Interesterification of Milk Fat with Oleic Acid Catalyzed by Immobilized *Rhizopus oryzae* Lipase

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ABSTRACT

Milk fat was interesterified with oleic acid by catalysis of an immobilized lipase in a microaqueous two-phase system. A commercial lipase from *Rhizopus oryzae* and a controlled pore glass carrier were selected for preparation of an immobilized lipase. The prepared immobilized lipase showed a Michaelis constant of 77 m*M* and a maximum velocity of 40 U/g of carrier on the hydrolysis of triolein. Conditions for interesterification catalyzed by the immobilized lipase were optimized by the reaction between trimyristin and oleic acid under various conditions. The interesterification of milk fat with oleic acid was performed in isooctane with .3% (vol/vol) water content. The fatty acid composition and thermal characteristics of the triglycerides of the interesterified milk fat were investigated. The interesterified milk fat had about 50% more oleic acid and a significantly lower palmitic acid content than those of the original milk fat. The crystallization and melting curves obtained by differential scanning calorimetry analysis showed that the transition temperature of the major milk fat peaks decreased by 7.6 and 5.4°C, respectively. The results suggest that the prepared immobilized lipase can induce rather specific interesterification between oleic acid and palmitic acid in the milk fat triglycerides, which produces a lower melting milk fat, without losses of the short-chain fatty acid composition.
(**Key words**: interesterification, immobilized lipase, milk fat, oleic acid)

Abbreviation key: **CPCS** = controlled pore ceramics silica carrier, **CPCSS** = controlled pore ceramics silica carrier silane coated, **CPG** = controlled pore glass carrier, **DG** = diglyceride, **FA** = fatty acid, **MG** = monoglyceride, **TG** = triglyceride.

INTRODUCTION

In recent years, there have been many reports (2, 8, 10, 17, 18, 21, 22, 23, 24) on enzymatic catalysis in nonaqueous or microaqueous reaction systems composed of an apolar solvent and a small amount of aqueous solvent. A particularly interesting example with considerable potential in the modification of fats involves the use of lipases in organic solvents to catalyze interesterification, which is a reversal of the lipolysis catalyzed by lipases in aqueous solvents (4, 9, 12, 14, 19, 20).

Sawamura (16) and Chang et al. (3) used such interesterification to produce a fat from vegetable oils that was similar to cocoa butter. Osada et al. (15) reported the modification of sardine oil by immobilized lipase. Kalo et al. (6) reported changes in the triglyceride composition of milk fat following interesterification. These and similar studies employed immobilized lipases for their advantages in allowing the recycling of the lipases and in reducing lipase inactivation by the apolar solvents. We are interested in modifying milk fat in butter and have therefore explored the potential of immobilized lipases in the preparation of modified milk fat.

Milk fat, which has a high melting temperature, contains mainly medium- and long-chain saturated fatty acids. The low content of unsaturated fatty acids and high cholesterol con-

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tent of milk fat is considered to be a health liability, and consumers have therefore preferred the low fat butter or margarine made from vegetable oils (1) despite the lack of the characteristic butter flavor of these products. Because the high content of unsaturated fatty acid of these products is considered to be healthful, interesterification of milk fat medium- and long-chain saturated fatty acids with unsaturated fatty acids should add value to such modified milk fat.

However, although interesterification might be used to lower the saturated fatty acid content of milk fat, it can also, depending on the reaction conditions and on the characteristics of the lipase used, produce acylglycerides having a fatty acid profile altered substantially from that of the original milk fat. Changes in the composition of the short-chain fatty acids will especially disturb the butter flavor, because of the characteristic flavors that occur when such acylglycerides are hydrolyzed. We have therefore initiated a study aimed at a specific interesterification to increase the unsaturated fatty acid content of fat in butter without altering the short-chain fatty acid composition.

In this paper, we report on the interesterification of milk fat with oleic acid, an unsaturated fatty acid with a low melting point. We tested several lipases and enzyme carriers and then selected a lipase from *Rhizopus oryzae*, immobilized on glass bead carriers, as a promising enzyme preparation. We used synthetic glycerol trimyristin as a milk fat analogue and tested the interesterification of oleic acid to this triglyceride under various conditions. Interesterification of milk fat was performed under the optimal conditions thus determined, and triglycerides were separated from the products for analysis. The changes of fatty acid composition and melting characteristics of the triglycerides formed by interesterification of the milk fat were investigated.

MATERIALS AND METHODS

Materials

Rhizopus oryzae lipase (lipase F), *Pseudomonas* sp. lipase (lipase PS), *Mucor javanicus* lipase (lipase M), and *Penicillium camembertii* lipase (lipase G) were donated by Amano Enzyme Europe Ltd. (Milton Keynes, England). Controlled pore glass 10-170Å carrier (**CPG**), controlled pore ceramics silica carrier (**CPCS**), and silane-coated CPCS (**CPCSS**) were purchased from Fluka AG (Buchs, Switzerland) to be used as carriers for lipase immobilization. Milk fat was donated by Koninklijke Buisman Zuivelexport (Leeuwarden, The Netherlands). Fatty acid standards, triglyceride standards, and other general reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Lipase-Catalyzed Hydrolysis

The hydrolysis of triolein by lipase was determined in a two-phase system containing a high proportion of aqueous solvent. Lipases were dissolved with .1 *M* bis-tris propane buffer, pH 7.0, containing 10 m*M* $CaCl_2$. Triolein was dissolved in hexane at concentrations of 100, 50, 25, and 12.5 m*M* to obtain kinetic parameters. Two milliliters of lipase solution and 1 ml of hexane solution of triolein were mixed in a sealed tube and shaken vigorously in a 37°C water bath for 1 h. When immobilized lipase was used, a suspension of immobilized lipase in the bis-tris propane buffer was employed for the reaction system. To stop the reaction, the suspension in the tube was centrifuged for a few minutes at 1000 × *g* to separate the two phases. A 50-μl sample was withdrawn from the upper hexane layer and methylated for GLC analysis.

Preparation of Immobilized Lipase

Lipases were adsorbed on CPG, CPCS, and CPCSS. Before the adsorption, the carriers were washed with 1*N* HCl and 1*N* NaOH, followed by rinsing with plenty of water, after which the carriers were dried in vacuum. Lipases were dissolved in 20 m*M* bis-tris propane, pH 7.0, and centrifuged for 10 min at 5000 × *g* to remove insoluble materials.

An immobilized lipase was prepared as follows: 10 g of a carrier were suspended in 100 ml of the lipase solution and stirred for 2 h at 1 to 5°C. Then the suspension was centrifuged for 2 min at 1000 × *g*, and the protein-adsorbed carrier was recovered. The protein contents of the original lipase solution and the supernatant were measured with the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and the

amount of protein adsorbed on the carrier was determined. The carrier with adsorbed protein was washed 10 times with 200 ml of distilled water, after which the carrier was dried in vacuum.

Lipase-Catalyzed Interesterification

Lipase catalyzes both hydrolysis and interesterification of acylglycerol in two-phase reaction systems in which the relative rates of two activities can be modulated through the influence of the apolar phase on the equilibria in the concentrations of substrates and products in the two phases. Martinek et al. (13) have discussed in detail equilibria in two-phase reaction systems, and Laane et al. (11) introduced the log P parameter, based on the partition coefficient of a given compound in octanol and water for the representation of the polarity of the compound in question.

We examined the interesterification of trimyristin with oleic acid by immobilized lipases in a microaqueous two-phase reaction system. The proportion of triglyceride (**TG**) to fatty acid (**FA**), the water content, and the organic solvent used were investigated because these parameters seemed to affect the interesterification strongly. The log P values of the organic solvents used were taken from work by Inoue and Horikoshi (5). One unit of interesterification activity was defined as the incorporation of 1 μmol of oleic acid/min into trimyristin.

The typical interesterification reaction was performed as follows: trimyristin and oleic acid were dissolved separately in hexane at concentrations of .1 *M* and mixed in a proportion 1:1. Ten milligrams of the carrier with immobilized lipase were wetted with .1 *M* bis-tris propane buffer pH 7.0 containing 10 m*M* $CaCl_2$ to attain a water content of .5% (vol/vol) in the whole reaction system and then suspended in 1 ml of the trimyristin/oleic acid solution. The suspension was sealed in a small vial and shaken vigorously in a 37°C water bath for 1 h.

A 10-μl sample was withdrawn and spotted on an aluminum TLC plate (.2-mm layer thickness, silica gel 60). The spotted samples were developed on the TLC plate with hexane:ether:acetic acid (80:30:1), and separated diglyceride (**DG**), monoglycerides (**MG**), TG, and FA fractions were detected by the fluorescent emission observed after the plate was sprayed with 2′,7′-dichlorofluorescein. The TG fraction was scraped off into a test tube, screw-capped, saponified, and methylated for subsequent GLC analysis. The interesterification activity was calculated from the amount of oleic acid found in the TG fraction.

Interesterification of Milk Fat with Oleic Acid

Interesterification of milk fat with oleic acid was performed under the conditions optimized for the interesterification activity. Before the interesterification, milk fat was kept at 37°C for 1 h, after which it was dried under vacuum at 20 to 25°C for 1 d. The dried milk fat was dissolved in isooctane at a concentration of .35 g/ml, corresponding to .5 *M*, based on an average molecular weight of about 700, calculated from the glyceride composition and the FA composition of milk fat. A .5 *M* oleic acid solution was also prepared with isooctane. The milk fat solution was mixed with the oleic acid solution in a 1:1 ratio.

The immobilized lipase was suspended in .1 *M* bis-tris propane buffer, pH 7.0, containing 10 m*M* $CaCl_2$. This suspension was then added to the milk fat and oleic acid mixture in a sealed vessel. The final mixture, which contained .3% (vol/vol) water and 1.0% (wt/vol) enzyme-coated glass beads, was vigorously shaken in a 37°C water bath for 1 to 4 h. A 2-μl sample was separated into TG, DG, MG, and FA fractions with TLC as described.

Methylester Fatty Acid Derivatives

The TG fractions separated on TLC were saponified and methylated as follows: the TG fraction in a screw-capped test tube was directly saponified in 1 ml of 3.75 *N* NaOH (H_2O-methanol, 1:1) at 80°C for 40 min. After rapid cooling to room temperature, 2 ml of 3.25 *N* HCl (H_2O-methanol; 1:1) was added in the tube, and the saponified FA was methylated at 80°C for 15 min. The tube was cooled again, and then 2 ml of hexane were vigorously mixed to extract the methylated fatty acids. The mixture was separated into two layers by centrifugation at 1000 × *g* for 2 min. The bottom water layer was pipetted out, and

then 3 ml of .3 *N* NaOH were mixed to remove residual HCl from the hexane. The hexane separated by the same centrifugation was transferred and sealed in a vial for GLC analysis. When hydrolysis activity was to be determined, the 50-μl sample was treated from the methylation step onward (without saponification) to derivatize the enzymatically hydrolyzed oleic acid only.

GLC Analysis

The fatty acid methylesters were analyzed with a capillary gas chromatograph (436S; Packard Becher, Delft, The Netherlands) equipped with a flame ionization detector and a capillary column Sil-5 CB (Chrompack, Middelburg, The Netherlands). The injector and detector temperatures were kept at 300°C. The column temperature was started and kept at 45°C for 2 min, after which it was increased to 220°C at 25°C/min and to 284°C at 8°C/min. The chromatograms were analyzed with a Shimadzu integrator CR3A (Kyoto, Japan).

Differential Scanning Calorimetry

Thermal analyses of triglycerides of the original milk fat and the interesterified milk fat were performed with a differential scanning calorimeter (DSC-7; Perkin-Elmer, Norwalk, CT). The triglyceride was prepared by preparative TLC separation followed by extraction with chloroform:methanol (1:1) and drying in vacuum at 40°C for 2 h.

The temperature program was applied following Kalo et al. (7). A sample kept at 50°C in the chamber of the apparatus was cooled to −15°C at 8°C/min and heated again to 50°C at the same rate. Then the sample was ready for analysis. The crystallization curve was measured by cooling to 60°C at 8°C/min, and the temperature was held there for 5 min. The melting curve was then measured by heating to 50°C at 8°C/min.

RESULTS AND DISCUSSION

Selection of Lipase

In this paper, we have examined the catalytic properties of four relatively crude, commercially available lipase preparations (Table 1), which can be separated in two groups by their substrate specificities from the supplied specifications. Lipases F and M preferentially attack 1,3-ester bonds or triglyceride ester bonds with medium- and long-chain fatty acids. Lipases PS and G attack all ester bonds to any fatty acid. The regio-specificity of the ester bonds is not so important, but specificity of the fatty acyl chain length is very important for this study. The protein content and kinetic parameters of each of the four lipases were determined (Table 1). Lipase F, which has a suitable specificity, showed the highest maximum velocity, 72.9 U/mg of protein, and the highest protein content, 10.9%.

Selection of Carrier

Before preparation of immobilized lipase, the protein adsorption capacity of the different carriers was investigated. For these experiments we measured adsorption of total protein in lipases F and PS, only a small percentage of which is lipase (see Table 1). The different carriers showed similar capacities for both lipases. The maximum capacity of the CPG was about 16 mg of protein/g of carrier, and that of the CPCS and the CPCSS was 8 mg of protein/g of carrier (Figure 1), perhaps because the CPG has a larger specific surface area than CPCS or CPCSS.

We tested the lipase activity adsorbed to the carriers and the retention of this enzyme after repeated washings. Figure 2 shows the residual lipase activities after adsorption to carriers and washing of the protein adsorbed carriers. The residual activities are related to the activity of the original lipase solution, which was taken as 100%. When CPCSS was used, the activity of the lipase preparations decreased strongly on adsorption to the carriers. The reason for this may be that lipases adsorb to the carrier in such a way that contact of the lipase active site with substrate in organic solvent is reduced. Kinetic parameters were also determined for the carriers dried after 10 times washing. Only lipases adsorbed to CPG showed some activity. No activity was found for lipases bound to CPCS or CPCSS. Kinetic measurements of the hydrolysis of triolein were carried out for the finally prepared immobilized lipase F on CPG. The Michaelis constant and the maximum velocity were 77 m*M* and 40 U/g of carrier.

TABLE 1. Protein content of commercial lipase preparations and their kinetic parameters of lipase activity on triolein.

Lipase	Origin	Protein	Michaelis constant	Maximum velocity
		(%)	(m*M*)	(U/mg of protein)
F	*Rhizopus oryzae*	10.9	105	72.9
PS	*Pseudomons* sp.	1.0	32	48.7
M	*Mucor javanicus*	6.7	64	12.4
G	*Penicillium camembertii*	1.1	20	2.2

Interesterification Activity of Immobilized Lipase

We compared the hydrolysis efficiency with the interesterification activity obtained for each reaction condition tested, because some conditions induced not only high interesterification but also high hydrolysis activities. Three sets of experimental conditions induced high interesterification activity and reasonably low hydrolysis efficiency, which might be useful in retaining the butter flavor. Triglyceride can be a substrate or a product in the interesterification reaction, and the maximum lipase activity was obtained with a proportion 5:5 to 4:6 of TG:FA (Figure 3). The hydrolysis efficiency was constant at 26%, independent of the proportion of TG:FA used. The maximum enzyme activity occurred in a very limited range of the water content (Figure 4). Lipase was inactive in the absence of water. With small amounts of water, lipase catalyzed lipolysis and interesterification; the interesterification activity increased significantly, and the hydrolysis also increased gradually in more than .1% water content. The maximum rate of interesterification was at .2 to .3% water and decreased rapidly upon further increase in water content.

Five different organic solvents were tested with this reaction system to select a good solvent (Table 2). No obvious relationship existed between the interesterification rate and the log P. However, the lipase was completely inactivated in toluene (log P = 2.8), but sol-

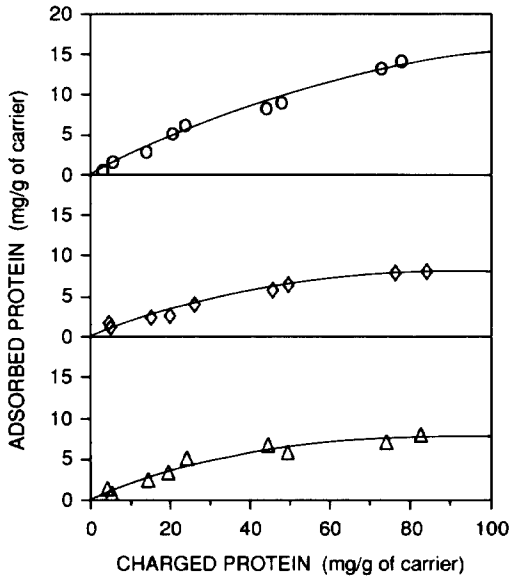

Figure 1. Protein adsorption onto carrier in lipase solutions: controlled pore glass carrier (CPG; ○), controlled pore ceramics silica carrier (CPCS; ◊), and silane-treated CPCS (CPCSS; △).

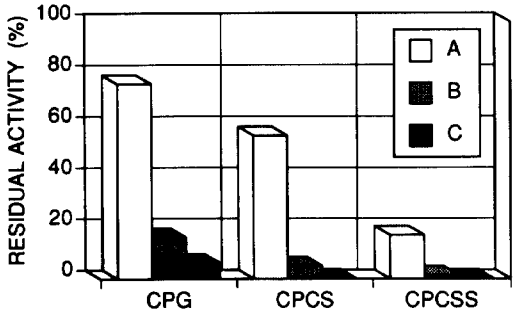

Figure 2. Residual activities after adsorption onto carriers. Ten grams of carrier were suspended in 100 ml of lipase F solution and then washed with 200 ml of water 10 times. The lipase activity of the original solution was taken to correspond to 100%. Lipase solution with carrier after adsorption (A), after the first washing (B), and after 10 washings (C). CPG = Controlled pore glass carrier, CPCS = controlled pore ceramics silica carrier, and CPCSS = silane-treated CPCS.

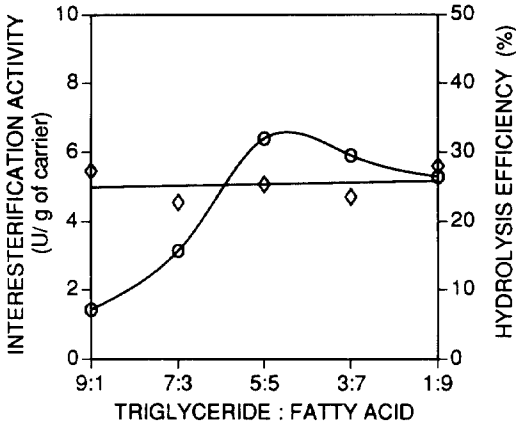

Figure 3. Effect of the proportion of triglyceride to fatty acid on interesterification activity (o) and hydrolysis efficiency (◊).

TABLE 2. Interesterification activities and hydrolysis efficiencies in different organic solvents.

Organic solvent	Interesterification activity	Hydrolysis efficiency	Log P[1]
	(U/g of carrier)	(%)	
Dodecane	5.99	34.7	7.0
n-Octane	4.08	28.6	4.9
Isooctane	9.42	31.5	4.8
Hexane	2.32	25.1	3.9
Toluene	0	6.6	2.8

[1]Values based on the partition coefficient to octanol and water were defined by Inoue and Horikoshi (5).

vents with log P >4 allowed adequate activities, which is in agreement with the observations of Laane et al. (11). Isooctane (log P = 4.8) induced high interesterification activity and reasonably low hydrolysis efficiency.

All subsequent experiments were therefore carried out with isooctane. For future pilot-scale application, additional solvents will have to be explored because isooctane is difficult to remove completely, leaving solvent residues in the interesterified product. One alternative is hexane, a solvent acceptable for food processing. However, because immobilized lipase preparations used here showed low interesterification activity in hexane, we employed isooctane to study interesterification under the best possible conditions.

The optimal conditions thus determined were applied for the interesterification of milk fat. The reaction system consisted of the immobilized lipase in a microaqueous two-phase system. The water molecules [.1 to .2% (vol/vol)] are probably mostly bound to the carrier and to the protein bound to the carrier (ca. 10 mg/g of carrier). The carrier-enzyme-H_2O system is suspended in the apolar phase. As the CPG particles are surrounded by a water-immiscible organic solvent, the lipase (protein) probably does not desorb from the particles. Catalysis can occur on contact of the triglycerides with lipase on the CPG surface. Such a system has several advantages over a two-phase system consisting of a direct dispersion of a solvent phase in a water phase containing enzyme. Thus, it is easy to recycle the immobilized enzyme, to use it in a continuous reaction system, and to separate the organic solvent from the biocatalyst after the reaction. Furthermore, enzyme immobilization decreases inactivation by contact of enzyme with the organic solvent and reduces lipase inhibition by the substrate and the products.

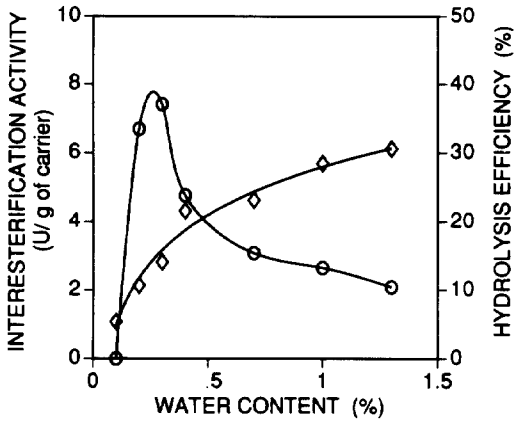

Figure 4. Effect of water content on interesterification activity (o) and hydrolysis efficiency (◊).

Interesterified Milk Fat

The fatty acid compositions of the triglycerides of original milk fat and interesterified milk fat are shown in Table 3. Oleic acid increased about 50% after the interesterifica-

TABLE 3. Fatty acid compositions of the triglycerides of original and interesterified milk fat.

Fatty acid	Milk fat	
	Original	Interesterified
	(g/100 g)	
Butyric acid	3.53	3.65
Caproic acid	2.25	2.36
Caprylic acid	1.56	1.32
Capric acid	3.63	3.52
Lauric acid	5.96	5.96
Myristic acid	13.04	12.59
Palmitic acid	34.12	25.39
Stearic acid	8.95	5.58
Oleic acid	22.55	33.15

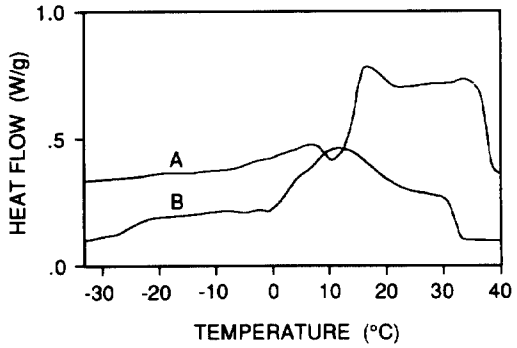

Figure 6. Melting curves of triglycerides of original (A) and interesterified milk fat (B) by differential scanning calorimetry analysis.

tion. The compositions of short- and medium-chain length fatty acids from $C_{4:0}$ to $C_{10:0}$ remained nearly the same, but longer chain fatty acids, especially palmitic acid ($C_{16:0}$), decreased as oleic acid was introduced. We expect this substrate specificity to be reasonable for this study, because of the limited specificity to short-chain fatty acids from milk fat triglycerides.

The results of thermal analysis are shown in Figures 5 and 6. The triglycerides of the original milk fat showed a small peak at 18.3°C and a main peak at 9.7°C in the crystallization curve (Figure 5). The crystallization curve of the triglycerides of the interesterified milk fat was shifted to a lower temperature; two clear peaks were detected at 10.8 and 2.0°C. The temperature shifts for the two peaks were identical: 7.6°C. The melting curves were also very similar. For the original milk fat, a main peak and a shoulder peak were observed at 16.8 and 34.0°C. These peaks shifted to 11.5 and 28.5°C, respectively, for the interesterified milk fat, resulting in differences of about 5.4°C. Thus, the interesterifications imparted milk fat with lower apparent melting characteristics.

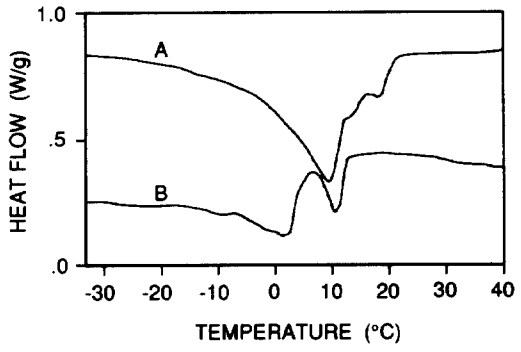

Figure 5. Crystallization curves of triglycerides of original (A) and interesterified milk fat (B) by differential scanning calorimetry analysis.

CONCLUSIONS

The lipase F from *R. oryzae* showed the highest hydrolysis activity to triolein among four commercial lipase preparations. The prepared immobilized lipase F on glass beads catalyzed both hydrolysis and interesterification in a two-phase reaction system. Interesterification occurred preferentially in a reaction mixture that consisted of an equimolar concentration of TG and FA, .2 to .3% (vol/vol) of water, and isooctane as the organic phase. The interesterification of milk fat with oleic acid by the *R. oryzae* lipase resulted in the exchange of palmitic acid in the milk fat triglyceride with oleic acid. This exchange lowered the melting temperatures of the milk fats and is expected to have only a limited effect on the development of undesirable flavors if such modified fats are subsequently hydrolyzed.

Further work is necessary to develop practical applications of these findings. Among

these, the selection of suitable solvents that are acceptable for food-grade applications and removable in subsequent postcatalytic processing is essential.

Our present findings indicate that the investment and energy needed to surmount these potential difficulties should be worthwhile, thus permitting the full development of two-liquid phase biocatalysis for food processing.

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