure quenched enzyme activity and no further changes in product profile took place. 2-Methyl-2-propanol was not reactive (detection limit of 0.1% mass fraction for any product component) in these mixtures (Yang et al., 1993a).

Analysis of Reaction Mixtures. The filtered extracts were analyzed for acylglycerol and fatty acid components using an HPLC method previously reported (Yang and Chen, 1991). A normal phase silica column (Econosil, 250 mm × 4.6 mm, 5-μm packing; Alltech Associates, Deerfield, IL) was used at 30 °C with gradient elution at 1.5 mL/min. Triacylglycerol (TAG), 1,3- and 1,2(2,3)-diacylglycerol (DAG), monoacylglycerol (MAG), and fatty acid (FA) components were resolved and detected by a light scattering detector (model ELSD II, Varex, Rockville, MD) operated at 81 °C, and the relative proportions of these components are reported as mass fractions. Initial rate data were derived from samples taken within the first 20–30 min of reaction. Data points represent the mean of four replicates; the coefficient of variation averaged about 4% and was always less than 10%.

Enzyme pH Adjustment. To evaluate the effect of pH on enzyme activity, 200 mg of lipase powder was combined with 0.6 mL of 0.1 M sodium phosphate buffer (pH 5.5–9.0) and then lyophilized. A control sample was prepared in a similar fashion except that water was substituted for buffer.

Analysis of Water Content. The water content of the glycerol reagent (range <1.0–6.3% water) and lipase preparations was determined by the Karl Fischer method.

Estimation of Molarity of Acyl Groups in Butteroil. The average molecular weight of a butteroil fatty acyl residue and TAG (and, thus, molar concentrations) was estimated from the data provided by Christie (1983) on the molar composition and distribution of FA along the glycerol backbone and the TAG content (97.1%) of butteroil. On the basis of our calculations, average molecular weight estimations for a fatty acyl group and TAG of butteroil are 219 and 699, respectively.

RESULTS AND DISCUSSION

Choice of Enzymes. The enzymes used in this study are reported to be regioselective (and not regiospecific) for the *sn*-1,3 positions along the glycerol backbone in terms of hydrolytic activity (Berger and Schneider, 1991) but nonspecific in terms of esterification activity between FA and glycerol (Berger et al., 1992). The enzymes were used without any further purification or modification except for the evaluation of the effect of enzyme pH on activity. These enzymes were chosen for study because, of those screened by us (Yang et al., 1993a), they best supported glycerolysis and did so equally well in the presence or absence of solvent. The relationship, if any, between hydrolytic and glycerolysis or other lipase activities is not clear or predictable (Yang et al., 1993a; Vorderwülbecke et al., 1992). Therefore, we routinely used these enzyme reagents at equal levels.

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

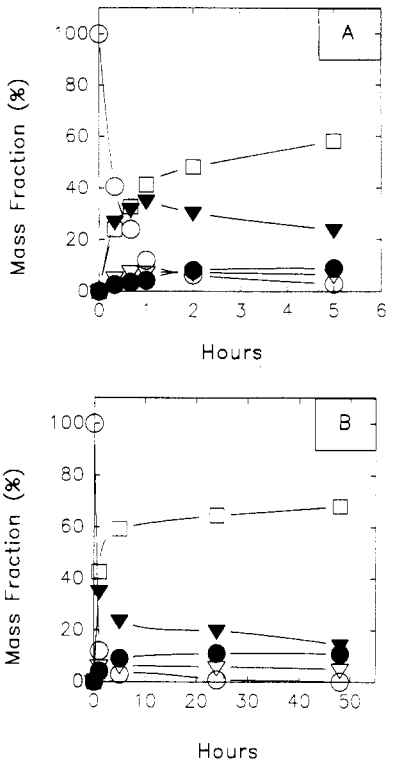

Figure 1. Time course of glycerolysis mediated by lipase AK after short- (A) or long-term (B) incubation. Reactive mixtures contained 10 mL of 25% butteroil in 2-methyl-2-propanol, 1.5 g of glycerol (6.6% water), and 200 mg of lipase AK and were incubated at 35 °C. Acylglycerol species are designated ○ (TAG), □ (MAG), ▼ (1,2-DAG), ▽ (1,3-DAG), and ● (FA).

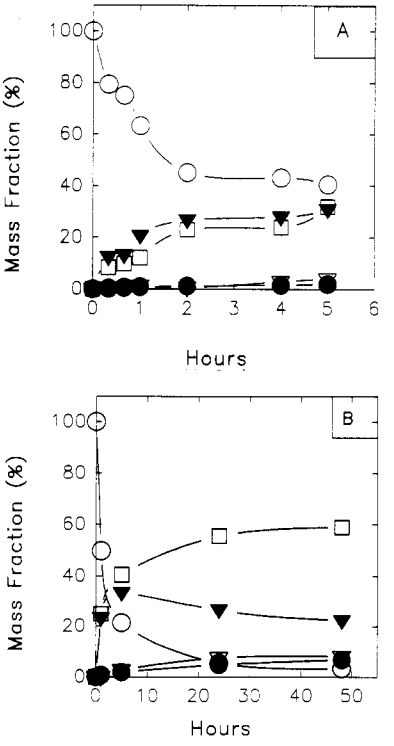

Figure 2. Time course of glycerolysis mediated by lipase PS-30 after short- (A) or long-term (B) incubation. Reactive mixtures contained 10 mL of 25% butteroil in 2-methyl-2-propanol, 1.0 g of glycerol (6.8% water), and 200 mg of lipase PS-30 and were incubated at 35 °C. Symbols are the same as for Figure 1.

indicated that TAG was converted primarily to 1,2(2,3)-DAG and MAG. TAG was almost 90% depleted within the first 1 h for lipase AK, and near-steady-state conditions

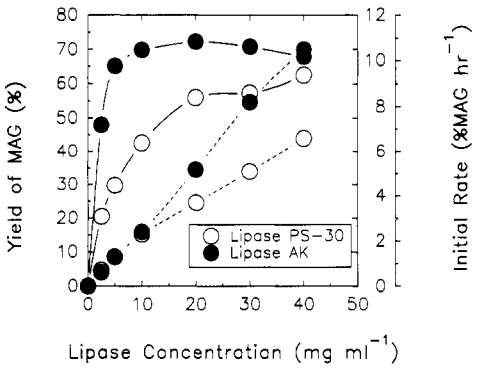

Figure 3. Effect of lipase concentration on the rate and extent of glycerolysis. Reactive mixtures contained 10 mL of 25% butteroil in 2-methyl-2-propanol and 1.0 or 1.5 g of glycerol (6.3% water) for lipase PS-30 and AK, respectively, and were incubated at 35 °C. Yields of MAG were determined after 24 h of incubation and are represented by the solid-line plots. Initial rates are indicated by the broken-line plots.

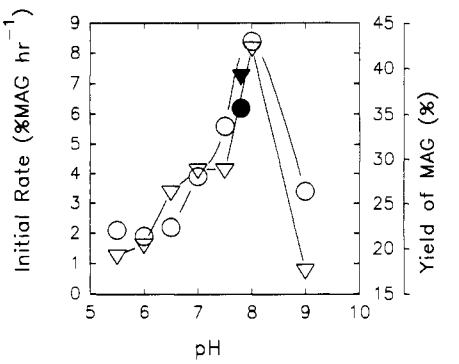

Figure 4. Effect of pH on rate and extent of glycerolysis for lipase PS-30. Reactive mixtures contained 10 mL of 25% butteroil in 2-methyl-2-propanol, 1.0 g of glycerol (<1.0% water), and 200 mg of lipase PS-30 and were incubated at 35 °C. Solid symbols represent enzyme dissolved in water and lyophilized without pH adjustment. Open symbols represent pH-adjusted enzyme. Circles indicate percent yield of MAG after 24 h, and triangles indicate initial rates of MAG formation.

were reached after 5 h (Figure 1A). After 48 h of incubation, FA and 1,3-DAG each remained at levels near 10% or less of the total reaction components (Figure 1B). Under the conditions used, glycerolysis was slightly faster for the AK (Figure 1) than for the PS-30 lipase (Figure 2). With lipase PS-30, 24 h was required to deplete the original levels of TAG by almost 90%, again with MAG and 1,2-(2,3)-DAG being the dominant products. As was observed for the AK lipase, 1,3-DAG and FA components were maintained at levels less than 10% each during the course of the reaction with the PS-30 lipase. The greater degree of accumulation of FA for the AK lipase relative to the PS-30 lipase was probably because of the greater water content of the former.

Effect of Lipase Concentration. For both lipases, initial reaction velocities and percent yield (mass fraction) of MAG production after 24 h were dependent on lipase concentration (Figure 3). Initial reaction rates increased linearly with increasing lipase levels to 40 mg/mL. Yields of MAG reached a maximum for lipase AK at 10 mg of enzyme/mL, and at 20–40 mg/mL for lipase PS-30. As the amount of lipase was increased to 40 mg/mL, the levels of FA accumulating progressively increased to 16% and 9% for lipases AK and PS-30, respectively, probably because of the increasing amount of water, originating from the enzyme reagent, added to the system. The greater tendency to accumulate FA for the AK compared to the PS-30 enzyme is likely because of the greater water content of the former. Enzyme levels of 20 mg/mL were judged to be best at maximizing rate and yield of MAG production while minimizing FA accumulation.

Effect of pH. The response to pH in terms of initial rates and percent yield of MAG production was evaluated only for lipase PS-30 (Figure 4). Both reaction velocity and yield of MAG production were optimum at pH 8.0, and activity was reduced substantially at pH 7 and 9. The pH of the enzyme reagent dissolved in water was 7.8. The enzyme reagent used without any pH adjustment (7.8), but similarly lyophilized, had activities 75–90% of those observed at pH 8.0. By comparison, the enzyme reagent used directly from the supplier without any modification had an initial reaction rate of 12% MAG/h and a reaction yield of 56% MAG after 24 h of incubation. Thus, there was no advantage to adjusting enzyme pH.

Effect of Glycerol Concentration. As glycerol concentration was increased to 100 mg/mL, the yield of MAG progressively increased to 50–60% for both lipases (Figure 5). At 25% butteroil in 2-methyl-2-propanol and 100 mg

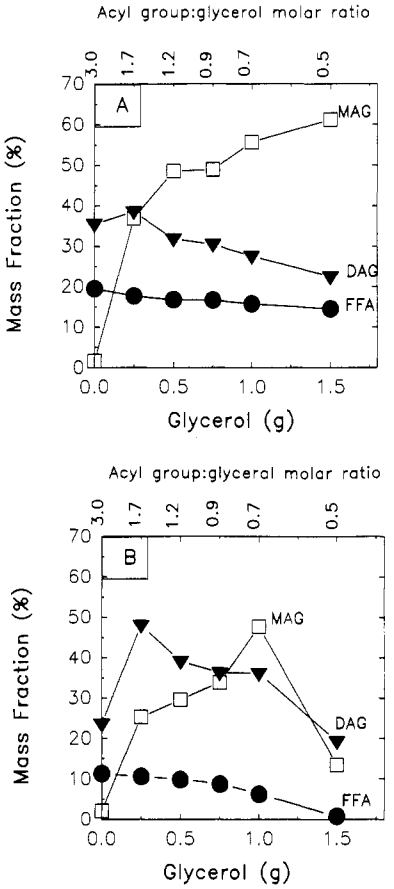

Figure 5. Effect of glycerol content on extent of glycerolysis. Reactive mixtures contained 10 mL of 25% butteroil in 2-methyl-2-propanol, 200 mg of lipase AK (A) or PS-30 (B), and various levels of glycerol (6.8% water) and were incubated for 24 h at 35 °C.

of glycerol/mL, glycerol is in moderate molar excess to fatty acyl groups (Table I). Maximum yield of DAG was observed at 25 mg of glycerol/mL, the lowest level of added glycerol evaluated. Above 100 mg of glycerol/mL, the yield of MAG remained near 60% for the AK lipase, but steady-state levels of MAG, DAG, and FA declined precipitously for lipase PS-30.

These observations indicated that an overall restriction of activity took place for lipase PS-30 at 150 mg of glycerol/mL. This may be caused by desorption of water from the enzyme by the elevated levels of glycerol. This was tested

Table I. Calculated Molar Ratios of Fatty Acyl Groups to Glycerol for Reactive Mixtures of Various Glycerol and Butteroil Levels[a]

various glycerol levels (mg/mL)	molar ratio of fatty acyl groups to glycerol: at 25% butteroil in 2-methyl-2-propanol, various glycerol	at 100 mg of glycerol/mL (PS-30 lipase), various butteroil	at 150 mg of glycerol/mL (AK lipase), various butteroil	various butteroil levels (%, w/w)
0	3.0	0.34	0.24	10
25	1.7	0.61	0.44	20
50	1.2	0.73	0.53	25
75	0.90	0.83	0.61	30
100	0.73	1.0	0.76	40
150	0.53	1.2	0.90	50

[a] Glycerol content, which is the sum of added glycerol and glycerol derived from butteroil TAG, is expressed as milligrams per milliliter of 2-methyl-2-propanol per butteroil substrate mixture.

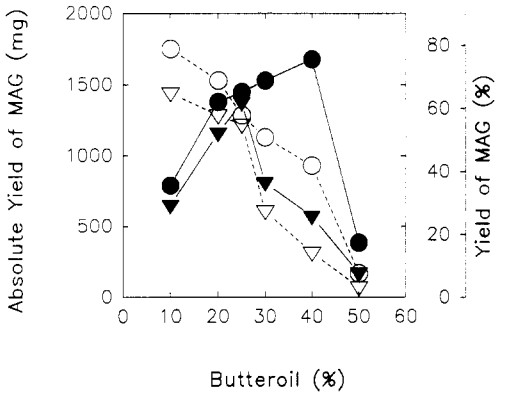

Figure 6. Effect of butteroil content on the extent of glycerolysis. Reactive mixtures contained a 10-mL portion of various amounts of butteroil in 2-methyl-2-propanol and 1.0 or 1.5 g of glycerol (<1.0% water) for lipase PS-30 (▼, ▽) and AK (●, ○), respectively, and were incubated at 35 °C. Open symbols and broken-line plots indicate (percent) yield (mass fraction) of MAG relative to other acylglycerol species formed under each condition after 24 h of incubation. Solid symbols and solid-line plots indicate absolute (mass) yields of MAG after 24 h of incubation for the full range of butteroil levels evaluated.

by adding 5.3% water (w/w based on added glycerol); a re-establishment of the steady-state yields of MAG, DAG, and FA to levels (51%, 31%, and 12%, respectively) similar to those under near-optimal conditions of MAG production was observed.

A limited degree of inhibition by increased glycerol levels is observed when glycerolysis reactions are achieved in reverse micelles using isooctane as the continuous phase (Holmberg et al., 1989). In this reaction configuration, a separate aqueous glycerol phase is confined within the micelle and in proximity to the enzyme. The greater overall reaction inhibition in our study is likely to be attributable to the fact that in the presence of 2-methyl-2-propanol a single liquid phase exists and, thus, water and glycerol are diluted throughout the full reaction volume.

Effect of Butteroil Concentration. Optimum butteroil levels for producing MAG were evaluated in terms of percent conversion or yield and absolute yield of MAG. Greatest percent yield of MAG was observed at 10% butteroil (Figure 6). This might be expected since at 10% butteroil the molar ratio of fatty acyl groups to glycerol was 0.24–0.34 (Table I), favoring the distribution of fatty acyl groups in the form of MAG rather than DAG or TAG. The yield of MAG, or any other acylglycerol component, is known to be dependent on the relative levels of TAG and glycerol (Ergan et al., 1990).

An optimum butteroil content, in terms of absolute yield of MAG after 24 h of incubation, was observed at 20–25% for lipase PS-30 and at 30–40% for lipase AK. The corresponding fatty acyl groups/glycerol molar ratios for both enzymes were similar at 0.61–0.73 and 0.61–0.76 for lipases PS-30 and AK, respectively (Table I).

The decline in both absolute and percent yields of MAG at the greater butteroil levels evaluated was manifest as a lack of TAG conversion. At 50% butteroil, the mass ratios of TAG/DAG/MAG/FA after 24 h of incubation were about 90:7:3:<1 and 82:11:7:<1 for lipases PS-30 and AK, respectively, indicative of a severe restriction in enzyme activity. However, at 50% butteroil, the ratios of acyl groups to glycerol are 1.2 and 0.90 for lipases PS-30 and AK, respectively (Table I), still stoichiometrically favorable for yielding MAG.

One factor that could cause the reduced yields of MAG at 50% butteroil was that a two-phase liquid system existed, probably compromising reaction efficiency. We also noted a caking or flocculation of the enzyme powder on the walls of the reaction vessels, and this probably also limited catalytic activity.

The nature of inhibition of glycerolysis in reaction mixtures with 50% butteroil was further evaluated by subsequently diluting these mixtures to 25% butteroil with additional 2-methyl-2-propanol and then extending the incubation period for another 24 h (enzyme level was also adjusted accordingly). After dilution to 25% butteroil and subsequent incubation, yields of 50–60% MAG were observed for lipase AK. However, for the PS-30 lipase, inhibition by 50% butteroil was not reversed after dilution. It is difficult to surmise why the PS-30 lipase was more and irreversibly sensitive to high substrate concentrations than the AK lipase.

Effect of Temperature. For both lipases, as reaction temperature was increased from 30 to 55 °C, a progressive decrease in percent yield of MAG after 24 h was noted (Figure 7). With lipase AK, the decreased yield of MAG corresponded to an increased yield of DAG. In contrast, for lipase PS-30, a decreased yield of both MAG and DAG was observed with increased temperature. For both enzymes, there was a decrease in the levels of FA accumulating with increasing temperature.

A temperature-dependent change in availability of water could be a factor affecting activity and yield of MAG for both lipases. Specifically, this could be manifest as a greater power of the 2-methyl-2-propanol/glycerol mixture to solubilize water and desorb water from the enzyme at elevated temperatures. When an additional 2% (lipase AK) and 10% (lipase PS-30) water (w/w in glycerol) was added to reaction mixtures incubated at 55 °C, yields of MAG (50–65%) and DAG (25–35%) were similar to those observed at the optimal reaction temperatures of 35–40 °C, suggesting that water availability was a primary factor limiting activity at elevated temperatures.

Effect of Added Water. The influence of water content on glycerolysis was evaluated at the optimum temperature of 35 °C under standardized reaction conditions. Added water of 5–10 μL [0.33–1.0% (w/w) in glycerol] had a slight activating effect with both lipases in terms of yield of MAG after 24 h of incubation (Figure 8). As added water was increased further within the range evaluated, an increase in the levels of FA accumulating was observed, although little effect was observed on the yield of MAG and DAG. For the PS-30 lipase, data for FA, MAG, and DAG yields at 55 °C are provided for comparison (Figure 8B) because

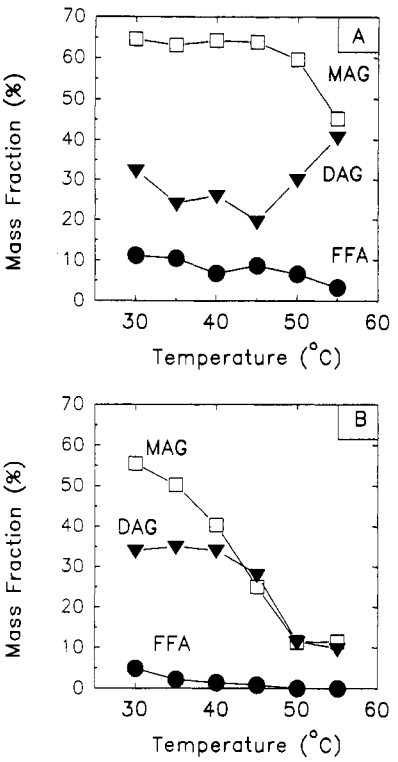

Figure 7. Effect of temperature on extent of glycerolysis. Reactive mixtures contained 10 mL of 25% butteroil in 2-methyl-2-propanol and 1.5 or 1.0 g of glycerol (6.3% water) for 200 mg of lipase AK (A) and PS-30 (B), respectively. Mass fractions of reactive components were determined after 24 h of incubation.

Table II. Effect of Molecular Sieves (MS) on Acylglycerol Profile of Glycerolysis Reaction Mixtures with Butteroil as Substrate

amt of MS added[a] (g)	mass fraction (%) of acylglycerol components[b]				
	TAG	FA	DAG	MAG	MAG + DAG
0	1.8	9.9	22	65	87
0.5	2.3	7.7	25	66	91
1.0	2.8	1.8	29	67	96
1.5	4.5	0.4	35	61	95
2.0	9.5	0	31	49	90

[a] Reaction mixtures, minus enzyme, were preincubated for 2 h at 35 °C with molecular sieves prior to initiation of the reaction by addition of enzyme. [b] After 24 h of incubation. Reaction conditions were 10 mL of 25% butteroil in 2-methyl-2-propanol, 1.5 g of glycerol (6.3% water), and 200 mg of lipase AK, with various amounts of molecular sieves suspended, at 35 °C.

of the temperature–water availability interaction suggested earlier (Figure 7B). These results indicate that progressive addition of water was effective in overcoming the temperature-dependent restriction of glycerolysis activity of lipase PS-30.

The role of water in glycerolysis reactions was tested by adding different levels of molecular sieves to reaction mixtures containing lipase AK (Table II). As the amount of molecular sieves added was increased, the amount of FA accumulating decreased (at the expense of less TAG being converted), and ultimately, the yield of MAG became reduced from over 60% to less than 50%.

The effect of preincubating reaction mixtures with molecular sieves prior to reaction of both lipases on several oil substrates also was evaluated (Table III). All oils tested were transformed to MAG with yields of 64–69% and 51–63% for lipases AK and PS-30, respectively. Preincubation with molecular sieves slightly increased the yields of MAG with lipase AK but reduced the yields of MAG

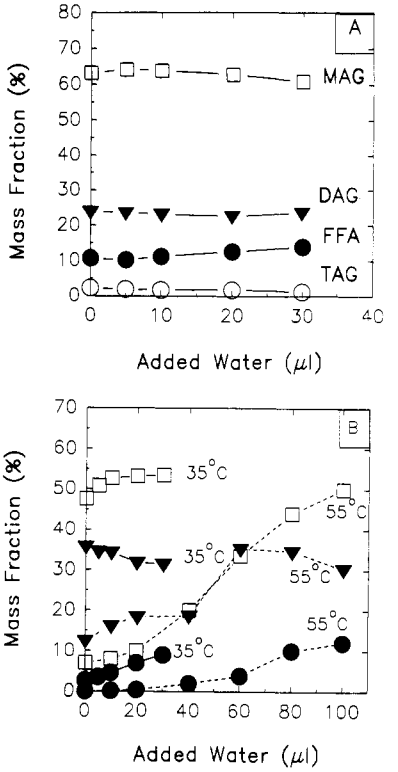

Figure 8. Effect of added water on extent of glycerolysis. Reactive mixtures contained 10 mL of 25% butteroil in 2-methyl-2-propanol and 1.5 or 1.0 g of glycerol (6.3% water) for 200 mg of lipase AK (A) and PS-30 (B), respectively, with various amounts of water added. Reactive mixtures were incubated at 35 °C and in (B) also at 55 °C (broken-line plots). Mass fractions of reactive components (□, MAG; ▼, DAG; ●, FA) were determined after 24 h of incubation.

Table III. Effect of Molecular Sieves (MS)[a] on Yield of Monoacylglycerols (MAG) of Glycerolysis Reaction Mixtures with Various Oils as Substrates

substrate	yield (%) of MAG[b]			
	with lipase AK		with lipase PS-30	
	with MS	without MS	with MS	without MS
butteroil	71	65	37	51
coconut oil	72	69	38	51
peanut oil	70	66	46	60
sunflower oil	70	64	45	60
corn oil	70	67	48	59
soybean oil	70	67	51	63
olive oil	70	65	43	60

[a] Reaction mixtures, minus enzyme, were preincubated for 2 h at 35 °C with molecular sieves prior to initiation of the reaction by addition of enzyme. [b] After 24 h of incubation. Reaction conditions were 10 mL of 25% butteroil in 2-methyl-2-propanol and 1.5 g of glycerol (6.3% water) and 200 mg of lipase AK or 1.0 g of glycerol (6.3% water) and 200 mg of lipase PS-30, with various amounts of molecular sieves suspended, at 35 °C.

by about 25% with lipase PS-30. The difference in effect of molecular sieves is probably because water became more limiting in the case of the PS-30 lipase and water necessary for catalysis may have been removed.

Conclusions. Conditions optimum for MAG production from butteroil in the presence of glycerol and 2-methyl-2-propanol as solvent using lipases from *Pseudomonas* sp. were established. Critical factors include (1) water content of the reaction mixture, (2) the molar ratio of fatty acyl groups to glycerol as well as actual butteroil and glycerol concentrations, and (3) temperature. These three parameters also interact, with the primary effect of this interaction apparently being the modulation of water

available for enzyme catalysis. In this regard the presence and level of organic solvent (and secondarily, glycerol) are particularly critical as the solvent may be capable of stripping essential water from the enzyme. In addition, adventitious sources of water are important.

Control of glycerolysis reactions can also be obtained by varying the fatty acyl group/glycerol ratios to yield various ratios of MAG to DAG with a net conversion of TAG to these emulsifying agents of over 80%. Although butteroil was used as the principal lipid substrate for these studies, our findings are likely to be applicable to the reactivity of other TAG-rich oils used under similar conditions. In reaction mixtures in which organic solvent is omitted, the same parameters influence reaction efficiency, but in a slightly different manner, as reported in the following paper (Yang et al., 1993b).

ABBREVIATIONS USED

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

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