Interesterification of Butterfat by Commercial Microbial Lipases in a Cosurfactant-Free Microemulsion System

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Lipase-catalyzed interesterification of butterfat was carried out in a cosurfactant-free microemulsion system containing mixtures of Span 60 and Tween 60 (ICI Specialty Chemicals Altemix Inc., Brantford, Ontario, Canada) as surfactants. Four commercial lipases were used—Lipozyme 10,000L (Novo Nordisk, Copenhagen, Denmark) and N, D and MPA (Amano Pharmaceutical Co. Ltd., Nagoya, Japan). Stereospecific analyses of fractionated selected high-molecular weight triacylglycerols were performed by enzymatic deacylation with commercial pancreatic lipase, random generation of *rac*-1,2-diacylglycerols by Grignard degradation, synthesis of *rac*-phosphatidylcholines and a stereospecific release of *sn*-1,2 diacylglycerols by phospholipase A_2. The results showed that the hydrolytic affinity of commercial lipases demonstrated an acyl-group specificity toward lower-molecular weight fatty acids C4–C14:0. Stereospecific analyses of fatty acids of interesterified selected triacylglycerols of butterfat catalyzed by lipase N demonstrated a 46% increase in the proportion of C18:1 *cis* Δ^9 at the *sn*-2 position, whereas those catalyzed by lipases MAP, D and Lipozyme 10,000L were enriched with C16:0 at the same position by 21, 35 and 41%, respectively.

KEY WORDS: Butterfat, cosurfactant-free microemulsion system, interesterification, lipase.

Because of their exceedingly high catalytic activity under mild conditions and exceptionally high substrate specificity, enzymes are widely used in biocatalysis in technological processes (1). Martinek *et al.* (2) suggested that further applications of enzymes could be impeded because nature has mostly designed them for functioning in aqueous solution. However, Walde and Luisi (3) indicated that the entrapment of an enzyme molecule in a reversed micelle system could protect it against denaturation (unfolding) in a way where the interface between the protein globule and the organic solvent could be stabilized by the surfactant molecules; these authors concluded that lipases are of particular interest because they act on water-insoluble substrates that could be dissolved at a relatively high concentration in the bulk organic phase.

Stead (4) indicated that the use of extracellular microbial lipases has promising potential applications due to the wide range of enzyme specificity. Bevinakatti and Newadkar (5) reported on the screening of lipases and their use in organic solvents for the biocatalysis of regio- and stereoselective esterification and transesterification reactions. Martinek *et al.* (2) reported that the use of a micelle-forming surfactant, solubilized in organic solvents, enhanced the stability and catalytic activity of certain lipases.

Kennedy (6) reported that use of microbial lipases of selected positional specificity resulted in the prodution of interesterified mixtures of triacylglycerols that could not be produced by simple chemical interesterification. Quinlan and Moore (7) reported that different lipases, however, can show preferences for both the nature of the acyl group and the positional distribution of fatty acids in the triacylglycerol molecules. Because butterfat contains appreciable quantities (27.8%) of oleic acid (8), which is randomly distributed at *sn*-1, *sn*-2 and *sn*-3 positions at 21, 14 and 15 mole%, respectively (9), the cholesterol-raising effect of butterfat could be suppressed by the interchange of oleic acid with palmitic acid at the *sn*-2 position by means of a specific lipase.

This work is a part of ongoing research (10–12) aimed at the optimization of lipase-catalyzed interesterification of selected fatty acids of butterfat. The objective of this study was to investigate the specificity of selected commercial lipases in terms of fatty acid positional distribution in interesterification reactions in a cosurfactant-free microemulsion system.

MATERIALS AND METHODS

Materials. Commercial lipases N, D and MAP, supplied kindly by Amano Pharmaceutical Co. Ltd. (Nagoya, Japan), were produced by a unique fermentation process from selected strains of *Rhizopus niveus, R. delemar* and *Mucor javanicus*, respectively. Lipozyme 10,000L, a commercial enzyme produced by submerged fermentation of a selected strain of *M. miehei*, was obtained from Novo Nordisk (Copenhagen, Denmark). Surfactants sorbitol monostearate (Span 60) and polyoxyethylene sorbitan monostearate (Tween 60) were generously provided by ICI Specialty Chemicals Altemix Inc. (Brantford, Ontario, Canada). Sodium xylene sulfonate was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Butterfat used throughout this study was obtained from the local market. Fatty acids and mono-, di- and triacylglycerol standards were purchased from Nu-Chek-Prep (Elysian, MN). Acetone, acetonitrile, chloroform, diethyl ether and hexane (Omnisolv grade) were supplied by BDH Inc. (Toronto, Ontario, Canada). Pancreatic lipase, used for the deacylation of triacylglycerol molecules, was obtained from Solvay Enzyme Inc. (Elkhart, IN). Thin-layer chromatography (TLC) plates of silica gel GF 254 were purchased from Merck (Darmstadt, Germany).

Preparation of microemulsion system. The microemulsion system was prepared according to a modification of the method described by Fletcher *et al.* (13). The lipase suspension (400 mg of enzyme preparation containing 80 mg protein) was prepared in 1 mL phosphate buffer solution (0.1 M, pH 7.0). Surfactant solutions of 0.01 M Span 60 (43%) and 0.33 M Tween 60 (42%) were prepared in hexane. Diluted surfactant mixture (3 mM) of 48% Span 60 and 52% Tween 60 was introduced into 25-mL screw-cap tubes, followed by addition of 100 μL (8 mg protein) of enzyme suspension. The addition of aqueous enzyme suspension to the surfactant solution spontaneously produced the microemulsion system.

Lipase-catalyzed interesterification reaction in microemulsion system. Five grams of butterfat was melted at 37°C and mixed with 40 mg sodium xylene sulfonate, used as a hydrotrope; the mixture was dissolved in 10 mL of the prepared microemulsion system. The interesterification

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reaction was carried out in a 50-mL vacuum-sealed flask in a reciprocal shaker water-bath (Model 25; Precision Scientific Inc., Chicago, IL) for 48 h of continuous shaking (37°C, 145 rpm). Two mL of the reaction mixture was withdrawn, and 4 mL of chloroform was added to terminate the interesterification reaction. The reaction mixture was then filtered through a 0.45-μm membrane filter (Costar nucleopor, Toronto, Ontario, Canada) to remove the lipase residue before high-performance liquid chromatography (HPLC) analysis (14).

Determination of free fatty acid composition. Qualitative and quantitative analyses of free fatty acids (FFA) were performed by gas–liquid chromatography (GLC). The preparation of free and bound fatty acid methyl esters were carried out according to the procedure described by Badings and De Jong (15). The GLC analyses of free and bound fatty acids of interesterified butterfat were carried out according to a previously described procedure (11).

Separation of triacylglycerols. The triacylglycerols of interesterified butterfat (100 mg) were dissolved in 0.5 mL hexane and separated by their application on a Supelclean LC-SI column (Supelco, Inc., Bellefonte, PA), pre-equilibrated with 5 mL hexane. The triacylglycerols were eluted with 2 × 2.5 mL of elution solvent, a mixture of hexane/diethyl ether (93:7, vol/vol).

HPLC fractionation of purified butterfat triacylglycerols. HPLC analyses of triacylglycerols were performed according to the procedure described by Kermasha *et al.* (10). The separation of selected high-molecular weight triacylglycerols, fractions number 8 to 16 (Fig. 1), was carried out with a gradient elution (Table 1) and demonstrated with a laser light-scattering detector (Varex Corporation, Burtonsville, MD).

Stereospecific analysis of triacylglycerol molecules. Stereospecific analysis of fractionated butterfat fractions 8 to 16 (Fig. 1) was performed for the determination of fatty acids on *sn*-1, *sn*-2 and *sn*-3 positions.

Generation of sn-*2 monoacylglycerol.* The generation of *sn*-2 monoacylglycerols was performed according to the

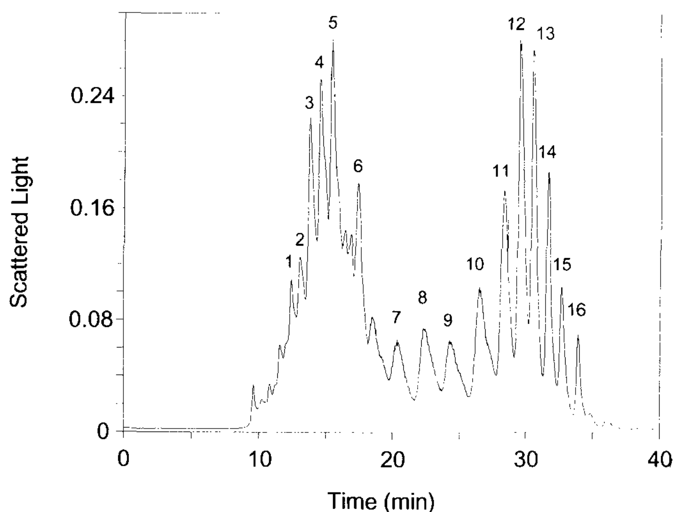

FIG. 1. Chromatogram of high-performance liquid chromatography triacylglycerol separation and fractionation of butterfat on a Spherisorb-ODS-2 reverse-phase column of 250 × 22 mm i.d. and pore size 5 μ (Alltech Associates, Inc., Deerfield, IL). The elution profile is described in Table 1.

TABLE 1

Conditions of High-Performance Liquid Chromatography (HPLC) Elution Profile for the Fractionation of Butterfat

		Elution gradient system[a]	
Time (min)	Flow rate (mL/min)	Chloroform (%)	Acetonitrile (%)
Initial[b]	7.0	40.0	60.0
0–11	7.0	50.0	50.0
12–17	5.0	50.0	50.0
18–26	3.0	50.0	50.0
27–39	7.0	50.0	50.0
40.0	0.0	40.0	60.0

[a]A gradient elution was performed on the preparative HPLC column; Spherisorb-ODS-2 reverse-phase 250 × 22 mm i.d., pore size 5μ (Alltech Associates, Inc., Deerfield, IL).
[b]The elution profile used to conditionate the column.

procedure described by Skipsi *et al.* (16). The purified selected triacylglycerols of interesterified butterfat samples were incubated (2 h, 40°C) in a buffer solution containing commercial procine pancreatic lipase. The 2-monoacylglycerols were isolated on TLC plates, coated with silica gel 60 F 254, with a mixture of chloroform/acetone (88:12, vol/vol) as developing solvent (17).

Generation of rac-*1,2-diacylglycerols.* The *rac*-1,2-diacylglycerols were produced according to the method described by Skipsi *et al.* (16). The selected triacylglycerols of butterfat were subjected to Grignard reaction; the *rac*-1,2-diacylglycerols and *rac*-1,3-diacylglycerols were resolved and recovered by preparative TLC of silica gel G of 0.5-mm thickness with a mixture of chloroform/acetone (97:3, vol/vol) as the developing solvent (18).

Preparation of phosphatidylcholines. The *rac*-phosphatidylcholines were synthesized by treating purified *rac*-1,2-diacylglycerols with phosphorus oxychloride and choline chloride, as described earlier by Kermasha *et al.* (10).

Stereospecific hydrolysis with phospholipase A_2. The hydrolysis of *rac*-phosphatidylcholine was performed according to the procedure of Robertson and Lands (19). The purified *rac*-phosphatidylcholines were incubated with phospholipase A_2, and the lysophosphatidylcholines were detected on a silica gel G TLC plate, which was developed first in a mixture of hexane/diethyl ether/formic acid (60:40:2, vol/vol/vol) and then redeveloped in a mixture of chloroform/methanol/acetic acid/water (25:15:4:2, by vol).

RESULTS AND DISCUSSION

Effect of lipase-catalyzed interesterification on hydrolysis rate. Quantitative GLC analyses of FFA were performed to determine the level of hydrolysis in lipase-catalyzed interesterification reaction mixtures. The results demonstrate that the interesterification of butterfat by lipase D resulted in the least hydrolysis, 0.45 mmol/g of butterfat, whereas the maximum hydrolytic activity occurred with Lipozyme 10,000L (5.32 mmol/g of butterfat). In addition, the interesterification of butterfat by lipases N and MAP resulted in an increase in the amount of FFA from 0.4 to 1.5 and 1.4 mmol/g, respectively. These findings suggest that the amount of FFA produced in a lipase-catalyzed interesterification reaction depends on the type of enzyme used.

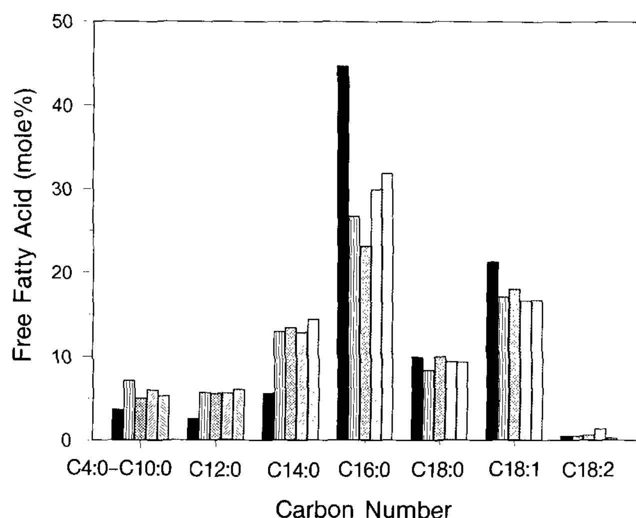

FIG. 2. Changes in free fatty acid composition of interesterified butterfat by commercial lipases in a cosurfactant-free microemulsion system: Untreated butter (■), lipase N (Amano, Nagoya, Japan) ('), lipase MAP (Amano) ('), lipase D (Amano) (■) and Lipozyme 10,000L (Novo, Copenhagen, Denmark) (').

Changes in FFA content of interesterified butterfat. The results (Fig. 2) show that the use of lipase N for the interesterification of butterfat resulted in an increase in the total proportions of free small-chain fatty acids (C4–C10), C12:0 and C14:0 by 51, 50 and 116%, respectively, with a concomitant decrease in the proportions of C16:0 and C18:1 $cis\Delta^9$ by 42 and 23%, respectively.

The results (Fig. 2) also demonstrate that the interesterification of butterfat by lipase MAP resulted in an increase in the proportions of free fatty acids C4–C10, C12:0 and C14:0 by 30, 50 and 60%, respectively, with a concomitant decrease in the proportions of C16:0 and C18:1 $cis\Delta^9$ by 48 and 19%, respectively. In addition, the results (Fig. 2) indicate that the use of lipase D and Lipozyme 10,000L for the interesterification of butterfat resulted in an increase in the amounts of free fatty acids C:4–C10:0, C12:0 and C14:0 by 50, 52 and 126%, respectively, with a concomitant decrease in the proportions of C16:0 and C18:1 $cis\Delta^9$, respectively, by 31 and 23%.

The results (Fig. 2) suggest that the hydrolytic affinity of commercial lipases in the microemulsion system, used throughout this study, demonstrate an acyl-group specificity toward lower-molecular weight fatty acids C4–C14:0.

Changes in positional distributions of selected fatty acids of interesterified butterfat. The results (Table 2) obtained from stereospecific analyses of selected triacylglycerols of butterfat, interesterified by lipase MAP, show that there was a 39% decrease in the total proportions of saturated small-chain fatty acids (C6–C10), with a concomitant increase of 21% in the amount of C16:0, on the *sn*-2 position of triacylglycerols. However, there were no considerable changes in the proportions of C12:0, C14:0, C18:0 and C18:1 $cis\Delta^9$ at the same position. The results (Table 2) demonstrate that the total proportions of fatty acids (C6–C10), C12:0 and C16:0 at the *sn*-1 position of triacylglycerols increased by 92, 38 and 28%, respectively, with a concomitant decrease of 52% in the amount of C18:1 $cis\Delta^9$ at the same position. The results (Table 2) also indicate that the proportions of relatively small-chain fatty acids (C4–C10:0), as well as C16:0, decreased by 17 and 62%, respectively, at the *sn*-3 position, with a concomitant increase in the proportions of C12:0, C14:0, C18:0 and C18:1 $cis\Delta^9$ by 52, 37, 37 and 44%, respectively. These findings suggest that an acyl exchange reaction took place at the three possible positions, mainly at *sn*-1 and *sn*-3 positions of triacylglycerols, wherein C18:1 $cis\Delta^9$, originally located at *sn*-1 position, exchanged with C16:0, originally located at *sn*-3 position.

The stereospecific analyses of interesterified butterfat catalyzed by lipase D show (Table 2) that the proportions of relatively small-chain fatty acids (C4–C10:0), as well as C18:0 and C18:1 $cis\Delta^9$, at the *sn*-2 position of triacyl-

TABLE 2

Stereospecific Analyses of Lipase-Catalyzed Interesterified Butterfat Triacylglycerols with Commercial Lipases in Cosurfactant-Free Microemulsion System

Carbon number[f]	Untreated butter[a]			Lipase N[b]			Lipase MAP[c]			Lipase D[d]			Lipozyme[e]		
	sn-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-3	*sn*-3	*sn*-1	*sn*-2	*sn*-3
C4:0	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]
C6:0	0.8	1.9	1.5	3.5	—[g]	—[g]	2.1	—[g]	—[g]	0.3	—[g]	—[g]	0.4	—[g]	—[g]
C8:0	0.5	1.5	1.2	1.8	0.2	—[g]	1.7	—[g]	—[g]	0.6	0.2	5.9	0.6	—[g]	—[g]
C10:0	2.9	3.4	9.2	4.7	0.7	21.0	4.2	4.3	10.0	1.3	5.5	9.0	2.5	1.2	18.0
C12:0	4.7	3.7	11.9	4.1	5.1	10.7	6.5	3.5	18.3	2.0	5.7	11.4	3.7	3.1	13.6
C14:0	15.9	12.9	10.6	15.6	10.7	20.7	16.0	13.1	14.8	4.2	18.3	11.7	12.5	18.1	9.3
C16:0	28.0	33.1	34.4	33.8	26.5	21.4	35.8	40.2	13.0	34.4	44.8	12.0	35.3	46.9	10.0
C18:0	17.4	10.7	15.8	17.7	12.9	13.2	16.5	10.0	22.3	26.8	8.0	18.0	16.1	8.5	20.4
C18:1 $cis\ \Delta^9$	27.7	28.7	9.0	16.1	42.0	8.0	13.4	27.0	13.2	22.3	16.5	23.0	26.5	20.0	15.9
C18:2 $cis\ \Delta^{9,12}$	2.2	3.4	5.5	0.6	1.8	4.1	3.0	1.0	4.4	2.1	0.1	4.9	2.0	1.9	9.4
C20:0	0.3	0.8	1.0	2.1	0.5	0.9	0.9	0.8	3.8	6.0	0.9	3.9	0.5	0.2	3.4

[a]Untreated butterfat fractionated by preparative high-performance liquid chromatography (HPLC) column, fraction numbers 8 to 16 of HPLC profile (see Fig. 1).
[b]Interesterified butterfat with lipase lipase N (Amano, Nagoya, Japan) from *Rhizopus niveus*.
[c]Interesterified butterfat with lipase MAP (Amono) from *Mucor javanicus*.
[d]Interesterified butterfat with lipase D (Amano) from *R. delemar*.
[e]Interesterified butterfat with lipozyme 10,000L (Novo, Copenhagen, Denmark) from *M. miehei*.
[f]Carbon number of fatty acid methyl esters obtained from stereospecific analysis of interesterified butterfat.
[g]Not detected.

glycerols, decreased by 17, 30 and 42%, respectively, whereas the amounts of C12:0, C14:0 and C16:0 increased by 54, 41 and 35%, respectively, at the same position. The results (Table 2) demonstrate that there was a reduction in the total proportions of small-chain fatty acids (C4–C10:0), as well as in that of C12:0, C14:0 and C18:1 *cis*Δ^9, located at *sn*-1 position, by 50, 57, 72 and 19%, respectively, with a concomitant increase in the amounts of C16:0 and C18:0 by 23 and 54%, respectively, at the same position. It was also indicated (Table 2) that there was a drastic decrease (62%) in the proportion of C16:0, with a concomitant increase in the amount of C18:1 *cis*Δ^9 (1.5-fold) at the *sn*-3 position.

The comparison between the positional distribution of fatty acids on selected triacylglycerol molecules of untreated butterfat (Table 2) and that of interesterified butterfat suggests that an acyl exchange reaction mainly occurred between C12:0 and C14:0, originally located at *sn*-1 position, with C18:0 and C18:1 *cis*Δ^9, originally located at the *sn*-2 position.

The results obtained from positional distribution of fatty acids in lipozyme 10,000L-interesterified butterfat triacylglycerol molecules show (Table 2) that the proportions of relatively small-chain fatty acids (C4–C10:0), C18:0 and C18:1 *cis*Δ^9, at the *sn*-2 position of triacylglycerol molecules, decreased by 82, 20 and 30%, respectively, with a concomitant increase in the amounts of C14:0 and C16:0 by 40 and 41%, respectively, at the same position. The results (Table 2) also demonstrate that the proportions of C12:0 and C14:0, at *sn*-1 position, decreased by 21 and 19%, respectively, with a concomitant 26% increase in the amount of C16:0 at the same position. However, the results show that there was a limited change in the amounts of relatively small-chain fatty acids (C4–C10:0), C18:0 and C18:1 *cis*Δ^9, located at the *sn*-1 position. The results (Table 2) also indicate that the proportions of relatively small-chain fatty acids (C4–C10:0), C12:0, C18:0 and C18:1 *cis*Δ^9, at the *sn*-3 position of triacylglycerols, increased by 50, 14, 29 and 77%, respectively, with a concomitant 71% decrease in the amount of C16:0 at the same position.

These findings suggest that there was an acyl exchange reaction between C16:0, previously located at the *sn*-3 position, and C18:1 *cis*Δ^9, previously located at the *sn*-2 position. The overall result indicates an increase in the amount of C16:0 at the *sn*-2 position of interesterified butterfat triacylglycerol molecules, with a concomitant decrease in the proportions of relatively small-chain fatty acids (C4–C10:0).

The stereospecific analyses of butterfat, interesterified by lipase N, show (Table 2, Fig. 3) that the proportions of relatively small-chain fatty acids (C4–C10), C14:0 and C16:0, located at the *sn*-2 position of triacylglycerol molecules, decreased by 87, 17 and 20%, respectively, with a concomitant increase in the amounts of C18:0 and C18:1 *cis*Δ^9 by 20 and 46%, respectively. The results (Table 2) also show that the interesterification resulted in an increase in the proportions of relatively small-chain fatty acids (C4–C10) and C16:0, located at *sn*-1 position, by 130 and 21%, respectively, with a concomitant 42% decrease in the proportion of C18:1 *cis*Δ^9; however, there were no considerable changes in the amounts C12:0, C14:0 and C18:0 on the same position. The results (Table 2) go on to demonstrate that there was a decrease in the proportion of relatively small-chain fatty acids (C4–C10), C16:0, C18:0 and C18:1 *cis*Δ^9 on the *sn*-3 position by 25, 37, 16 and 11%, respectively, with a concomitant drastic increase (100%) in the amount of C14:0.

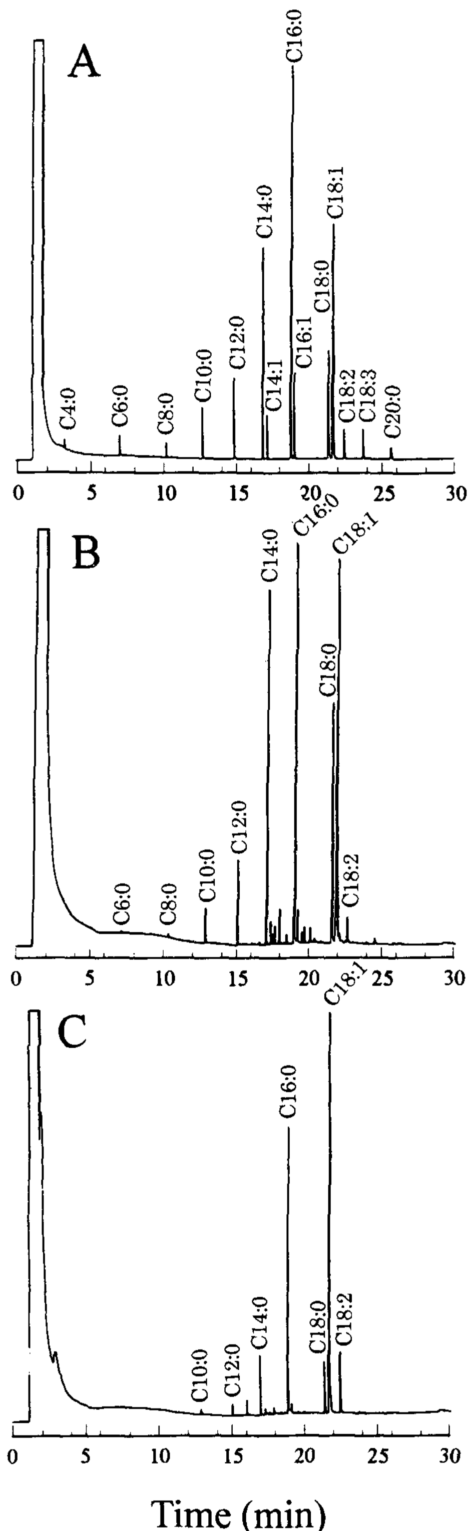

FIG. 3. Chromatogram of gas–liquid chromatography fatty acid profile of *sn*-2 fatty acid composition of (A) fatty acid standard, (B) untreated butterfat and (C) interesterified butterfat with lipase N (Amano, Nagoya, Japan) in a cosurfactant-free microemulsion system.

TABLE 3

Changes in Positional Distributions of Selected Fatty Acids in Triacylglycerols of Interesterified Butterfat by Lipase N (Amano) in Cosurfactant-Free Microemulsion System

	Relative fatty acid (%)[a]					
	Untreated butter			Treated butter		
Carbon number[b]	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3
C12:0–C16:0[c]	16.2	16.6	19.0	11[d]	15[e]	7[e]
C18:0–C18:1 *cis* Δ^9[f]	22.5	19.7	12.4	25[e]	39[d]	14[e]

[a]Fatty acid composition (mole %) of untreated and interesterified butterfat.
[b]Carbon number of fatty acid methyl esters obtained from stereospecific analysis of interesterified butterfat.
[c]Changes in percentage of hypercholesterolemic fatty acids C12:0, C14:0 and C16:0 on *sn*-2 and *sn*-1,3 positions of lipase-catalyzed interesterified butterfat triacylglycerols.
[d]The relative percentage increase in the proportion of selected fatty acids.
[e]The relative percentage decrease in the proportion of selected fatty acids.
[f]Changes in percentage of hypocholesterolemic fatty acids C18:0 and C18:1 on *sn*-2 and *sn*-1,3 positions of lipase-catalyzed interesterified butterfat triacylglycerols.

These results suggest that there was an acyl exchange reaction among all three possible positions of triacylglycerol molecules, wherein C18:1 *cis*Δ^9, previously located mainly on *sn*-1 position, exchanged with C16:0, previously located on *sn*-2 position. The net results indicated that the interesterified butterfat, catalyzed by lipase N, contained mainly C18:1 *cis*Δ^9 fatty acid on the *sn*-2 position.

The results (Table 3) also show that the total amounts of hypercholesterolemic fatty acids (C12:0, C14:0 and C16:0), at the *sn*-2 position, decreased by 15%, with a concomitant increase of these fatty acids by 11% at the *sn*-1 position. However, they demonstrate that there was an important increase (39%) in the amount of hypocholesterolemic fatty acids (C18:0 and C18:1 *cis*Δ^9) at the *sn*-2 position, with a concomitant decrease in these fatty acids at the *sn*-1 and *sn*-3 positions by 25 and 14%, respectively.

The interesterification of butterfat by selected commercial lipases in a cosurfactant-free macroemulsion system indicated that these enzymes exhibited an acyl-group specificity toward lower-molecular weight fatty acids. The stereospecific analyses of selected interesterified triacylglycerols catalyzed by lipase N demonstrated that there was an important increase in the proportion of C18:1 *cis*Δ^9 at the *sn*-2 position at the expense of the *sn*-1 position, whereas the interesterified butterfats catalyzed by lipases MAP, D and Lipozyme 10,000L contained C16:0 at the same position. These findings suggest that, although the four commerical lipases used throughout this study have 1,3-specificity, these enzymes could exhibit, under certain conditions, different positional specificity.

ACKNOWLEDGMENT

This research was supported by a grant-in-aid award from Dairy Bureau of Canada.

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[Received January 18, 1994; accepted May 31, 1994]