Modification of Selected Indian Vegetable Fats into Cocoa Butter Substitutes by Lipase-Catalyzed Ester Interchange[1]

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A few solid and semi-solid fats of tree origin in India, namely sal (*Shorea robusta*), kokum (*Garcinia indica*), mahua (*Madhuca latifolia*), dhupa (*Vateria indica*) and mango (*Mangifera indica*), were chosen for modification into cocoa butter substitutes by lipase-catalyzed ester interchange with methyl palmitate and/or stearate. Hexane solutions of mixtures of fat and methyl ester(s) in various molar proportions were passed through a column of Lipozyme™, a lipase from *Mucor miehei* immobilized on a macroparticulate ion-exchange resin. The interesterified fats were purified by extraction with 95% ethanol followed by silica column chromatography. Interesterified dhupa, kokum and sal fats compared well with cocoa butter in the total fatty acid composition and the 2-position of triacylglycerols, as well as glyceride composition. In particular, interesterified kokum fat resembled cocoa butter well in solid fat content and peak melting temperature as determined by differential scanning calorimetry.

KEY WORDS: Cocoa butter substitutes, ester interchange, fatty acid composition, 2-monoacylglycerols, packed-bed interesterification, solid fat content, 1,3-specific *Mucor miehei* lipase, tree-borne vegetable fats, triacylglycerols.

The world production of cocoa butter was about 800,000 metric tons in 1987. A number of reformulated vegetable oils are currently produced as cocoa butter substitutes (CBS). The potential market for the CBS could be up to 10% of the total cocoa butter market (1).

Attempts have been made to prepare cocoa butter-like fats by interesterification of hydrogenated cottonseed oil and olive oil and subsequent fractionation (2). Edible beef tallow also has been fractionated by acetone crystallization to yield cocoa butter-like fractions (3). The preparation of CBS by means of lipase-catalyzed interesterification has attracted much attention in recent years (4–7) due to availability of 1,3-specific microbial lipases that catalyze regioselective interesterification at the 1- and 3-positions of triacylglycerols (TAG). The CBS thus produced have been granted GRAS (generally recognized as safe) status (8).

Good potential exists for some solid or semi-solid fats of tree origin in India for modification into CBS because of their physical and chemical characteristics, as well as their fatty acid and glyceride compositions (9). Prominent among these are sal (*Shorea robusta*), kokum (*Garcinia indica*), mahua (*Madhuca latifolia* or *M. indica*), dhupa (*Vateria indica*) and mango (*Mangifera indica*) fats, which have a combined annual production potential of about one million metric tons (9). Attempts have been made to modify these fats, except dhupa, to CBS by solvent fractional crystallization (10–12), chemical interesterification (13) and/or hydrogenation (14), but they have met with only limited success. Catalytic hydrogenation produces isomeric fatty acids due to geometrical (*trans*) and positional isomerization of double bonds, whereas chemical interesterification produces randomized TAG, which affects the quality of CBS (12). Solvent fractionation is a tedious process that involves a series of crystallizations of fats to obtain a simple mixture of TAG of desired structures suitable for use as CBS. Hence, lipase-catalyzed interesterification by an ester interchange reaction was investigated to obtain CBS from sal, kokum, mahua, dhupa and mango fats.

MATERIALS AND METHODS

Mahua and kokum fats were purchased from reliable commercial sources. Dhupa fat was extracted from dhupa kernels received from Surya Kanti Soap Centre (Shimoga, Karnataka, India). Refined sal fat was donated by Hindustan Lever Ltd. (Bombay, India). Mango fat was extracted from dry mango kernels of the "Neelam" variety purchased from a local market. Cocoa butter was donated by Cadbury India Ltd. (Thane, Maharashtra).

To determine optimum mole ratios of fat to methyl ester, interesterification was done initially by a batch-stirred reaction on 100- to 200-mg fat samples. The mixtures of fat, fatty acid methyl esters (FAME) and 6% by wt. of Lipozyme™ [a lipase from *Mucor miehei* immobilized on macroparticulate ion exchange resin donated by Novo Industry (Copenhagen, Denmark)] were magnetically stirred in hexane (2–3 mL) at 60°C for 4 hr. The enzyme was filtered off and the TAG were isolated by preparative thin-layer chromatography (TLC) on silica gel G (Acme Synthetic Chemicals, Bombay, India) with hexane/diethyl ether (80:20, v/v). The optimum mole ratios of fat to FAME obtained by the batch-stirred reaction (mentioned in Table 1) were employed in a packed-bed continuous reaction on 2- to 5-g fat samples. Lipozyme (10 g) without any treatment, was packed to a height of 27 cm in a water-jacketed column (1.2 cm i.d. × 65 cm) and maintained at 60°C. The mixture of FAME and fat was dissolved in hexane (1 g/10 mL) and allowed to flow through the column. The contact time was 1 hr. The eluate was freed of solvent.

A typical procedure for purification of the product is as follows. The interesterified product (7.5 g) was stirred with 95% ethanol (1:6, w/w) at room temperature for 10 min and kept at 4°C for 90 min, followed by immediate filtration. This procedure was repeated with the insoluble fraction. The final insoluble product was subjected to column (2.5 cm i.d. × 65 cm) chromatography on silicic acid (160 g, 60–120 mesh, Acme Synthetic Chemicals) with a solvent mixture (800 mL) of hexane/diethyl ether (95:5, v/v) for eluting the pure TAG. The composition of the interesterified product at different stages of purification was determined by the TLC-FID method (Iatroscan-TH 10, Iatron Laboratories Inc., Tokyo, Japan) on Chromarod-S II. Chromarod-S II is a quartz rod (0.9 mm o.d. × 152 mm) of silicic acid-based frit of 5-μm particle size. The Chromarod was impregnated with 5% copper sulfate (15). The sample was applied to the Chromarod and developed

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with a solvent system of hexane/chloroform (84:11, v/v), the latter containing 5% isopropanol and 0.5% formic acid. The developed rods were scanned in a hydrogen flame from hydrogen and air at flow rates of 160 and 2000 mL/min, respectively. The recorder sensitivity was 100 mv and the scan speed was either 3.14 or 4.17 mm/sec corresponding to speed gear number 30 or 40, respectively. The pure TAG were analyzed for total fatty acid composition and for the 2-position of TAG, as well as for glyceride composition and solid fat content.

Fatty acid composition was determined by gas-liquid chromatography (GLC) in a Hewlett-Packard 5840 A unit fitted with a hydrogen flame ionization detector and a data processor (Hewlett-Packard Co., Palo Alto, CA). A glass column (4.8 mm i.d. × 1.8 m) packed with 10% Silar 10 C on Chromosorb W (60–80 mesh) was used. The column, detector and injection port were maintained at 190, 300 and 250°C, respectively. Nitrogen was used as carrier gas (35 mL/min). Peaks were identified with reference FAME and quantitated with methyl heptadecanoate as internal standard. FAME were obtained by methanolysis of acylglycerols with 1% methanolic solution of sodium methoxide (16).

Reversed-phase high-performance liquid chromatography (HPLC) of TAG was carried out in a Shimadzu LC-6A unit coupled with a Shimadzu SPD-6AV UV-VIS spectrophotometric detector and a Chromatopak C-R3A integrator (Shimadzu Corporation, Tokyo, Japan). A Macherey-Nagel column (Macherey-Nagel GmbH & Co. KG., Düren, Germany) packed with 5-μ Nucleosil 120-5 C18 and a solvent system of acetonitrile/tetrahydrofuran (73:27, v/v) were used. The total system pressure was 80 kg/cm^2 with a flow rate of 1 mL/min. The analysis was carried out isocratically and the UV spectra of TAG were determined at 220 nm (17). The TAG sample (25 mg/mL for standard mixture and 50 mg/mL for experimental samples) was dissolved in tetrahydrofuran. Reference TAG mixtures for HPLC analysis were prepared by interesterification of pure triolein (Sisco Research Laboratories Pvt. Ltd., Bombay, India) with palmitic and stearic acids (Godrej Soaps Pvt. Ltd., Bombay, India) by means of Lipozyme.

Pancreatic lipase hydrolysis of TAG was carried out according to Brockerhoff (18), and the resultant 2-monoacylglycerols (MAG) were isolated by TLC on silica gel G impregnated with boric acid (5% by wt.). Hexane/diethylether (1:1, v/v) was used as developer and 2′,7′-dichlorofluorescein as detector. The MAG were converted to FAME by methanolysis (16) and analyzed by GLC for fatty acid composition.

Differential scanning calorimetry (DSC) of TAG was carried out in a Mettler TA-3000 DSC with a PT-100 sensor (Mettler Instrument Corporation, Hightstown, NJ). The heat flow of the instrument was calibrated with indium. The PT-100 sensor was calibrated with indium, zinc and lead. To ensure homogeneity, the TAG sample was heated to 60°C to destroy all crystal nuclei and a sample of about 20 mg was accurately weighed into a standard aluminum pan and the cover was crimped in place. The pan containing the sample was chilled at 0°C for 1 hr prior to introducing it in the DSC cell. An empty aluminum pan with a pierced lid was used as a reference. The thermogram of the sample was recorded by heating at a constant rate of 2°C/min from 0°C to 45°C. The percent liquid fraction at various temperatures, peak melting temperature and the heating curves were recorded.

RESULTS AND DISCUSSION

Cocoa butter essentially contains oleic, stearic and palmitic acids in about equal proportions. The indigenous vegetable fats of tree origin, namely kokum, dhupa, sal, mango and mahua, contain major proportions of oleic (35–44%) and stearic (27–58%) acids (Table 1). Palmitic and linoleic acid contents in these fats (except in mahua) are low. Other fatty acids are not present in significant amounts in these fats, except arachidic acid in sal fat. Keeping this in view, these fats were chosen for modification into CBS by incorporation of palmitic acid through lipase-catalyzed interesterification. This can be done by interesterification of the fat with either palmitic-rich fat or with palmitic methyl/ethyl ester (ester interchange) or palmitic acid (acidolysis). Interesterification of a fat with another fat has the limitation of producing a wide range of TAG. The ester interchange method was observed in our preliminary investigations to be better than acidolysis for incorporation of palmitate. The ester interchange is also reported to be faster than acidolysis (19). Hence, the ester interchange method was employed in the present studies. Not much difference was observed between methyl and ethyl esters in the ester interchange reaction, except for a slightly better incorporation with methyl ester (unpublished observations).

The interesterified fat was freed of methyl esters and the partial glycerides by extraction with ethanol followed by chromatography. A typical purification of interesterified fats is as follows. An interesterified kokum fat sample containing 63.1% TAG, 10.5% diacylglycerols (DAG), 1.2% MAG and 25.2% FAME was stirred with 95% ethanol (1:6, w/w) at room temperature, cooled at 4°C for 90 min and filtered to remove FAME and MAG completely. The procedure was repeated on the precipitate. The loss of TAG in the combined ethanol filtrates amounted to 21.4% of the original TAG due to a solubility effect of FAME, DAG and MAG. The DAG content was reduced from 10.0 to 3.5% and the residual DAG were eliminated by silica gel column chromatography. The yield of CBS, *i.e.*, pure TAG, was 70% on the basis of original TAG used for ester interchange.

The data obtained for fatty acid composition of the interesterified fats, using the optimum ratios of fat to FAME, are shown in Table 1. It was observed that in addition to palmitate, incorporation of stearate was necessary, except in the case of kokum, to restore the depletion of stearate during ester interchange. Only then can products be obtained with fatty acid composition similar to cocoa butter. The stearate content in TAG of dhupa, sal and kokum after ester interchange was changed from 42–57% to 32–33% while incorporating palmitate to the desired level of cocoa butter (25%). The arachidate content in sal fat was reduced to 3.9% from 7.7% after ester interchange. In mahua TAG, linoleate was reduced only slightly, but oleate was decreased to an undesirably low level (29.7%). Hence, the interesterified mahua TAG were not further analyzed for suitability as CBS. In mango TAG, oleate content decreased to the desired level, but the linoleate content did not decrease

TABLE 1

Fatty Acid Composition of Original and Interesterified Triacylglycerols of Selected Vegetable Fats

Fat	Fat/methyl ester (mole ratio)		Fatty acid (mol %)					
	Fat/16:0	Fat/18:0	16:0	18:0	18:1	18:2	20:0	Other
Mahua	—	—	21.3	26.6	35.2	16.5	—	0.4
IE[a]	0.5	1.0	23.2	31.9	29.7	15.2	—	—
Mango	—	—	8.2	36.3	43.9	9.1	1.9	0.6
IE	1.7	1.6	23.8	32.0	34.9	8.0	0.9	0.4
Dhupa	—	—	10.6	42.0	43.6	1.3	1.7	0.8
IE	1.6	1.4	25.7	32.5	39.4	1.2	0.8	0.4
Sal	—	—	5.6	44.3	40.4	1.5	7.7	0.5
IE	1.9	1.7	24.3	32.7	37.7	1.2	3.9	0.2
Kokum	—	—	2.0	57.5	39.0	1.3	—	0.2
IE	1.7	—	26.9	33.2	38.8	0.9	—	0.2
Cocoa[b]			25.2	35.5	35.2	3.2	—	0.9

[a]Interesterified.
[b]Reported value (ref. 21).

TABLE 2

Fatty Acid Composition in the 2-Position of Original and Interesterified Triacylglycerols of Selected Vegetable Fats

Fat	Fatty acid (mol%)			
	16:0	18:0	18:1	18:2
Mango	1.1	1.7	78.9	18.0
IE[a]	5.8	4.7	72.2	16.6
Dhupa	2.1	4.4	90.1	2.6
IE	7.1	6.7	83.3	2.4
Kokum	0.4	1.3	96.3	2.0
IE	1.7	3.1	93.7	1.4
Sal	1.7	1.3	90.5	3.9
IE	3.0	5.3	87.4	3.4
Cocoa[b]	2.4	1.6	89.0	6.4

[a]Interesterified.
[b]Reported value (ref. 21).

to the required level. Mango fat from other varieties (20) with lower linoleic acid content (*e.g.*, "Alfonso", "Badami") could be a better starting material. The purified TAG of interesterified kokum, dhupa and sal fats were similar to cocoa butter in fatty acid composition.

The 2-position in cocoa butter TAG is occupied by oleic acid to the extent of more than 80% (21). Similar amounts (*ca.* 90%) are present in the 2-position of kokum, dhupa and sal TAG, while a slightly lower amount (79%) is present in mango TAG (Table 2). That oleic and linoleic acid contents in the 2-position were reduced only slightly in these fats after ester interchange confirms the 1,3-specificity of Lipozyme.

The TAG with one oleic and two saturated fatty acid moieties (SOS), such as 1,3-dipalmitoyl-2-oleoyl glycerol (POP), 1(3)-palmitoyl-3(1)stearoyl-2-oleoyl glycerol (POSt) and 1,3-distearoyl-2-oleoyl glycerol (StOSt), contribute about 72% of total TAG content of cocoa butter (22). The proportion of these TAG together in cocoa butter is responsible for its sharp, exclusive, melting characteristics. In cocoa butter, the POP, POSt and StOSt contribute about 12.0, 34.8 and 25.2%, respectively, of the SOS. The TAG compositions of sal, dhupa, mango and kokum fats (Table 3) indicate that these fats are chiefly made of glycerides containing one unsaturated and two saturated fatty acyl groups (S_2U). Mango fat has considerably higher amounts of glycerides containing one saturated and two unsaturated fatty acyl groups (SU_2) followed by dhupa, sal and kokum fats. StOSt is the predominant glyceride in kokum, making it a higher-melting fat than cocoa butter. Ester interchange of kokum fat caused a significant reduction in the StOSt content from 77.3 to 29.4% while generating 16.2% of POP and an increase in POSt content from 8.1 to 36.9% (Fig. 1). In dhupa fat, ester interchange reduced the StOSt content from 46.0 to 19.0% while increasing the POP and POSt contents from 4.1 and 19.7% to 15.1 and 33.0%, respectively.

Some sal fat samples are reported to contain epoxy and hydroxy glycerides (23). The sal fat sample used in the present investigation was a refined fat and was not found to contain any oxygenated fatty acids. The sal fat after ester interchange registered an increase in the POP and POSt contents from 4.8 and 16.0% to 18.8 and 31.2%, respectively, whereas the StOSt content was reduced from 36.3 to 17.9%. Mango kernel fat contains high amounts of a triunsaturated (U_3) type of glycerides along with SU_2 and S_2U (Table 3). Considerable reduction in the

TABLE 3

Triacylglycerol Composition of Selected Original and Interesterified Vegetable Fats

Fat	Glyceride type (wt%)			
	S_2U	SU_2	U_3	S_3
Sal	76.4	19.6	2.0	1.7
IE[a]	73.9	22.6	1.2	1.9
Dhupa	72.0	24.2	0.6	3.2
IE	69.6	24.1	—	6.2
Mango	62.2	30.1	7.7	—
IE	72.8	17.3	9.3	0.5
Kokum	87.9	12.1	—	—
IE	82.5	16.1	—	1.5
Cocoa[b]	78.3	19.0	0.5	2.2

[a]Interesterified.
[b]Reported value (ref. 22).

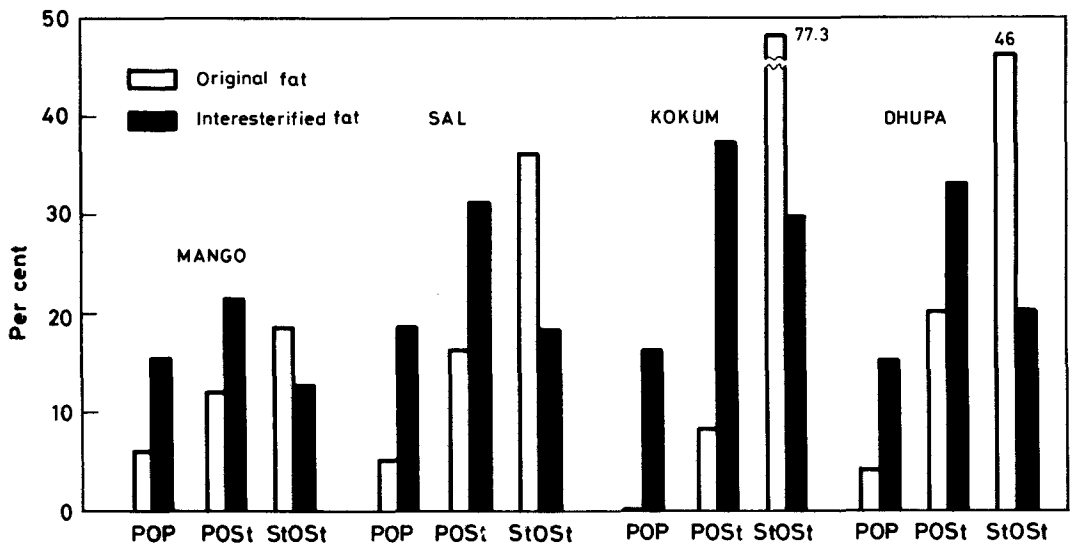

FIG. 1. Triacylglycerol (SOS) content (%) of selected original and interesterified vegetable fats.

SU_2 type of glycerides was seen in the interesterified mango TAG, whereas the S_2U type of glycerides, such as POP, were found to increase from 37.0 to 49.4% (Fig. 1). However, the total S_2U content was considerably lower than in cocoa butter. Because this composition was not suitable for CBS, the interesterified mango TAG were not analyzed further.

Cocoa butter is hard and brittle at room temperature and has a relatively short melting range, with more than four-fifths of it melting between 27 and 35°C (24). The solid fat content (SFC) is an important factor in judging the physical properties of cocoa butter. It characterizes the melting properties, giving an indication of how a fat performs at various temperatures. SFC can be calculated indirectly from the percentage liquid fraction estimated by DSC. Table 4 gives the SFC of original and interesterified TAG of dhupa, sal and kokum fats, determined at various temperatures from 20 to 42°C. After ester interchange, the peak melting temperatures of dhupa and sal TAG were lowered from 36.2 and 35.3° to 31.2 and 32.1°C, respectively. The peak melting temperature of kokum TAG was lowered significantly from 41.2 to 32.8°C, which was almost the same as that of the reference cocoa butter sample (32.7°C). The SFCs of interesterified dhupa and sal fats were reduced significantly at temperatures above 25°C, but the presence of solid fats at 42°C could have been due to the high melting glycerides formed during ester interchange. Kokum TAG had a sharp melting range, with the SFC declining from 91.8 to 4.5% between 37.0 and 40.0°C. The high melting kokum TAG were altered considerably after ester interchange. The SFC and the heating curve (Fig. 2) of interesterified kokum TAG, the most satisfactory of the products obtained, compared well with those of cocoa butter. The results show the feasibility of obtaining satisfactory CBS from tree-borne vegetable fats, namely dhupa, sal and kokum, by ester

TABLE 4

Solid Fat Content of Original and Interesterified Triacylglycerols of Selected Vegetable Fats

Fat	Temperature (°C)								Peak melting temp. (°C)
	20	25	30	32.5	35	37	40	42	
Dhupa	89.8	85.2	76.6	63.5	34.8	2.6	—	—	36.2
IE[a]	84.6	71.2	50.7	34.5	23.6	15.1	8.0	4.6	31.5
Sal	100	93.3	78.6	54.0	16.0	—	—	—	35.3
IE	90.0	69.4	44.4	20.0	12.6	8.7	4.6	2.0	32.1
Kokum	100	100	100	100	98.2	91.8	4.5	—	41.2
IE	91.9	81.7	58.6	24.1	6.4	3.2	1.2	—	32.8
Cocoa	96.6	93.8	71.2	22.0	4.8	1.9	0.3	—	32.7

[a]Interesterified.

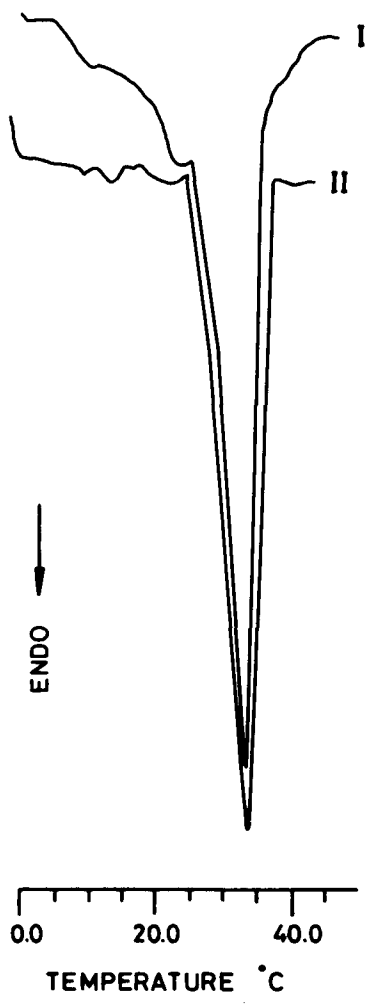

FIG. 2. DSC-heating curves of I, kokum triacylglycerols (interesterified); and II, cocoa butter.

interchange catalyzed by 1,3-specific lipase from *Mucor miehei.* Of these, interesterified kokum TAG possessed physical and chemical properties nearest to cocoa butter.

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