Hydrolysis of Milk Fat By Lipase in Solvent-free Phospholipid Reverse Micellar Media

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ABSTRACT

Butterfat was hydrolyzed with lipases contained in lecithin reverse micelles. The influence of pH, temperature, molar ratio of surfactant to water (R) and surfactant concentration on the hydrolytic reaction indicated linear, quadratic, and interactive effects for reaction systems mediated by *R. javanicus* and *C. rugosa* lipases. The initial reaction rate was dependent on reaction parameters; however, the degree of hydrolysis was independent of pH. Both enzymes exhibited high thermal stability. The content and composition of milk fat hydrolysates prepared by *R. javanicus* lipase were most influenced by reaction temperature and R. The optimum conditions for production of free fatty acids, monoacylglycerols, diacylglycerols and specific regio-isomers were defined.

Key Words: butterfat, lipase, lecithin, reverse micelles, fat hydrolysates

INTRODUCTION

CHANGES IN AMERICAN DIETARY HABITS have led to a surplus of milk fat (butter oil). Research that may lead to unique uses of milk fat is needed. A key attribute of milk fat is its unique flavor, which can be increased in use with conversion to functional ingredients using lipolytic enzymes. This aspect of enzyme-modified lipid ingredients was reviewed by Kilara (1985). Conventionally, milk fat has been hydrolyzed by lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) in aqueous emulsion systems to yield various types of flavors. Lipase-mediated bioconversion of triacylglycerols (TAG) and other sparingly water-soluble compounds in low aqueous media (such as reverse micelles) is of interest for production of food ingredients (Bello et al., 1987; Holmberg and Osterberg, 1987, 1988; Holmberg et al., 1989; Kim and Chung, 1989; Osterberg et al., 1989; Hayes and Gulari, 1990; Nagao and Kito, 1990). Reverse micellar medium is the micellar solution of a surfactant in organic solvent, where enzyme and water are solubilized in the core of the micelles and water-insoluble substrate is solubilized in the bulk solvent. Lipase reactions in such media have several advantages over reactions in aqueous systems. These include increased reaction rates and yields, increased thermal stability, lower costs and lower reaction volumes. They also have good protection from protection from microbial contamination, product inhibition, and provide reuse of enzyme.

Reverse micellar media comprised of *bis*(2-ethylhexyl) sodium sulfosuccinate (Aerosol-OT) and *iso*-octane have been reported (Han and Rhee, 1985; Han et al., 1987, 1990; Chang and Rhee, 1990; Schmidli and Luisi, 1990; Stark et al., 1990) to study hydrolysis of TAG. The hydrolysis of two model substrates (olive and coconut TAG) has also been reported in a novel solvent-free reverse micellar media composed of a purified lecithin as surfactant and substrate TAG as continuous nonpolar medium (Chen and Pai, 1991). The solvent-free lecithin reverse micellar medium is more suitable than the Aerosol-OT/*iso*-octane medium for food application because of possible toxicity associated with the organic solvent and the use of synthetic surfactant. Further studies on hydrolysis of milk fat in solvent-free reverse micellar media using commercial grade lecithin (soy lecithin) and commercial enzymes were conducted. The objective was to study this system for applications and to identify conditions for converting milk fat to ingredients with multiple functionalities.

MATERIALS & METHODS

Materials

Commercial grade lipases from *Rhizopus javanicus* (FAP-15) and *Candida rugosa* (AY-30) were a gift from Amano International Enzyme Co. (Troy, VA). Specific activity of the lipases under standard aqueous (bulk) conditions were 58.9 units and 8.9 units per mg, respectively. One unit is the amount of enzyme required to hydrolyze 1 μM triglyceride/minute. Clarified milk fat (saponification value = 229.2) was purchased from Level Valley Dairy (West Bend, WI). Soybean lecithin (Centrophase-C, ⋮ 6002) containing 55% phospholipids (acetone insolubles) was a gift from Central Soya Co. (Fort Wayne, IN). HPLC grade acetone was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Oleic acid of 99.9% purity was obtained from Sigma Chemical Co. (St. Louis, MO), and was used as a standard. Benzene and pyridine of spectrophotometric grade were purchased from Aldrich Chemical Co. (Milwaukee, WI). Anhydrous glycerol (calibration standard) and n-tetradecane (internal standard) were purchased from Fluka (Ronkonkoma, NY). The other calibration standards for free fatty acids (FFA), monoacylglycerols (MAG) and diacylglycerols (DAG) were purchased from NuCheckPrep (Elysian, MN). All other reagents were analytical grade.

Preparation of reverse micellar media

A 55.6 mM stock solution of soybean lecithin (avg mol wt 780) in milk fat was prepared and stored at room temperature until further use. Reverse micellar media of various compositions were prepared by mixing appropriate volumes of milk fat and the stock solution of lecithin in milk fat in batch reactors of 10 mL volume. The desired enzyme concentration and molar ratios of water to phospholipids (R) were obtained by injecting appropriate volumes of buffer solution and the concentrated buffered enzyme stock solution using a positive displacement micropipettor. The true value of R may be higher due to hydrated state of phospholipids in commercial preparations. After adding enzyme solution, the micellar solutions were mixed on a Vortex stirrer until a clear single-phase medium was formed.

Hydrolysis of milk fat in reverse micellar media

The reaction media with substrate were equilibrated to appropriate temperatures before addition of enzyme solution. The hydrolysis of milk fat was initiated by injecting the enzyme solution and Vortex-mixing until the media became clear. At predetermined time intervals, appropriate volumes (10–100 μL) of reaction mixtures were transferred into 15-mL screw-cap tubes containing a predetermined volume of benzene, producing a total volume of 5 mL. The tubes were immediately Vortex-mixed to stop the enzyme reaction and stored until later measured for liberated fatty acids. Also, at predetermined time intervals, 0.2 mL reaction mixtures were transferred into 4-mL screw-cap glass vials containing 1 mL acetone (containing 3 mg/mL of internal standard, n-tetradecane) and stored in freezer until later analysis of hydrolysates by gas chromatography.

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Table 1—Variable-level combinations for 3-variable rotatable composite design

			Reaction parameters (independent variables)		
				Temperature (°C)	
				Lipase FAP-15	Lipase AY-30
	pH	R = [water] [surfactant]		(*R.javanicus*)	(*C. rugosa*)
Coded levels					
−1.682 (−α)	3.0	4.6		46.5	36.5
−1	4.0	8.0		52.0	42.0
0	5.5	13.0		60.0	50.0
1	7.0	18.0		68.0	58.0
1.682 (α)	8.0	21.4		73.5	63.5
Experimental design points[a]					
Cubic points (n_c)					
1	−1	−1		−1	
2	1	−1		−1	
3	−1	1		−1	
4	1	1		−1	
5	−1	−1		1	
6	1	−1		1	
7	−1	1		1	
8	1	1		1	
Axial points (n_a)					
9	−1.682	0		0	
10	1.682	0		0	
11	0	−1.682		0	
12	0	1.682		0	
13	0	0		−1.682	
14	0	0		1.682	
Center point (n_o)					
15	0	0		0	
Additional points					
1	−1	0		0	
2	1	0		0	
3	0	−1		0	
4	0	1		0	
5	0	0		−1	
6	0	0		1	

[a] Center point (n_o) was replicated six times and entire experiment was replicated twice. The additional points were excluded from second-order model fitting.

Table 2—Regression coefficients and coefficients of determination (R^2) of second-order fitted equation for lipase-catalyzed hydrolysis of milk fat in phospholipid/TAG reverse micellar media[a]

| | Initial rate | | Degree of hydrolysis | |
| | Lipase FAP-15 (*R. javanicus*) | Lipase AY-30 (*C. rugosa*) | Lipase FAP-15 (*R. javanicus*) | Lipase AY-30 (*C. rugosa*) |
Variable				
Intercept (β_0)	32.614	13.822	27.298	10.700
Linear				
R (β_1)	4.492**	0.598	2.728**	0.232
P (β_2)	−1.544	1.543**	−0.483	0.387
T (β_3)	−1.625	0.136	−3.816**	−1.359*
Quadratic				
R × R (β_{11})	−5.025**	−2.450**	−2.727**	−1.415*
P × P (β_{22})	−4.905**	−2.810**	−0.268	−1.538**
T × T (β_{33})	−7.053**	−1.186*	−3.958**	−0.910*
Interaction				
R × P (β_{12})	−0.104	−0.480	−0.672	−0.253
P × T (β_{23})	−3.000*	−0.339	−1.214	−0.360
T × R (β_{31})	−2.073*	0.209	−3.600**	−0.465
Lack of fit	NS	NS	NS	**
R^2	0.92**	0.92**	0.90**	0.76**

[a] The second-order function is: $\hat{Y} = \beta_0 + \beta_1 R + \beta_2 P + \beta_3 T + \beta_{11} R^2 + \beta_{22} P^2 + \beta_{33} T^2 + \beta_{12} RP + \beta_{23} PT + \beta_{31} TR$; where, P = pH, R = [water]/[surfactant], and T = temperature.
* Significant ($P < 0.05$); ** Significant ($P < 0.01$); NS Nonsignificant ($P > 0.05$).

Table 3—Lipase-catalyzed hydrolysis of milk fat in phospholipid/TAG reverse micellar media at various reaction conditions

| Reaction parameters[a] | | | Initial rate[b] (µmoles FFA/min) | | Degree of hydrolysis[b] (%) | |
| | | | Lipase FAP-15 | Lipase AY-30 | Lipase FAP-15 | Lipase AY-30 |
T (°C)	pH	R	(*R. javanicus*)	(*C. rugosa*)	(*R. javanicus*)	(*C. rugosa*)
	4	8	9.6 ± 0.2	4.0 ± 0.0	18.6 ± 0.4	6.1 ± 0.0
52 (42)[c]		18	16.4 ± 0.3	5.5 ± 0.1	28.1 ± 0.5	6.6 ± 0.1
	7	8	9.7 ± 0.1	8.9 ± 0.2	18.5 ± 0.3	8.3 ± 0.2
		18	23.1 ± 0.4	7.0 ± 0.1	29.3 ± 0.5	7.4 ± 0.0
	4	8	16.8 ± 0.4	5.5 ± 0.1	21.0 ± 0.5	5.7 ± 0.1
68 (58)[c]		18	22.4 ± 0.1	6.4 ± 0.1	20.1 ± 0.1	3.9 ± 0.1
	7	8	12.0 ± 0.2	7.5 ± 0.1	20.0 ± 0.3	6.0 ± 0.1
		18	10.0 ± 0.2	8.0 ± 0.1	12.5 ± 0.3	3.7 ± 0.0

[a] Reaction parameters: phospholipid concentration = 50 mM, enzyme concentration = 5 mg/10 mL reaction mixture, substrate concentration = 95.4% v/v at R = 8 and 94.5% v/v at R = 18; buffers: 50 mM citrate-phosphate buffer for pH 4 and 50 mM Na-K-phosphate buffer for pH 7.
[b] Mean ± standard deviation.
[c] Values in parenthesis are the reaction temperature for Lipase AY-30.

Determination of initial rate and degree of hydrolysis

The liberated free fatty acids were quantified using a method described by Lowry and Tinsley (1976). Cupric acetate-pyridine reagent (1 mL) was added to 15-mL screw-cap tubes containing 5 mL mixture of reaction medium and benzene. The tubes were then shaken for 2 min using an oscillating shaker. After 5 min centrifugation at 3300 rpm (1200 × g), absorbance of the supernatant was measured at 715 nm. The concentration of liberated fatty acid was calculated from the standard curve generated using oleic acid as calibration standard.

Unless otherwise stated, the initial rate (activity) is expressed as µmole of free fatty acid liberated/min under the assay condition based at 1 hr reaction time. The kinetics of hydrolysis was linear up to 75–90 min under the conditions we used. The degree of hydrolysis or percent conversion was calculated as follows:

$$\% \text{ Degree of hydrolysis} = \frac{\text{Liberated fatty acids}}{\text{Total number of ester bonds}} \times 100$$

The total number of ester bonds in TAG was estimated from the saponification value determined by standard procedure (AOAC, 1984). Unless otherwise stated, the degree of hydrolysis is expressed for 24 hr reaction.

Gas chromatography of milk fat hydrolysates

A microprocessor-controlled PERKIN-ELMER 8410 gas chromatograph (Norwalk, CT) with on-column injector and flame ionization detector was used. The chromatograms were run with 2 mm i.d. and 1.83 m long glass column packed with 3% OV-17 on 100/120 mesh Supelcoport (Supelco, Bellefonte, PA). The flow rate of N_2 carrier gas was 20 mL/min with inlet pressure 275 kPa.

The trimethylsilyl derivatives of FFA, MAG, DAG, and glycerol in hydrolyzed fat were prepared using a 90:1 mixture of bis-(trimethysilyl)trifluroacetamide:trimethylchlorosilane (Sylon-BFT, Supelco, Bellefonte, PA)) reagent. About 0.25 mL of the mixtures, containing milk fat hydrolysate and internal standard, were transferred to 2-mL screw-cap reaction vials. The solvents were evaporated to dryness under a stream of N_2. Sylon-BFT (0.25mL), a silylating reagent, was added and after closing the vials, they were briefly warmed on a heating block, stirred vigorously for a few sec, and heated at 85°C for 25 min. The vials were allowed to cool to room temperature. After 1 hr, about 1 µL (≈50 µg hydrolysates) of derivatized mixtures was injected at 75°C. After an isothermal period of 2 min, the column temperature was raised to 225°C at 25°C/min and then to 315°C at 9°C/min. Finally, temperature was raised to 355°C at 1°C/min and was held at 355°C for 6 min. The injector was 370°C and detector was 375°C.

Qualitative and quantitative chromatographic analyses were performed by internal standardization using n-tetradecane as internal standard. The peaks in the chromatogram were identified and quantified according to acyl carbon number (ACN) for each class of lipid: FFA, MAG, and DAG. The relative retention time (RRT) and response factor (RF) were determined using various classes of calibration mixture from Nu Check Prep. Inc. (Elysian, MN). The FFA mixture contained standards from AC4 to AC18. The MAG mixture contained standards from AC12 to AC18. The DAG mixture contained AC16, AC24, AC28, AC32, and AC36 standards. The RRT and RF for other peaks were determined by interpolation.

The relative retention time was calculated as follows:

RRT(I) = retention time of ith component/retention time of internal standard

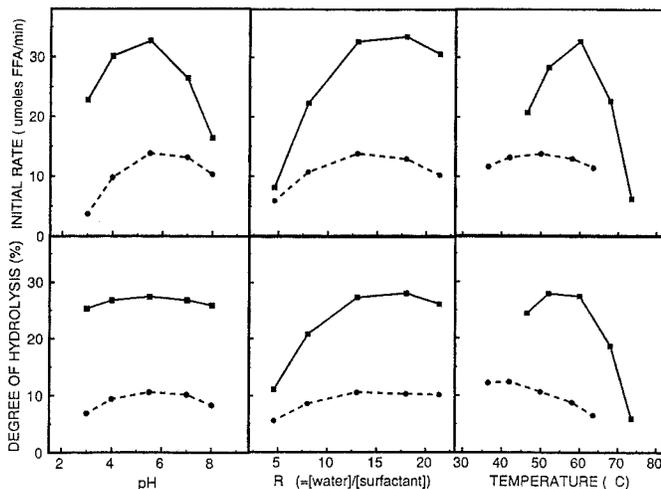The response factors were calculated as follows:

Fig. 1—Effect of pH, molar ratio of water to surfactant (R), and temperature on the initial rate and degree of hydrolysis for _R. javanicus_ and _C. rugosa_ lipase catalyzed hydrolysis of milk triacylglycerols in solvent-free phospholipid reverse micellar media. Reaction parameters: pH 5.5 (50 mM citrate-phosphate buffer), R = 13, temperature = 60°C for _R. javanicus_ lipase and 50°C for _C. rugosae_ lipase, phospholipid concentration = 50 mM, enzyme concentration = 5 mg/10 mL reaction mixture. _R. javanicus_, (——) lipase, _C. rugosa_ (----).

$$RF(I) = W(I) \times A(is)/W(is) \times A(I)$$

where W(I) = weight of ith component; W(is) = weight of internal standard; A(I) = peak area of ith component; A(is) = peak area of internal standard. These RF values were used to calculate the composition of sample as follows:

$$\%W(I) = A(I) \times RF(I) \times W(is) \times 100/A(is) \times W(s)$$

where %W(I) = weight percent of ith component; W(s) = weight of sample.

Experimental design

Validation of initial reaction velocities was reported (Patel et al., 1995). To examine the effects of temperature, pH, and molar ratio of water to surfactant (R) on the lipase-catalyzed hydrolysis and on the content and composition of end products, a rotatable central composite design (Box and Wilson, 1951) was adopted with three-variable and five-level pattern (Table 1). The total number of observations was 20, which included eight factorial (cubic) points, six axial points, and six center-point replicates. The axial points represented the extreme high and low values of independent variables (reaction conditions). The entire design was replicated twice. The data were fit to the second order function as follows:

$$Y_i = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ii} X_i X_j$$

where Y_i = initial rate or degree of hydrolysis; X_i and X_j = independent variables (pH, temperature, or R); β_0 = center point of system; β_i = coefficient of linear effects of reaction; β_{ii} = coefficient of quadratic effects of reaction parameters; β_{ij} = coefficient of interactive effects of reaction parameters.

In addition to the 20 experimental points, six additional points (Table 1) representing +1 and −1 coded levels of each reaction condition were studied to provide a complete set of five observations. These observations were made to assess the effects of each variable on the lipase reaction, as studied over the entire range of variable levels at a fixed level of all other variables.

Statistical analysis

The coefficient of each parameter in the model was determined by General Linear Model (GLM) procedure using Minitab (1990) statistical software. The three-dimensional surface plots, showing effects of two-reaction conditions (holding all others constant), were generated by Axum (1989) graphical software using the GLM regression model. Optimum reaction condition ranges were determined by the 3-factor Taylor
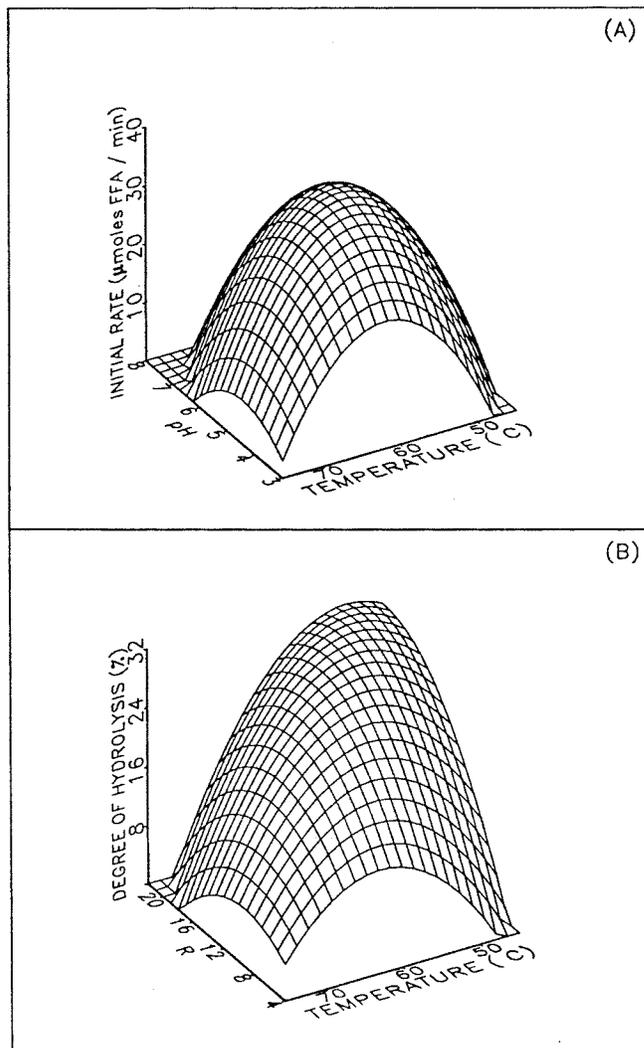

Fig. 2—Interactive effects of reaction parameters on hydrolysis of milk triacylglycerols by _R. javanicus_ lipase in solvent-free reverse micellar media. Reaction parameters: phospholipid concentration = 50 mM, substrate concentration = 94.5%, v/v, enzyme concentration = 5 mg/10 mL.

Table 4—Optimum reaction conditions for lipase-catalyzed hydrolysis of milk fat in phospholipid/TAG reverse micellar media[a]

Reaction conditions	Lipase FAP-15 (_R. javanicus_)	Lipase AY-30 (_C. rugosa_)
R = [Water] / [Phospholipids]	14–16	13–14.5
pH	5–6	5.5–6
Temperature (°C)	58–60	45–47

[a] Determined using three-factor Taylor polynomial optimization for the initial rate and degree of hydrolysis.

polynomial optimization program using Design-Expert (1988) statistical software. Where applicable, results were analyzed by the ANOVA procedure using Minitab (1990) software, and significant differences were determined using Fisher's test.

RESULTS & DISCUSSION

TRIACYLGLYCEROLS can be hydrolyzed in solvent-free reverse micellar media comprising a highly purified lecithin as a surfactant, TAG as continuous non-polar phase and substrate, and purified lipase as a biocatalyst (Chen and Pai, 1991; Patel, 1993). Further possibility of using available, natural lecithin to form reverse micelles for bioconversion of milk fat to multi-
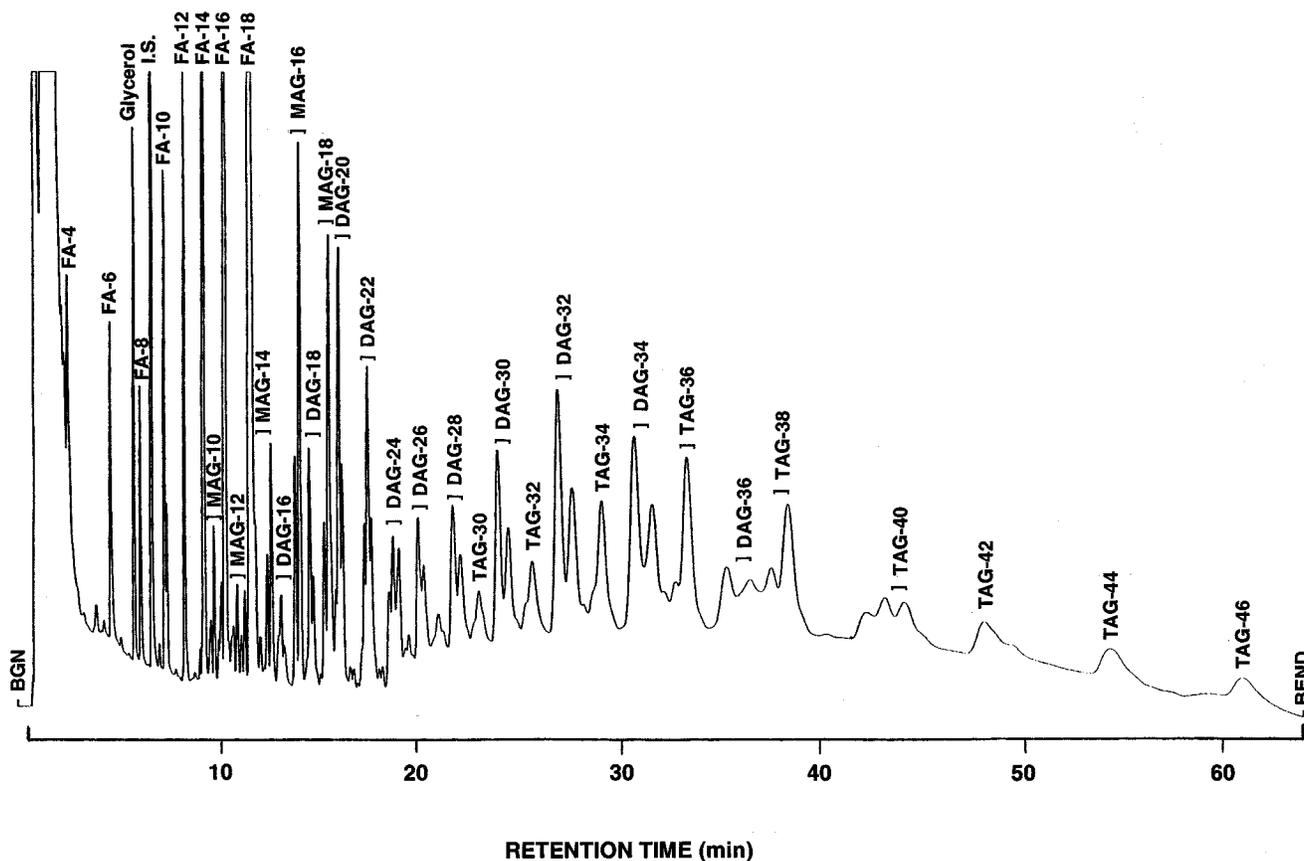
Fig. 3—Gas chromatogram of milk triacylglycerol hydrolyzate prepared by *R. javanicus* lipase catalyzed hydrolysis in solvent-free phospholipid reverse micellar media. Chromatography on 1.83 × 2 mm i.d. glass column packed with 3% OV-17. Peak designations: FA = fatty acids, MAG = monoacylglycerols, DAG = diacylglycerols, TAG = triacylglycerols. The numbers indicate the acyl carbon number components.

functional food ingredients was examined. Natural lecithin occurs as a mixture of phospholipids. Because of the difficulty in separation of its components, natural lecithin is used as a mixture. Lipase could be solubilized in the reverse micelles of soybean lecithin in milk fat, yielding clear isotropic solutions.

Milk fat hydrolysis in that system did not require continuous agitation; and the system remained clear throughout the reaction without any visible phase separation.

Effect of reaction parameters on milk fat hydrolysis

Lipase-mediated hydrolytic reactions in reverse micellar media have been critically influenced by molar ratio of water to surfactant (R), reaction temperature and reaction pH (Scmidli and Luisi, 1990; Stark et al., 1991). The effects of such conditions on the initial rate and degree of hydrolysis for *R. javanicus* and *C. rugosa* lipase-catalyzed hydrolysis of milk fat in phospholipid reverse micellar media were compared (Fig. 1).

The pH-activity profiles for both enzymes in the range pH 3 to 8 were the typical bell-shaped profiles for most enzymes in aqueous systems. The reaction pH is pH of the buffer for preparation of enzyme solution and medium. Both enzymes exhibited maximum reaction rates and degree of hydrolysis at about pH 5.5 for hydrolysis of milk fat. However, no difference was observed in degree of hydrolysis in pH range 3 to 8 when milk fat was hydrolyzed by *R. javanicus* lipase. The pH maximum in the solvent-free phospholipid reverse micellar medium was considerably lower than that in Aerosol-OT reverse micellar medium or the aqueous emulsion system (Han and Rhee, 1986).

Lipase activity in reverse micellar medium is dependent on R because properties of water in the aqueous pool of and in the enzyme microenvironment differ considerably at different R values (Gupte et al., 1995). The function of water in the enzymatic reactions in nonpolar solvents is not fully understood; however, it has been discussed in terms of hydration of enzyme (Klibanov, 1986; Yamane et al., 1988; Zaks and Klibanov, 1988; Han et al., 1990; Hirata et al., 1990). The R-activity profile for both enzymes exhibited a bell-shaped curve with maximum rate at R = 18 for *R. javanicus* lipase and R = 13 for *C. rugosa* lipase. Chen and Pai (1991) reported that an R value of 10 was necessary for the maximum activity of enzyme in an lecithin-butteroil-water reverse micellar system. The higher value in our study may have been due to the commercial grade lecithin and enzyme used. The compounds included in the water pool as impurities may complete for the water, thereby reducing available water. The R-activity profiles in solvent-free phospholipid reverse micellar media are considerably different from those in Aerosol-OT reverse micellar media (Han and Rhee, 1986; Kim and Chung, 1989).

The rate and the degree of hydrolysis of milk fat were maximum between 50° and 60°C for *R. javanicus* lipase and between 40° and 50°C for *C. rugosa* lipase. Activity above those temperatures was reduced, presumably due to thermal denaturation of enzyme. Activity below those temperatures was reduced, possibly due to an increase in viscosity of oil phase and co-existence of another phase at the lower temperature extreme. Both enzymes exhibited unusually high thermal stability as evidenced by the temperature optimum at a considerably higher value and the significant activity at the extreme temperature of about 74°C for *R. javanicus* lipase and 64°C for *C. rugosa* lipase. Enhanced thermal stability in this medium could be due to a difference between properties of water in the core of micelles and those of bulk water in aqueous solution. These could also be due to the protection of enzyme from temperature-induced denaturation by nonspecific interactions with lipids or phospholipids in the medium.

Table 5—Yield of various components for hydrolysis of milk fat by lipase FAP-15 (*R. javanicus*) in the solvent-free reverse micellar media of phospholipids in TAG

				Components in milk fat hydrolysates[b]						
Variable[a]	Glycerol	SCFFA	LCFFA	Total FFA	*sn*-2 MAG	*sn*-1(3) MAG	Total MAG	*sn*-1,2(2,3) DAG	*sn*-1,3 DAG	Total DAG
Intercept (β_0)	0.394	2.528	22.697	25.224	0.857	3.918	4.778	10.349	14.106	24.465
Linear										
R(β_1)	0.058*	0.226*	1.588*	1.814*	0.053	0.283*	0.336*	−0.007	0.440	0.434
P (β_2)	0.006	−0.068	−0.060	−0.129	−0.008	−0.072	−0.080	0.040	−0.166	−0.127
T (β_3)	−0.043*	−0.299**	−2.752**	−3.052**	−0.386**	−0.366**	−0.754**	−2.519**	0.563	−1.956**
Quadratic										
R × R (β_{11})	−0.053*	−0.312**	−2.752**	−3.063**	−0.093**	−0.642**	−0.737**	−0.766*	−1.771**	−2.541**
P × P (β_{22})	−0.014	−0.075	−0.371	−0.446	0.011	−0.154	−0.142	−0.219	−0.638	−0.862
T × T (β_{33})	−0.088**	−0.445**	−3.316**	−3.761**	0.013	−0.829**	−0.817**	0.111	−2.245**	−2.136**
Interaction										
R × P (β_{12})	−0.010	0.046	−0.128	−0.084	0.012	−0.079	−0.066	−0.600	−0.806	−1.408
P × T (β_{23})	−0.018	−0.149	−1.098	−1.249	−0.040	−0.179	−0.219	−0.505	−0.794	−1.298
T × R (β_{31})	−0.050	−0.406**	−2.302*	−2.709**	−0.142*	−0.421*	−0.564**	−0.305	−0.296	−0.602
Lack of fit	*	NS	NS	NS	NS	NS	NS	NS	*	*
R^2	0.81	0.90	0.91	0.91	0.95	0.92	0.92	0.90	0.87	0.81

[a] Regression coefficients and coefficients of determination (R^2) of second-order fitted equation. The second-order function is: $\hat{Y} = \beta_0 + \beta_1 R + \beta_2 P + \beta_3 T + \beta_{11} R^2 + \beta_{22} P^2 + \beta_{33} T^2 + \beta_{12} RP + \beta_{23} PT + \beta_{31} TR$; where, P = pH, R = [water]/[surfactant], and T = temperature.

[b] FFA = free fatty acids, SCFFA = short chain (C:4 − C:10) FFA, LCFFA = long chain (C:12 − C:18) FFA, MAG = monoacylglycerols, and DAG = diacylglycerols.

* Significant ($P < 0.05$); ** Significant ($P < 0.01$); NS = Nonsignificant ($P > 0.05$).
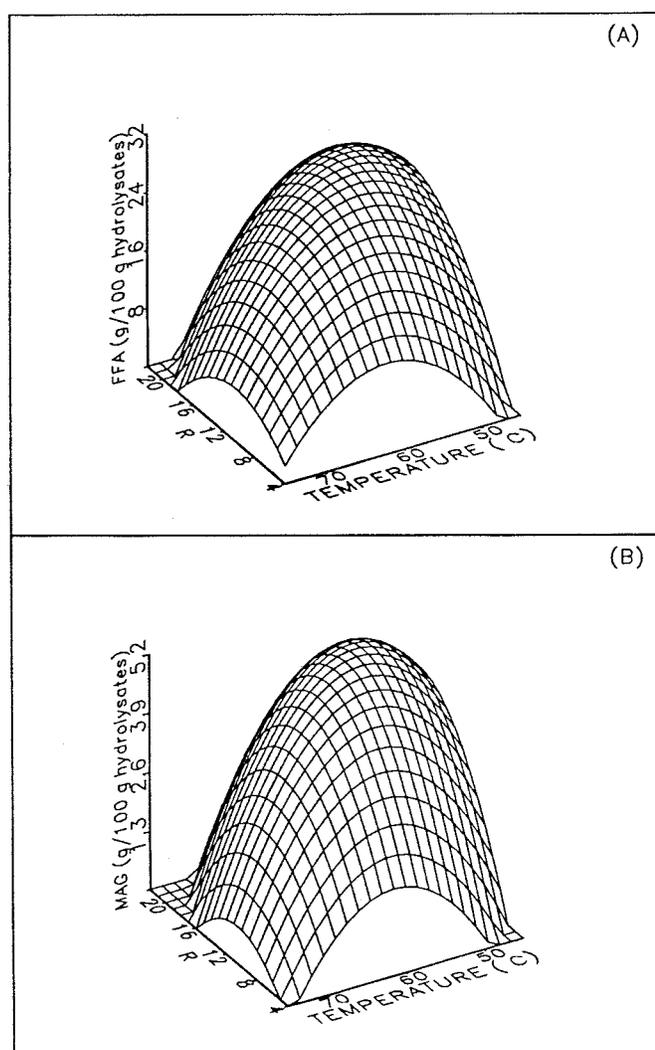

(A)

(B)

Fig. 4—Interactive effects of temperature and R on free fatty acid and monoacylglycerol contents of milk triacylglycerol hydrolyzates prepared by *R. javanicus* lipase catalyzed hydrolysis in solvent-free reverse micellar media. Reaction parameters: phospholipid concentration = 50 mM, substrate concentration = 94.9% v/v, pH = 5.5, enzyme concentration = 5 mg/10 mL reaction mixture.
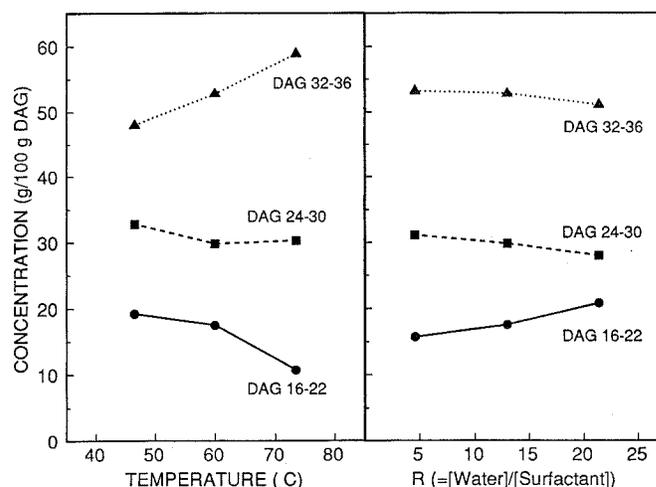

Fig. 5—Effect of temperature and R on diacylglycerol contents of different acyl carbon number in milk triacylglycerol hydrolyzates prepared by *R. javanicus* lipase catalyzed hydrolysis in solvent-free reverse micellar media. Reaction parameters: pH = 5.5 (50 mM citrate-phosphate buffer). Temperature = 60°C, phospholipid concentration = 50 mM, substrate concentration = 95.7, 94.9, and 94.2%, respectively at R = 4.6, 13, and 21.4, respectively, enzyme concentration = 5 mg/10 mL reaction mixture. Diacylglycerol (DAG) 16–22, DAG 24–30 and DAG 32–36 represent total diacylglycerol contents from 16 to 22, 24 to 30, and 32 to 36 acyl carbon number, respectively.

Table 6—Optimum conditions for products of FAP-15 *R. javanicus* lipase-catalyzed hydrolysis of milk fat in phospholipid/TAG reverse micellar media[a]

	Reaction conditions		
Reaction products[b]	R = $\dfrac{[H_2O]}{[Phospholipid]}$	pH	Temp (°C)
Glycerol	16	5-6	57
Total FFA	16	5-6	55
Short-chain FFA	20-21	7-8	49-51
Monoacylglycerol	15-16	5-6	55
Diacylglycerol	11-13	6-7	55
sn-1(3) MAG	13-15	4-6	57-60
sn-2 MAG	18-20	6-8	46-49
sn-1,3 DAG	13-16	4-6	60-63
sn-1,2 (2,3) DAG	11-16	6-8	46-49

[a] Determined by using three-factor Taylor polynomial optimization.

[b] FFA = free fatty acids, MAG = monoacylglycerols, and DAG = diacylglycerol.

Dependence of milk fat hydrolysis on the interactive effects of reaction conditions

Linear, quadratic and interactive influence of the reaction conditions on milk fat hydrolysis by *R. javanicus* and *C. rugosa* lipases were compared (Table 2) in the solvent-free phospholipid reverse micellar systems. Information on interactive effects on milk fat hydrolysis, by investigating several variables simultaneously, is very useful for commercial applications.

The initial rate and degree of hydrolysis for *R. javanicus* and *C. rugosa* lipase-catalyzed hydrolysis of milk fat at different reaction conditions were compared (Table 3). The interactive effects of reaction conditions are evident. In the case of *R. javanicus* lipase-mediated hydrolysis, the initial rate was lower at pH 4 (16.4 μmoles/min) than that at pH 7 (23.1 μmoles/min) when the reaction was carried out at 52°C (at R = 18). The initial rate was higher at pH 4 (22.4 μmoles/min) than that at pH 7 (10.0 μmoles/min), when the reaction was carried out at 68°C (at R = 18). The interactive influence of pH and temperature on the lipase reaction was evident (Fig. 2). The pH at which maximum activity occurs shifted towards higher values as temperature decreased.

Optimization of reaction conditions for milk fat hydrolysis

The GLM regression models were used to optimize reaction conditions maximizing both initial rate and degree of hydrolysis. The coefficients of linear, quadratic, and interactive effects of the reaction variables for the second-order fitted GLM regression model were compared (Table 2). The optimum ranges for conditions determined by three-factor Taylor polynomial optimization for milk fat hydrolysis in solvent-free phospholipid reverse micellar media (Table 4). The optimum ranges of R and reaction temperature by *R. javanicus* lipase were considerably higher than those by *C. rugosa* lipase. The optimum R for milk fat hydrolysis by *R. javanicus* lipase by single-variable was about 18; whereas the same observed by multi-variable study was 14–16.

Gas chromatography of milk fat hydrolysates

Milk fat is characterized by a broad range of fatty acids and a substantial proportion of short-chain fatty acids. Reaction products include glycerol, FFA of different chain lengths and types, and MAG and DAG of different acyl carbon number (ACN) and regio-isomers.

The GC column separated FFA with 4–18, MAG with 10–18 and DAG with 16–36 ACN (Fig. 3). For each class of products, components with the same ACN, but different level of unsaturation, were not separated. The *sn*-2 MAG were separated from *sn*-1(3) MAG and *sn*-1,3 DAG were separated from *sn*-1,2(2,3) DAG. Also, impurities originating from the decomposition of the silylating reagent interfered with the C4 FFA. The separation efficiency observed in this study was in general agreement with that reported for the components in enzymatically interesterified milk fat using a similar column (Kalo et al., 1989).

Effect of conditions on composition of hydrolysates

Three reaction parameters—temperature, pH, and R—had effects on composition of milk fat hydrolysates prepared by *R. javanicus* lipase. Those prepared under combinations of reaction conditions contained 0.1 to 0.5% glycerol, 6.4 to 26.9% FFA, 0.6 to 5.2% MAG, 12.7 to 27.8% DAG, and 39.7 to 77.1% TAG. Linear, quadratic, and interactive effects of the parameters were compared (Table 5).

Among parameters studied, pH had the least influence on composition of hydrolysates. Reaction temperature and R had significant linear and quadratic effects on the content of all major classes of components. Only FFA and MAG were influenced by interactive effects of R and temperature (Fig. 4).

Temperature and R, had a considerable effect on composition of partial acylglycerols in terms of ACN and regio-isomers (Fig. 5). The effect of reaction temperature on the regio-isomer contents of MAG and DAG was mixed. The concentration of *sn*-1(3) MAG and *sn*-1,3 DAG increased with the increase in the reaction temperature. On the other hand, the concentration of *sn*-2 MAG and *sn*-1,2(2,3) DAG decreased with increase in reaction temperatures. The observed effect of temperature on content of specific regio-isomers of MAG or DAG may be attributed to altered specificity of enzyme as a result of conformational changes. They may also be due to the increased rate of spontaneous acyl migration from the *sn*-2 position to the *sn*-1(3) position at higher temperatures, or to differential temperature effects on reaction kinetics.

Optimization of conditions for various end products

The optimum reaction conditions for maximization of specific components were determined based on the GLM regression models (Table 6). There were sharp differences in conditions necessary for production of a certain group of FFA, class of partial acylglycerols and regio-isomers of partial acylglycerols. Short-chain FFA could be optimally produced when the lipase reaction was carried out at lower temperature, higher pH, and higher R values. Milk fat hydrolysate with a higher proportion of MAG over DAG could be prepared at 55°C (reaction temperature) by conducting the reaction at higher R and lower pH values. Also, different optimum reaction condition ranges were observed for different regio-isomer contents of partial acylglycerols. For instance, *sn*-1 MAG were favored over *sn*-2 MAG as reaction end-products and would be optimally produced when the reaction was carried out at higher temperature, higher R, and lower pH.

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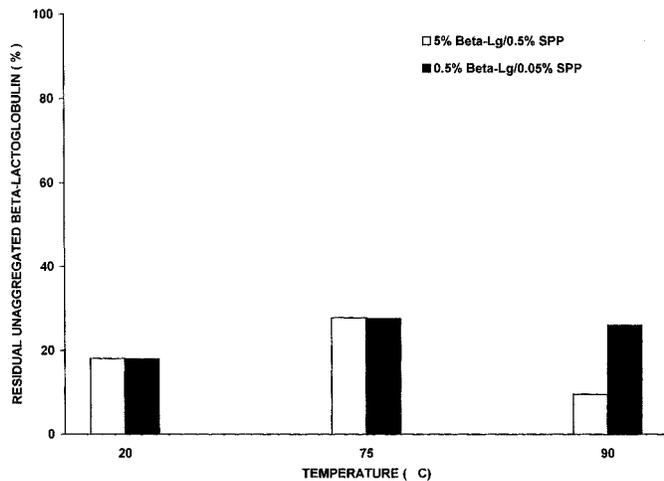
Fig. 9—Percentage unaggregated β-Lg in the filtrate of β-Lg/SPP mixtures at pH 3.5.

trostatic forces between oppositely charged gelatin and kappa-carrageenan molecules resulted in precipitate formation.

Due to precipitate formation between β-Lg and SPP at pH 3.5, chromatographic analysis was conducted only on the filtrate from unheated and heated β-Lg/SPP (10:1) mixtures. At 20°C, ≈20% of unheated β-Lg molecules did not interact with SPP to form a precipitate (Fig. 9). After heating the β-Lg/SPP mixtures to 75°C, the percentage of unaggregated β-Lg in the β-Lg/SPP filtrate increased unexpectedly to 30% (Fig. 9). This increase in unaggregated β-Lg suggested that the magnitude of β-Lg/SPP association forces was reduced during heating to 75°C. No significant difference was found in percentage of unaggregated β-Lg in the filtrates of 0.5% β-Lg/0.05% SPP mixtures heated to 75 and 90°C. However, the percentage of unaggregated β-Lg in the filtrate of 5% β-Lg/0.5% SPP mixtures was significantly greater at 75 than at 90°C. This was consistent with the observation that β-Lg was more susceptible to thermal aggregation at 5% than at 0.5% in the 75 to 90°C temperature range.

CONCLUSION

Thermal aggregation transitions of β-Lg were related to β-Lg concentration and pH. At pH 6.5, SPP did not affect the rate of β-Lg aggregation but influenced the formation of high-molecular-weight β-Lg aggregates. At pH 3.5, SPP interacted with β-Lg to form a cloudy white complex that precipitated on heating. Since aggregation is a prerequisite for gelation, SPP could be used to alter β-Lg aggregation and diversify the use of β-Lg and/or whey proteins as gelling ingredients in processed foods.

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