Modification of Milkfat Physical Properties by Immobilized *Pseudomonas fluorescens* Lipase

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A bifunctional fusion protein was constructed by fusion of the streptavidin gene from *Streptomyces avidinii* to the lipase gene from *Pseudomonas fluorescens*, and the resulting streptavidin−lipase was expressed in *Escherichia coli*. Immobilized streptavidin−lipase was prepared by direct bioselective adsorption from crude cell lysates on biotinylated controlled-pore glass and used to catalyze interesterification of anhydrous butteroil. Changes in the triacylglycerol composition indicated that those with equivalent carbon numbers (ECN) ranging from 36 to 42 decreased, while those with ECN values from 48 to 50 increased following interesterification for 120 h in hexane at 42 °C. Both the melting temperatures and the solid fat content at various temperatures were lower as compared to those of the unmodified butteroil. Addition of unsaturated fatty acids, linoleic and linolenic, yielded modified butteroils with lower melting points and solid fat content, whereas addition of saturated fatty acids, palmitic and stearic, increased the solid fat content of the modified butteroil. The liquid butteroil could function as a solvent as well as the substrate; however, the interesterification reaction rate was much slower than in hexane.

Keywords: *Milkfat; immobilized lipase; streptavidin−lipase; fusion protein; interesterification*

INTRODUCTION

Bovine milk contains about 4% (w/w) of fat, in which >95% of the milkfat is triacylglycerols followed by diacylglycerols (∼2%) and phospholipids (∼1%) (Swaisgood, 1996). Over 400 different fatty acids have been identified in bovine milkfat; however, only 10 fatty acids are quantitatively significant [>1% (w/w); Jensen, 1992]. Palmitic acid (26%), oleic acid (25%), stearic acid (12%), and myristic acid (10%) are the predominant fatty acids in milkfat. Due to a positive correlation between high-fat diets, particularly those high in saturated fatty acids, and atherosclerosis, milkfat consumption in the United States has been reduced dramatically. To extend milkfat uses and improve its nutritional properties, processing steps such as fractionation (Fouad *et al.*, 1990), hydrolysis (Chen and Pai, 1991; Ha and Lindsay, 1993), and interesterification (Kalo *et al.*, 1986,1990; Elliott and Parkin, 1991; Christensen and Holmer, 1993; Bornaz *et al.*, 1994; Kermasha *et al.*, 1995) have been applied to milkfat. The resulting products have distinguishing melting points, solid fat contents, flavor, and fatty acid distributions on the glycerol backbone as compared with those of unmodified milkfat.

Interesterification catalyzed by lipase from *Candida cylindracea* yielded products similar to those formed with sodium methoxide (Kalo *et al.*, 1986). Significant changes occurred in the concentrations of monounsaturated triacylglycerols with 36 and 38 acyl carbons, which are decreased by 45 and 52%, respectively, and in the concentrations of trisaturated triacylglycerols with carbon numbers ranging from 44 to 50, which are increased by 26% after a 14-day reaction period (Kalo *et al.*, 1986). The triacylglycerol compositions of *Pseudomonas fluorescens* lipase-treated butterfat are similar to those calculated for a random distribution (Kalo *et al.*, 1990). Adding high-melting trimyristin or tripalmitin to butter resulted in an increase of the solid fat content by 114% after 48 h of interesterification catalyzed by *sn*-1,3-specific *Mucor miehei* lipase (Bornaz *et al.*, 1994). Christensen and Holmer (1993) prepared a human milkfat analogue that had the same total fatty acid composition and positional distribution as those found in human milk using a *Rhizomucor miehei* lipase-catalyzed modification of butteroil.

Lipases catalyze both reversible hydrolysis and synthesis of acylglycerols. By using organic solvents and limiting water content in the reaction medium, lipase-catalyzed hydrolysis of lipids can be minimized, whereupon interesterification becomes the dominant reaction (Macrae, 1983; Sonnet *et al.*, 1986; Lilly and Dunnill, 1987; Chulalaksananukul *et al.*, 1990; Elliott and Parkin, 1991; Lie and Molin, 1991; Miller *et al.*, 1991; Haas *et al.*, 1993, 1995; Triantafyllou *et al.*, 1993; Valivety *et al.*, 1994). Besides reducing the water content in the reaction medium, organic solvent also increases the solubilities of lipids, improves thermostabilities of lipases, and possibly "freezes" the active conformation of the lipases (Neidleman and Geigert, 1984; Zaks and Klibanov, 1984, 1985; Ahearn and Klibanov, 1986; Russell and Fersht, 1987; Reetz *et al.*, 1996). Recently, interesterification of milkfat in the absence of an organic solvent was reported by Kalo *et al.* (1990), Elloitt and Parkin (1991), and Bornaz *et al.* (1994), which indicates that butteroil could act as dispersant as well as substrate in the reaction mixture (Elliott and Parkin, 1991). By not using organic solvents, requirements for their removal after interesterification could be eliminated and their possible toxicity to humans thus avoided.

The purpose of this study was to investigate the feasibility of modifying the physical properties of milkfat by *P. fluorescens* lipase immobilized by bioselective adsorption. Previously, we have shown that biotinylated *C. cylindracea* lipase immobilized on biotinylated

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matrices through an avidin spacer exhibited better interesterification activity than did soluble lipase in an organic solvent (Lee and Swaisgood, 1997a). Also, we have constructed a streptavidin–lipase expression vector and demonstrated that the streptavidin–lipase chimeric protein produced by *Escherichia coli* could be simultaneously purified and immobilized on biotinlyated matrices in a single step (Lee and Swaisgood, 1997b). In this study, the immobilized streptavidin–lipase fusion protein was used to catalyze the interesterification of milkfat with a variety of fatty acids either in the presence or in the absence of organic solvent, and the resulting compositional changes were determined by reversed-phase HPLC, while the melting points and solid fat contents were analyzed by differential scanning calorimetry.

MATERIALS AND METHODS

Preparation of Biotinylated Beads. Controlled-pore glass beads with a particle size of 120/200 mesh and a mean pore diameter of 302 nm (CPG-3000) were obtained from CPG Inc. (Fairfield, NJ). The beads were derivatized with (3-aminopropyl)triethoxysilane according to the methods of Janolino and Swaisgood (1982). The aminopropyl beads were biotinylated using sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) (Pierce, Rockford, IL) dissolved in 50 mM sodium bicarbonate buffer, pH 8.5, at a concentration of 5 mg/mL.

Immobilization of Streptavidin–Lipase Fusion Protein. The construction of plasmid pSTLP2 was described previously by Lee and Swaisgood (1997b). Briefly, the lipase gene from *P. fluorescens* B52 (Tan and Miller, 1992) was modified by a polymerase chain reaction (PCR) to introduce *Sal*I and *Kpn*I restriction sites into 5′ and 3′ ends of the gene, respectively. The PCR-modified lipase gene was cloned inframe at the 3′-end of streptavidin gene in plasmid pStp4 (Walsh and Swaisgood, 1994). The constructed plasmid, pSTLP, was used to transform *E. coli* NM 522 (Invitrogen, San Diego, CA). Lipase-producing colonies were identified by screening on tributyrin and trioleoylglycerol-rhodamine B agar plates. Expression of the streptavidin–lipase fusion gene was induced by adding 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Ambion, Austin, TX) to the lipase-producing *E. coli* cell cultures. Induced cell cultures were further incubated overnight before harvesting by centrifugation. Cell pellets were resuspended in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.5 M NaCl, and 10 mM $MgCl_2$ (lysis buffer), and the cells were disrupted using a sonic dismembrator (Fisher Scientific, Atlanta, GA). Cell lysate was centrifuged at 8000*g* at 4 °C for 15 min, and the supernatant was collected as soluble protein fraction 1. The cell precipitate was washed twice with the lysis buffer supplemented with 0.1% Triton X-100, and a supernatant (soluble protein fraction 2) was collected by centrifugation and combined with the soluble protein fraction 1 to give the total soluble protein fraction. The combined soluble protein fraction was circulated through the biotinylated CPG-3000 overnight at 4 °C. Immobilized enzyme beads were washed, first with 2 M urea followed by 4 M NaCl, to remove any nonspecifically bound proteins and finally with 0.1 M Tris-HCl, pH 7, containing 0.02% sodium azide.

Modification of Butteroil by Immobilized Lipase-Catalyzed Interesterification. Anhydrous butteroil (0.5 g; Danish Maid, Chicago, IL) with or without 100 mg of free fatty acid (myristic acid, palmitic acid, stearic acid, linoleic acid, or linolenic acid; Sigma Chemical, St. Louis, MO) was dissolved in 20 mL of hexane. Immobilized streptavidin–lipase beads (0.2 g) were added to the mixture, and the mixture was incubated in a 42 °C shaker operating at 200 rpm (New Brunswick Scientific Inc., Edison, NJ). For interesterification conducted in the absence of hexane, 10 g of butteroil was preincubated in the 42 °C shaker for 30 min before 0.2 g of immobilized enzyme beads was added to the melted butteroil. A small amount (∼60 μL) of sample was withdrawn from the reaction mixture every 24 h. The samples were filtered through a 0.2-μm filter disk, dried under nitrogen gas, redissolved in diethyl ether/acetonitrile (90:10, v/v), and analyzed by reversed-phase HPLC. The HPLC system was composed of a SSI 222B HPLC pump (SSI Scientific Inc., State College, PA), two in-series 250-mm × 4.6-mm Zorbax ODS columns (Mac-Mod Analytical Inc., Chadds Ford, PA), and a Waters differential refractometer R401 (Millipore Corp., Milford, MA). The mobile phase used was acetone and acetonitrile (63.5:36.5, v/v) at a flow rate of 1 mL/min (El-Hamdy and Perkins, 1981; Frede and Thiele, 1987).

After 120 h of incubation, the added free fatty acid was separated from mono-, di-, and triacylglycerols of the butteroil by thin-layer chromatography (TLC) according to the method of Linfield *et al.* (1984). The butteroil was filtered through a 0.45-μm filter disk, dried under nitrogen gas, and redissolved in methylene chloride at a concentration of ∼60 mg of acids and acyglycerols/mL of solvent. One milliliter was applied on a 20- × 20-cm silica gel G plate (Fisher Scientific). The developing solution was prepared fresh daily by combining 210 mL of petroleum ether, 90 mL of anhydrous ethyl ethee, and 0.4 mL of formic acid. The separated bands were visualized by exposing the plate to iodine vapor, and the bands corresponding to mono-, di-, and triacylglycerols were scraped from the TLC plate, extracted twice with diethyl ether, dried under nitrogen gas, and stored at −20 °C. These samples were used for thermal analyses.

Thermal Analysis. A Perkin-Elmer 7 series differential scanning calorimeter (DSC) (Norwalk, CT) was used to measure the melting curves of the butteroil. The machine was calibrated against indium (156.61 °C) and dodecane (−9.6 °C) according to the manufacturer's instruction manual. Before the melting curve was measured, the butteroil samples were cooled from 30 to −60 °C at 8 °C/min and heated to 50 °C at the same rate to destroy any previous crystalline structures (Kalo *et al.*, 1986). The DSC was programmed to cool the samples to −60 °C at 8 °C/min. After a 10-min holding period at −60 °C, the melting curves were measured during heating of the samples to 50 °C at 8 °C/min. The solid fat content of the butteroils as a function of temperature ranging from 0 to 50 °C was determined by integration of the DSC melting curve using the analysis program supplied by Perkin-Elmer.

RESULTS

Triacylglycerol Composition of Butteroils. The triacylgylcerols of butteroil were separated by reversed-phase HPLC according to their equivalent acyl carbon number (ECN; El-Hamdy and Perkins, 1981) and degree of saturation. Typical HPLC profiles of butteroil triacylglycerols, similar to those observed by Frede and Thiele (1987) and Bornaz *et al.* (1994), were obtained. Forty different peaks, which correspond to triacylglycerols with ECNs from 30 to 50, could be distinguished in the chromatogram. Because the separation of butteroil triacylglycerols by reversed-phase HPLC was conducted at room temperature, the highest ECN of butteroil triacylglycerols that eluted from the reversed-phase HPLC was 50, compared with an ECN of up to 52 at elution temperatures of 30 or 35 °C (Frede and Thiele, 1987). Peaks in the chromatogram were observed in groups of four, each representing a single class of triacyglycerols having the same ECN. In each class, the first peak is triunsaturated triacylglycerols, whereas the second, third, and fourth peaks are mainly diunsaturated, monounsaturated, and trisaturated triacylglycerols, respectively (Frede and Thiele, 1987; Bornaz *et al.*, 1994). The percentages and the changes of butteroil triacylglycerol compositions before and after immobilized lipase-catalyzed interesterification in hexane are shown in Figure 1. Changes resulting from interesterification were mainly in the triacylglycerols with ECN values ranging from 36 to 42 (peaks 9–24)

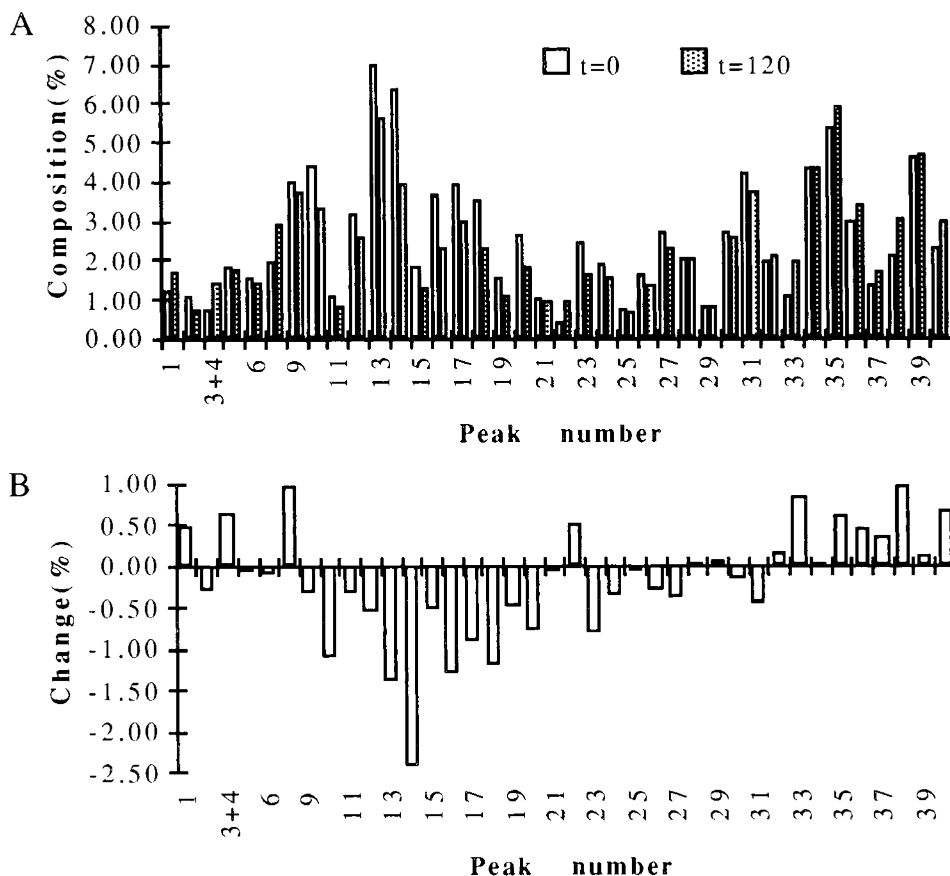

Figure 1. Composition (A) and percentage change (B) of the triacylglycerols of butteroil modified by immobilized lipase-catalyzed interesterification at reaction times of 0 and 120 h. The interesterification reaction mixture contained 0.2 g of immobilized lipase beads and 0.5 g of butteroil dissolved in 20 mL of hexane. The reaction temperature was 42 °C.

and from 48 to 50 (peaks 33–40) (Figure 1B). A decrease in product percentages was observed for the triacylglycerols with ECN values of 36–42 (peaks 9–21, 23, and 24), whereas an increase was observed for those with ECN values of 48–50 (peaks 33–40). Similar changes in milkfat composition were reported by Kalo *et al.* (1986) when they used *C. cylindracea* lipase or sodium methoxide to catalyze interesterification. Also, similar but less intense compositional changes of butteroil triacylglycerols were observed when butteroil was used as both a reaction substrate and a dispersed solvent (data not shown). Although reversed-phase HPLC analyses do not identify the fatty acid component in each peak, those triacylglycerols whose quantities were increased after interesterification corresponded to the nature of the fatty acid added to the reaction mixture. El-Hamdy and Perkins (1981) reported that the presence of each double bond in a triacylglycerol would decrease the ECN of that triacylglycerol by 2. Thus, triacylglycerols containing linolenic (octadecatrienoic) acid would be eluted from the reversed-phase HPLC together with those containing lauric (dodecanoic) acid [$18 - (2 \times 3) = 12$]. The effects of adding linolenic acid to the interesterification reaction mixture on the triacylglycerol composition of butteroil are shown in Figure 2. The main changes were in the triacylglycerols with 36–48 acyl carbons (peaks 9–36). Triacyglycerols with ECN values of 36–40 (peaks 9–20) decreased, and those with ECN values of 42–46 (peaks 21–32) increased after 120 h of interesterification. The increased amounts of triacylglycerols (ECN from 42 to 46; peaks 21–32) after interesterification corresponded to those that incorporated linolenic acid into glycerol backbone. Similarly, a decrease of triacylglycerols with ECN values ranging from 36 to 44 (peaks 9–28) and an increase of triacylglycerols with ECN values ranging from 46 to 50 (peaks 29–40) were observed when palmitic (hexadecanoic) acid was added to the interesterfication reaction mixture (Figure 3). The ECN of palmitic acid (C16:0) is 16; thus, triacylglycerols whose concentrations were increased after interesterification were observed in those with ECN values of 46 or higher (peaks 29–40).

Melting Properties and Solid Fat Content of Butteroil. The melting temperatures of interesterification-modified and untreated butteroil determined by DSC are listed in Table 1. There were noticeable differences between the melting curves observed for interesterification-modified and untreated butteroils. Three peaks were discernible in the melting curves of unmodified butteroil and those that had been modified by interesterification with immobilized lipase in the presence of an added fatty acid. However, only two peaks were observed in the melting curves of butteroil interesterified in hexane without any added fatty acid in the reaction mixture. The untreated butteroil had mean melting points of 8.99, 15.76, and 32.43 °C in the low-, medium-, and high-melting ranges, respectively. The medium- and high-melting points of butteroil were changed from 15.76 to 11.43 °C and from 32.43 to 29.15 °C, respectively, following immobilized lipase-catalyzed interesterification in the presence of hexane. Decreases of melting temperature were also observed for butteroil modified by immobilized lipase-catalyzed interesterification in the presence of unsaturated fatty acids such as linoleic (octadecadienoic) acid or linolenic (octadec-

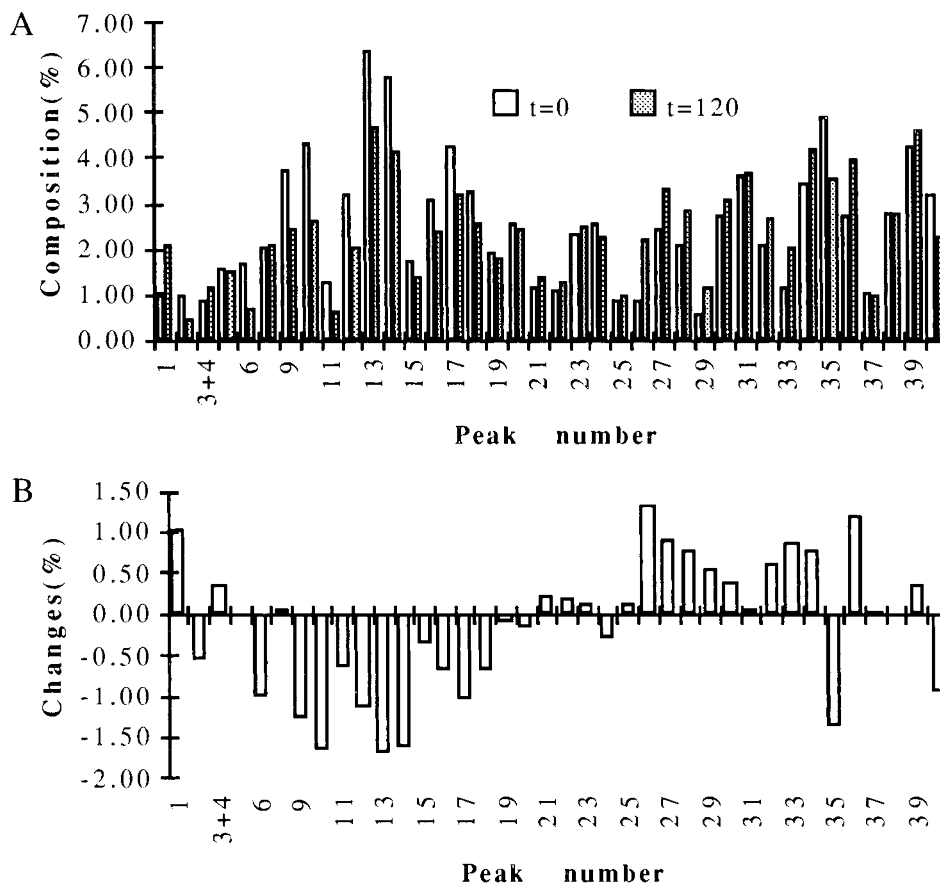

Figure 2. Composition (A) and percentage change (B) of the triacylglycerols of butteroil modified by immobilized lipase-catalyzed interesterification at reaction times of 0 and 120 h. The interesterification reaction mixture contained 0.2 g of immobilized lipase beads, 0.5 g of butteroil, and 100 mg of linolenic acid dissolved in 20 mL of hexane. The reaction temperature was 42 °C.

atrienoic) acid (Table 1). The more double bonds the fatty acid contained, the lower the melting temperature of the modified butter after interesterification. By contrast, the medium-melting temperature of butteroil was increased from 15.76 to 17.41 °C after interesterification in the presence of myristic (tetradecanoic) acid. Adding palmitic (hexadecanoic) acid to the interesterification mixture increased both medium- and high-melting temperatures from 15.76 and 32.43 °C for unmodified butteroils to 24.07 and 41.40 °C after interesterification, respectively. Only the high-melting temperature of modified butteroil was increased by interesterification in the presence of stearic (octadecanoic) acid.

The solid fat contents of untreated and modified butteroils interesterified in the presence of fatty acids at temperatures ranging from 10 to 50 °C are shown in Figure 4 and Table 2. Untreated butteroil contained ~26% solid fat at 25 °C and was completely melted at temperatures above 35 °C. After interesterification, the solid fat content of butteroil that was modified by immobilized lipase in the presence of stearic acid was increased by 1.7-fold (~44%) at 25 °C and still contained 33% solid fat at 35 °C. On the other hand, adding linoleic acid or linolenic acid to the interesterification mixture decreased the solid fat content of modified butteroil as compared with that of untreated butteroil at temperatures ranging from 10 to 25 °C. Butteroils that were modified by immobilized lipase in the presence of linoleic acid or linolenic acid contained only 8 and 1% solid fat, respectively, at 25 °C. Furthermore, those butteroils were completely melted at temperatures above 30 °C, whereas untreated butteroil still contained ~10% solid fat. In the absence of added fatty acids, our results showed a 35% decrease in the solid fat content of butteroil at 20 °C after interesterification by immobilized streptavidin−*P. fluorescens* lipase in hexane after 120 h.

DISCUSSION

Interesterification catalyzed by bioselectively adsorbed streptavidin−lipase bifunctional fusion protein changed the triacylglycerol composition of butteroil and thus its functional properties. The compositional changes that occurred depended upon the reaction conditions, such as the addition of fatty acids and the type of acid added. The major changes in triacylglycerol composition due to immobilized lipase-catalyzed reactions in hexane solutions were for those with ECN values ranging from 36 to 42 and from 48 to 50. Similar observations were made for interesterification reactions catalyzed by *C. cylindracea* lipase and sodium methoxide (Kalo *et al.,* 1986), probably because both *C. cylindracea* and *P. fluorescens* lipases are nonspecific and therefore the acyl exchanges among the triacylglycerols are random (Kalo *et al.,* 1990). Although the percentages of triacylglycerols within the ECN range of 48−50 increased after 120 h of reaction in hexane, the modified butteroil exhibited lower melting points in both the medium- and high-melting point ranges as compared with unmodified butteroil. This apparent contradiction has been attributed to the presence of mono- and diacylglycerols in the final product, which yields a softer butteroil (Kalo *et al.,* 1986). Butteroil has been fractionated into solid and liquid fractions on the basis of

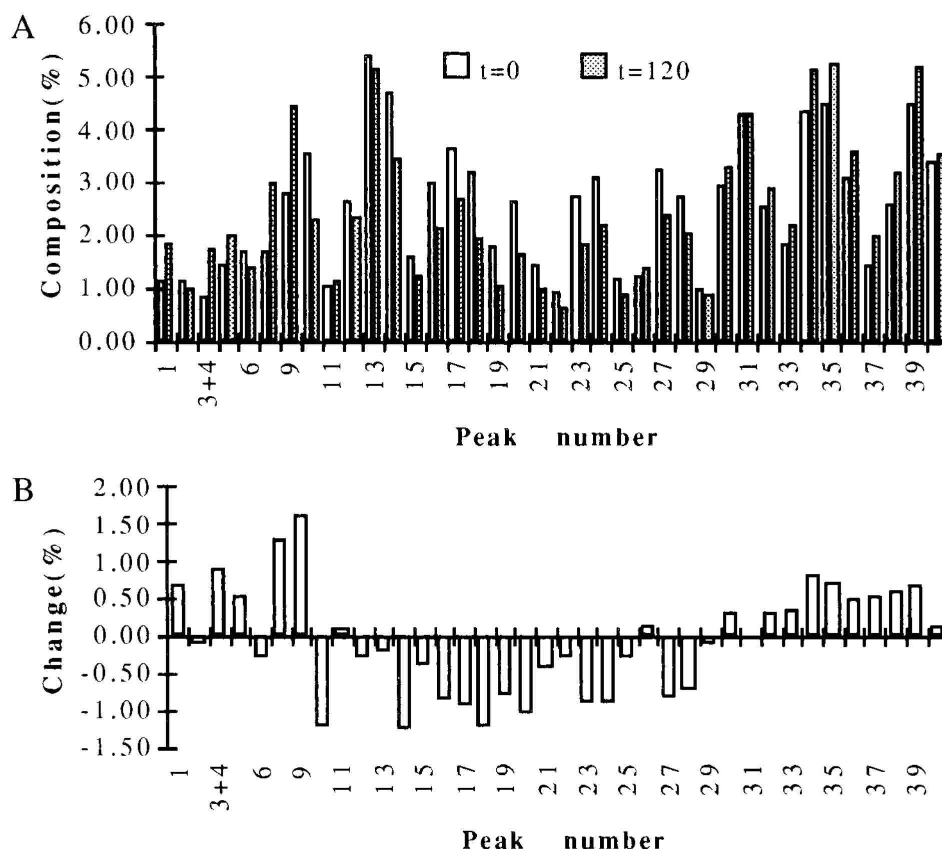

Figure 3. Composition (A) and percentage change (B) of the triacylglycerols of butteroil modified by immobilized lipase-catalyzed interesterification at reaction times of 0 and 120 h. The interesterification reaction mixture contained 0.2 g of immobilized lipase beads, 0.5 g of butteroil, and 100 mg of palmitic acid dissolved in 20 mL of hexane. The reaction temperature was 42 °C.

Table 1. Mean Melting Temperatures[a] of Untreated and Immobilized Lipase-Modified Butteroils As Determined by DSC[b]

sample	T_1 (°C)	T_2 (°C)	T_3 (°C)
untreated butteroil	8.99 ± 0.35	15.76 ± 0.13	32.43 ± 0.37
treated in hexane	NP[c]	11.43 ± 0.97	29.15 ± 0.10
treated without solvent	7.22 ± 3.06	15.25 ± 0.19	29.45 ± 1.76
C14:0 added	8.11 ± 0.76	17.41 ± 0.43	28.47 ± 4.33
C16:0 added	6.35 ± 1.26	24.07 ± 1.92	41.40 ± 3.83
C18:0 added	1.76 ± 1.49	17.29 ± 1.07	41.14 ± 0.96
C18:2 added	3.58 ± 0.47	10.66 ± 0.62	28.55 ± 0.62
C18:3	−1.62 ± 0.40	8.05 ± 0.49	27.84 ± 0.08

[a] Average of three measurements. [b] Reaction mixture compositions and reaction conditions are described under Materials and Methods. [c] Not present.

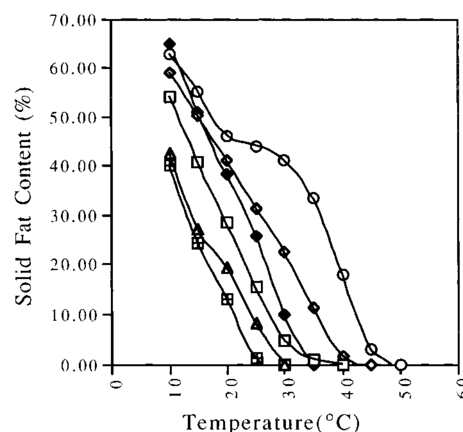

Figure 4. Solid fat contents of untreated butteroil and butteroil interesterified with added fatty acids by immobilized lipase as determined from their melting curves by DSC at temperatures ranging from 10 to 50 °C: (□) C14:0; (◇) C16:0; (○) C18:0; (△) C18:2; (box with cross) C18:3; (◆) untreated.

temperature in the range of 17−29 °C (Fouad *et al.*, 1990). The liquid fractions produced at temperatures ranging from 21 to 29 °C have two transition temperatures (DSC) corresponding to the medium- and high-melting ranges. Consequently, the melting properties of the softer butteroil produced by immobilized lipase-catalyzed interesterification are similar to those of the butteroil liquid fraction produced by thermal fractionation.

Interesterification can be carried out in liquid butteroil without dissolution in an organic solvent (Kalo *et al.*, 1990; Elliott and Parkin, 1991; Bornaz *et al.*, 1994). Acyl exchanges of butteroil triacylglycerols catalyzed by immobilized lipase in the absence of hexane were similar to those dissolved in hexane; however, the reaction rate was much slower. The slower change in percentage composition and corresponding change in functional properties was partly due to the presence of a larger quantity of butteroil (10 g) in the reaction without hexane as compared with that for the hexane solution (0.5 g); thus, the amount yielding a 10% change in the butteroil composition in hexane would yield only a 0.5% change in the composition for the reaction in butteroil. Nevertheless, the liquid butteroil was quite viscous at 42 °C. Organic solvents are frequently used because they enhance the reaction rate both by lowering the viscosity of the medium and by solubilizing nonpolar substrates (Dordick, 1989). Elliott and Parkin (1991) observed a decline in the initial rate of esterification of hendecanoic acid (C11:0) to butteroil in 100% butteroil as compared with that in organic solvent, which they attributed to an increase in viscosity. These authors also found that the optimum temperature for acyl

Table 2. Solid Fat Contents[a] of Untreated Butteroil and Butteroil Modified by Immobilized Lipase-Catalyzed Interesterification As Determined by DSC

butteroil	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C	45 °C
untreated	65 ± 2.1	50.9 ± 2.4	38.3 ± 2.6	25.7 ± 1.6	9.9 ± 1.0	0		
treated in hexane	61.2 ± 2.4	28.1 ± 2.9	24.7 ± 0.8	8.7 ± 1.1	0			
no solvent	56.5 ± 3.2	38.3 ± 2.7	32.8 ± 3.4	23.5 ± 3.1	10.8 ± 2.5	1.5 ± 0.7	0	
C14:0 added	54.2 ± 5.1	40.7 ± 4.4	28.5 ± 4.3	15.5 ± 2.7	4.7 ± 1.8	0.9 ± 0.7	0	
C16:0 added	59.0 ± 4.4	50.3 ± 3.9	41.4 ± 3.8	31.5 ± 4.2	22.5 ± 3.8	11.3 ± 6.0	1.5 ± 0.7	0
C18:0 added	62.8 ± 3.1	55.2 ± 2.0	46.0 ± 4.2	44.1 ± 3.7	41.0 ± 1.0	33.3 ± 1.0	17.9 ± 3.6	2.8 ± 1.8
C18:2 added	42.4 ± 3.1	27.1 ± 2.3	19.4 ± 2.5	8.2 ± 1.8	0			
C18:3 added	40.0 ± 1.5	24.2 ± 0.9	13.2 ± 1.6	1.1 ± 0.3	0			

[a] Average of three measurements.

exchange between hendecanoic acid and butteroil was 70 °C, at which the viscosity is considerably lower.

Adding unsaturated fatty acids to the interesterification reaction mixture, such as linoleic or linolenic acid, decreased melting temperatures of the modified butteroils in all three melting ranges. On the other hand, adding saturated fatty acids to the reaction mixture significantly increased either the medium or high, or both, melting temperatures of the modified butteroils, depending on the fatty acids added. For example, myristic acid, which is a medium-chain-length acid, increased the medium-range melting point by nearly 2 °C, whereas palmitic acid, which is two carbons longer, increased both the medium- and high-range melting points by approximately 8 and 9 °C, respectively. However, stearic acid, which is a long-chain fatty acid, affected only the high-range melting point, increasing it by about 9 °C. Lowering of the melting points in the low-melting range of the modified butteroil, even when saturated fatty acids were in the reaction mixture, has been attributed to hydrolysis of the butteroil (Kalo *et al.*, 1986; Bornaz *et al.*, 1994). Previously, we have shown that hydrolysis occurred during immobilized streptavidin−lipase-catalyzed interesterification of the model substrate mixture of tricarylin and oleic acid (Lee and Swaisgood, 1997b).

The solid fat content of butteroil at 20 °C (38%) decreased to 25% after interesterification catalyzed by immobilized streptavidin−lipase in hexane. However, Kalo *et al.* (1986) observed a 2% increase in the solid fat content of butteroil at 20 °C after interesterification for 10 days with *C. cylindracea* lipase, and Kalo *et al.* (1990) found a 39% increase after interesterification of butteroil in isooctane with immobilized *P. fluorescens* lipase. These differences are probably caused by a greater amount of hydrolysis in this study that resulted from higher water content of the immobilized enzyme. However, addition of palmitic or stearic acid to the reaction mixture increased the solid fat content of the modified butteroil, as would be expected when these medium- and long-chain saturated fatty acids are exchanged into the triacylglycerols. The amount of incorporation should depend upon the concentration of the added fatty acids, as was shown for immobilized lipase-catalyzed interesterification of tricaprylin with oleic acid (Lee and Swaisgood, 1997a,b) and the lipase-mediated acyl exchange between C11:0 and butteroil (Elliott and Parkin, 1991). Also, the solid fat content of modified butteroil was increased by adding high-melting triacylglycerols, such as trimyristin and tripalmitin, to a lipase-mediated interesterification reaction mixture (Bornaz *et al.*, 1994).

Addition of unsaturated fatty acids to the interesterification reaction mixture resulted in a modified butteroil that contained less solid fat at various temperatures than the unmodified fat or that modified in hexane without any added fatty acid. As would be expected when unsaturated fatty acids were exchanged into the triacylglycerols, as the number of double bonds in the fatty acid increased, the solid fat content decreased. Presumably both hydrolysis and interesterification of the triacylglycerols contributed to the lowering of the solid fat content of butteroils treated with immobilized lipase in the presence of unsaturated fatty acids.

These studies are the first to demonstrate that a recombinant bifunctional streptavidin−lipase, immobilized via bioselective adsorption directly from the crude cell lysate, could be used in a bioreactor for interesterification of butteroil either as the liquid butteroil or dissolved in hexane. Furthermore, the functional properties such as the softness or melting points can be designed by choice of interesterification reaction conditions including addition of appropriate fatty acids.

LITERATURE CITED

Ahern, T. J.; Klibanov, A. M. The mechanism of irreversible enzyme inactivation at 100 °C. *Science* **1985**, *228*, 1280−1284.

Bornaz, S.; Fanni, J.; Parmentier, M. Limit of the solid fat content modification of butter. *J. Am. Oil Chem. Soc.* **1994**, *71*, 1373−1380.

Chen, J.-P.; Pai, H. Hydrolysis of milk fat with lipase in reversed micelles. *J. Food Sci.* **1991**, *56*, 234−237.

Christensen, T. C.; Holmer, G. Lipase catalyzed acyl-exchange reactions of butter oil. Synthesis of a human milk fat substitute for infant formulas. *Milchwissenschaft* **1993**, *48*, 543−547.

Chulalaksananukul, W.; Condoret, J. S.; Delorme, P.; Willemot, R. M. Kinetic study of esterification by immobilized lipase in *n*-hexane. *FEBS Lett.* **1990**, *276*, 181−184.

Dordick, J. S. Enzymatic catalysis in monophasic organic solvents. *Enzyme Microb. Technol.* **1989**, *11*, 194−211.

El-Hamdy, A. H.; Perkins, E. G. High performance reversed phase chromatography of natural triglyceride mixtures: critical pair separation. *J. Am. Oil Chem. Soc.* **1981**, *58*, 867−872.

Elliott, J. M.; Parkin, K. L. Lipase-mediated acyl-exchange reactions with butteroil in anhydrous media. *J. Am. Oil Chem. Soc.* **1991**, *68*, 171−175.

Fouad, F. M.; van de Voort, F. R.; Marshall, W. D.; Farrell, P. G. A critical evaluation of thermal fractionation of butter oil. *J. Am. Oil Chem. Soc.* **1990**, *67*, 981−988.

Frede, E.; Thiele, H. Analysis of milkfat by HPLC. *J. Am. Oil Chem. Soc.* **1987**, *64*, 521−528.

Ha, J. K.; Lindsay, R. C. Release of volatile branched-chain and other fatty acids from ruminant milk fats by various lipases. *J. Dairy Sci.* **1993**, *76*, 677−690.

Haas, M. J.; Cichowicz, D. J.; Phillips, J.; Moreau, R. The hydrolysis of phosphatidylcholine by an immobilized lipase: optimization of hydrolysis in organic solvents. *J. Am. Oil Chem. Soc.* **1993**, *70*, 111−117.

Haas, M. J.; Cichowicz, D. J.; Jun, W.; Scott, K. The enzymatic hydrolysis of triglyceride-phospholipid mixtures in an organic solvent. *J. Am. Oil Chem. Soc.* **1995**, *72*, 519−525.

Janolino, V. G.; Swaisgood, H. E. Analysis and optimization of methods using water-soluble carbodiimide for immobilization of biochemicals to porous glass. *Biotechnol. Bioeng.* **1982**, *24*, 1069−1080.

Jensen, R. G. Fatty acids in milk and dairy products. In *Fatty Acids in Food and Their Health Implication*; Chow, C. K., Ed.; Dekker: New York, 1992.

Kalo, P.; Parviainen, P.; Vaara, K.; Ali-Yrrko, S.; Antila, M. Changes in the triglyceride composition of butter fat induced by lipase and sodium methoxide catalysed inter-esterification reactions. *Milchwissenschaft* **1986**, *41*, 82−85.

Kalo, P.; Huotari, H.; Antila, M. *Pseudomonas fluorescens* lipase-catalysed interesterification of butter fat in the absence of a solvent. *Milchwissenschaft* **1990**, *45*, 281−285.

Kermasha, S.; Safari, M.; Goetghebeur, M. Interesterification of butter fat by lipase from *Rhizopus niveus* in cosurfactant-free microemulsion system. *Appl. Biochem. Biotechnol.* **1995**, *53*, 229−244.

Lee, P.; Swaisgood, H. E. Characterization of a chemically conjugated lipase bioreactor. *J. Agric Food Chem.* **1997a**, *45*, 3350−3356.

Lee, P.; Swaisgood, H. E. Cloning and expression of a streptavidin-lipase fusion gene in Escherichia coli and characterization of the immobilized fusion protein. *Enzyme Microb. Technol.* **1997b**, submitted for publication.

Lie, E.; Molin, G. Hydrolysis and esterification with immobilized lipase on hydrophobic and hydrophilic zeolites. *J. Chem Tech. Biotechnol.* **1991**, *50*, 549−553.

Lilly, M. D.; Dunnill, P. Use of immobilized biocatalysts for conversions of water-insoluble reactants: interesterification of fats. *Ann. N. Y. Acad. Sci.* **1987**, *501*, 113−118.

Linfield, W. M.; Barauskas, R. A.; Sivieri, L.; Serota, S.; Stevenson R. W., Jr. Enzymatic fat hydrolysis and synthesis. *J. Am. Oil Chem. Soc.* **1984**, *61*, 191−195.

Macrae, A. R. Lipase-catalyzed interesterification of oils and fats. *J. Am. Oil Chem. Soc.* **1983**, *60*, 291−294.

Miller, D. A.; Prausnitz, J. M.; Blanch, H. W. Kinetics of lipase-catalyzed interesterification of triglycerides in cyclohexane. *Enzyme Microb. Technol.* **1991**, *13*, 98−103.

Neidleman, S. L.; Geigert, J. Biotechnology and oleochemicals: changing patterns. *J. Am. Oil Chem. Soc.* **1984**, *61*, 290−297.

Reetz, M. T.; Zonta, A.; Simpelkamp, J. Efficient immobilization of lipases by entrapment in hydrophobic sol-gel materials. *Biotechnol. Bioeng.* **1996**, *49*, 527−534.

Russell, A. J.; Fersht, A. R. Rational modification of enzyme catalysis by engineering surface charge. *Nature* **1987**, *328*, 496−500.

Sonnet, P. E.; Pfeffer, P. E.; Wise, W. B. Evaluation of some approaches to liquified tallow: sterochemical consequences of interesterification. *J. Am. Oil Chem. Soc.* **1986**, *63*, 1560−1564.

Swaisgood, H. E. Characteristics of milk. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; Dekker: New York, 1996; pp 841−878.

Tan, Y.; Miller, K. J. Cloning, expression, and nucleotide sequence of a lipase gene from *Pseudomonas fluorescens* B52. *Appl. Environ. Microbiol.* **1992**, *58*, 1402−1407.

Triantafyllou, A. O.; Adlercreutz, P.; Mattiasson, B. Influence of the reaction medium on enzyme activity in bio-organic synthesis: behavior of lipase from *Candida rugosa* in the presence of polar additives. *Biotechnol. Appl. Biochem.* **1993**, *17*, 167−179.

Valivety, R. H.; Halling, P. J.; Peilow, A. D.; Macrae, A. R. Relationship between water activity and catalytic activity of lipases in organic media. *Eur. J. Biochem.* **1994**, *222*, 461−466.

Walsh, M. K.; Swaisgood, H. E. An *Escherichia coli* plasmid vector system for production of streptavidin fusion proteins: expression and bioselective adsorption of streptavidin-β-galactosidase. *Biotechnol. Bioeng.* **1994**, *44*, 1348−1354.

Zaks, A.; Klibanov, A. M. Enzymatic catalysis in organic media at 100 °C. *Science* **1984**, *224*, 1249−1251.

Zaks, A.; Klibanov, A. Enzyme-catalyzed processes in organic solvents. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192−3196.

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