Enzymatic Interesterification of Tallow-Sunflower Oil Mixtures

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In an effort to improve the physical and/or thermal characteristics of solid fats, the enzymatic interesterification of tallow and butterfat with high-oleic sunflower oil and soybean oil was investigated. The two simultaneously occurring reactions, interesterification and hydrolysis, were followed by high-performance liquid chromatography of altered glycerides and by gas-liquid chromatography of liberated free fatty acids. The enzymes used in these studies were immobilized lipases that included either a 1,3-acyl-selective lipase or a *cis*-9-C_{18}-selective lipase. The degree of hydrolysis of the fat/oil mixtures was dependent upon the initial water content of the reaction medium. The extent of the interesterification reaction was dependent on the amount of enzyme employed but not on the reaction temperature over the range of 50–70°C. Changes in melting characteristics of the interesterified glyceride mixtures were followed by differential scanning calorimetry of the residual mixed glycerides after removal of free fatty acids. Interesterification of the glyceride mixes with the two types of enzymes allowed for either a decrease or increase in the solid fat content of the initial glyceride mix.

KEY WORDS: Butter, DSC, HPLC, hydrolysis, interesterification, lipases, melting points, soy oil, sunflower oil, tallow.

In recent years, lipase-catalyzed acyl exchange reactions of fats and oils have been investigated as a means of either altering the physical properties, producing a margarine or specialty fat or improving the nutritional quality of a fat or oil (1). Lipases are generally divided into the following three groups according to their specificity: (i) 1,3-selective lipases, which are positionally specific; (ii) lipases that are selective for a given acyl group of a given structure; and (iii) nonselective lipases, which act in a random manner. Of the nonspecific lipases, *Candida cylindracea* and *Pseudomonas fluorescens* lipases have been the most commonly studied for acyl glycerol synthesis, alcoholysis of glycerides and interesterification of glycerides (2,3). Of the 1,3-specific lipases, porcine pancreatic lipase (4) and *Mucor miehei* lipase (5) have been cited most frequently. The latter lipase has received considerable recent attention because it has been immobilized onto an anion exchange resin that is commercially available (6). This supported lipase has been used in the interesterification of vegetable fats for the production of cocoa butter-like fats (7,8) and in the alcoholysis/glycerolysis of triolein (9). More recently, it has been used for the interesterification of tallow-rapeseed oil mixtures (10). In this study, the effects of reaction parameters on the acyl interchange reaction were studied in detail with the goal of improving the melting properties of this fat/oil blend. Of the acyl-specific lipases, that from *Geotrichum candidum* (11) has received the most attention, especially in the alcoholysis of selected oils that contain polyunsaturated fatty acids (12). In the present study, we have examined the lipase-catalyzed interesterification of solid fats, tallow and butter oil, with sunflower or soybean oil, by means of either an immobilized 1,3-selective lipase or acyl-specific lipase. The work was carried out in an effort to provide interesterified blends of glycerides that have a wider range of thermal and melting properties than the starting fats and oils used.

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MATERIALS AND METHODS

The tallow used in this study was an edible grade obtained from Miniat Inc. (Chicago, IL). High-oleic sunflower oil was obtained from SVO Enterprises (Eastlake, OH), and low-linolenic acid soybean oil was obtained from Pioneer Hybrid International Inc. (Des Moines, IA). Butterfat was prepared in the dairy pilot plant of the Eastern Regional Research Center (Philadelphia, PA). The enzymes used included a commercial immobilized 1,3-specific lipase preparation of *Mucor miehei* (Lipozyme) obtained from Novo Nordisk (Danbury, CT), with an activity of 1613 μmol free fatty acid $min^{-1}g^{-1}$ expressed as μeq of oleic acid released from olive oil per gram of supported lipase. A second 1,3-specific lipase (13) used in this study was from *Rhizopus delemar*, isolated in this laboratory and immobilized on either Celite, Fisher Scientific (King of Prussia, PA), activity 577 μmol free fatty acid $min^{-1}g^{-1}$, or CM Sephadex-25, Pharmacia (Piscataway, NJ), activity 572 μmol free fatty acid $min^{-1}g^{-1}$ (T.A. Foglia, unpublished results). The acyl-specific lipase used was that obtained from *Geotrichum candidum* supported on Accurel EP_{100} (Akzo Chemie, Mc Cook, IL), activity 63 μmol free fatty acid $min^{-1}g^{-1}$ (P.E. Sonnet, unpublished results). Moisture content of the supported enzymes was adjusted by drying the enzyme preparations at 60°C for 4 h in a Perkin-Elmer (Norwalk, CT) infrared drying balance and adding water to a level of 10% by weight of supported enzyme.

Interesterification reactions were carried out with 10-g samples of the fat/oil mixtures and 0.5 g of immobilized lipase preparation in 50-mL glass-stoppered Erlenmeyer flasks. The mixtures were heated at 60°C in a shaker bath (100 rev min^{-1}) for a given time period, and reactions were stopped by removal of the enzyme by filtration. The reacted fat was transferred to a 50-mL volumetric flask and brought to volume with dichloromethane for subsequent analysis as outlined in Figure 1. Separation of interesterified fats into glyceride subclasses was done by normal-phase high-performance liquid chromatography (HPLC). Free fatty acids were quantitated with 0.1 N NaOH in ether/ethanol/water (3:3:2) solvent mixture (30 mL per 0.5 g) by titrating the samples to an endpoint of pH 12. The titrated samples were diluted with water (30 mL) and separated, as outlined in Figure 1, into free fatty acid (FFA) and glyceride fractions. The FFA fraction was converted to methyl esters (FAMEs), with BF_3/methanol reagent (14), and acyl composition was determined by gas-liquid chromatography (GLC). Analysis of the triglyceride (TG) fraction for its molecular species composition was accomplished with reverse-phase HPLC.

FAMEs were analyzed by GLC on a Hewlett-Packard (Avondale, PA) Model 5895 chromatograph equipped with a split capillary injector, a flame-ionization detector and

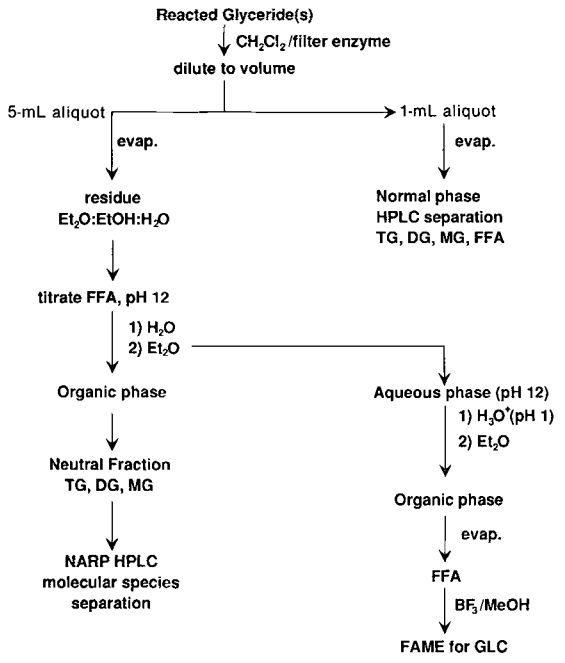

FIG. 1. Flow scheme for the analysis of interesterified glycerides. FFA, free fatty acids; TG, triglycerides; DG, diglycerides; MG, monoglycerides; HPLC, high-performance liquid chromatography; FAME, fatty acid methyl esters; GLC, gas-liquid chromatography.

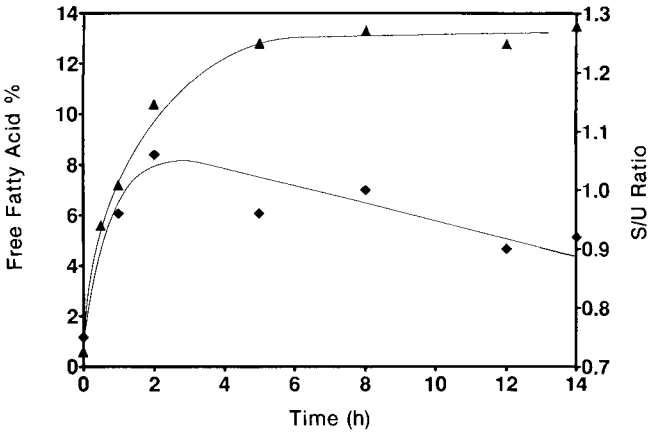

FIG. 2. Lipase-catylized hydrolysis of tallow with time (solid triangle); Lipozyme (10% H_2O); 50 mg/g tallow. Saturated fatty acid to unsaturated fatty acid ratio (S/U ratio) of liberated fatty acids (solid diamond).

a Hewlett-Packard Model 3396 integrator. Separations were obtained on a .25 mm i.d. × 30 m SP-2340 column from Supelco Inc. (Bellefonte, PA). The carrier gas was helium with linear velocity of 22.9 cm sec^{-1} at an 80:1 split ratio. Elution of FAMEs was carried out with temperature programming from 140°C to 155 at .5°C min^{-1}; 155 to 200°C at 2°C min^{-1}. FAMEs were identified by comparison with standards, mix M-100, obtained from Nu-Chek-Prep (Elysian, MN).

HPLC was carried out with a Hewlett-Packard Model 1090 ternary solvent delivery module equipped with a Rheodyne (Rainin, Woburn, MA), Model 7125 injector with a 20-μL loop and a Varex (Burtonville, MD) ELSD II mass detector. The detector output was routed to a Hewlett-Packard Model 3393A integrator. Reacted fat mixtures were separated into individual lipid subclasses by normal-phase HPLC on a Microsorb 5 μm silica column (4.6 mm × 25 cm) and identified by comparison with standards. Separations were obtained isocratically with a mobile phase of isooctane/isopropanol (90:10) by means of the following flow program: time 0.0–10.0 min, flow rate 0.5 mL min^{-1}; time 10.0–15.0 min, increase flow rate to 1.5 mL min^{-1} at 0.2 mL min^{-1}; time 15.0–18.0 min, decrease flow rate to 0.5 mL min^{-1} at 0.33 mL min^{-1}. TG mixtures were analyzed by nonaqueous reversed-phase HPLC on a Beckman/Altex (Rainin) Ultrasphere ODS 5 μm, (4.6 mm × 25 cm) column. Separations were obtained with acetone (solvent A): acetonitrile (solvent B) as eluent at a flow rate of 0.8 mL min^{-1}, and the following gradient profile: initial condition 70:30, A:B, hold 5 min; to 100 A over 18 min; hold for 5 min; return to original conditions. Total run time was 30 min. Identification of individual TG molecular species was made by comparison with standard glyceride mix G-1 obtained from Nu-Chek-Prep.

Melting profiles and percent solid fat content of the glyceride fractions, after removal of FFA (see above and Fig. 1), were obtained by differential scanning calorimetry (DSC) on a Perkin-Elmer Model DSC7. Samples were prepared by heating at 60°C for 1 h prior to measurement. The samples were cooled to −40°C at 10°C min^{-1}, held for 5 min at −40°C and heated at 5°C min^{-1} to a final temperature of 60°C. Melting points were determined by the closed capillary technique (15). Samples were prepared by tempering at 5°C overnight prior to measurement.

RESULTS AND DISCUSSION

Initial experiments were directed to the interesterification between the TG of tallow alone by the supported lipase from *Mucor meihei* at 5 wt% immobilized lipase with respect to tallow. Before interesterification can begin, partial hydrolysis of tallow to FFA and diglycerides (DG) must occur. In the present work, hydrolysis was initiated by addition of a given amount of water to the supported lipase or by the initial presence of water bound to the resin. Moreover, at a given water content, the degree of hydrolysis and esterification reactions approach each other and the system should come to equilibrium. Accordingly, the time course of this reaction was followed by measuring the extent of hydrolysis through titration. The effect of water on the interesterification of tallow with this enzyme has been reported recently (10). We found that, upon drying at 60°C for 16 h, the lipozyme preparation lost about 10% of its weight as water. At this water content, equilibrium was reached at the 13% hydrolysis level after 5 h at 60°C (Fig. 2 and Table 1). The same level was reached over the temperature range of 40–60°C, albeit at longer reaction times at the lower temperatures (data not shown). Accordingly, this time (5 h) and temperature (60°C) were selected as being optimum for all subsequent reactions. The dried enzyme retained its activity; however, its reactivity with tallow as measured by hydrolysis was signifi-

TABLE 1

Interesterification of Glycerides with Supported Enzymes

Glyceride(s)	Enzyme[a]	Hydrolysis[b] (%)	S/U Ratio[c]
Tallow	L	12.8	0.96
Tallow	L[d]	2.4	1.00
Tallow	RD1	12.3	0.94
Tallow	RD2	11.9	0.75
Tallow	GC	4.8	0.30
Sun oil	L	7.0	0.14
Sun oil	RD1	8.5	0.15
Tallow and sun oil[e]	L	7.2	0.44
Tallow and sun oil	RD1	11.2	0.94
Tallow and sun oil	GC	4.0	0.30
Tallow and soy oil	RD1	9.9	0.60
Butter and sun oil	L	7.4	0.55

[a]L = Lipozyme; *Mucor miehei*; RD1 = *R. delemar* on Celite RD2 = *R. delemar* on Sephadex-25; GC = *Geotrichum candidum* on polyproplene.

[b]Free fatty acid content after 5 h at 60°C, glyceride/enzyme ratio/20:1 (w/w) at 10% H_2O content of enzyme.

[c]Saturated fatty acids/unsaturated fatty acids released, original S/U ratio of starting glycerides: tallow/0.75; sunflower oil/0.08; soybean oil/0.20; butterfat/0.82.

[d]Lipozyme dried at 60°C for 24 h, H_2O content <1%.

[e]Fat and oil mixtures are 1:1 by wt.

cantly slower (Table 1). Also shown in Figure 2 are the ratios of saturated to unsaturated fatty acids released over the same time course (S/U ratio). The latter ratios were determined by GLC of FAMEs of the FFA fraction after isolation as shown in Figure 1. As expected for this 1,3-selective lipase, saturated fatty acids were released preferentially in the early stages of the reaction, as shown by the increase in the S/U ratio with time. As with percent hydrolysis, this ratio seemed to reach a maximum of about 1 after 5 h. However, the S/U ratio slowly decreased as reaction time increased, implying continued interesterification of tallow glycerides with the initially released FFA. Results obtained for tallow hydrolysis with other supported lipases (at 10% H_2O) are given also in Table 1. For *R. delemar* lipase on Celite, another 1,3-selective lipase (13), the percent hydrolysis increased slightly and the S/U ratio also approached unity, whereas with *R. delemar* on Sephadex, the S/U ratio was unchanged. On the other hand, with the supported *G. candidum* lipase, a *cis*-9-unsaturated-selective lipase (11,12), the percent hydrolysis decreased and, as expected, the S/U ratio decreased because of the preference of this enzyme for oleic acid over the saturates in tallow. Also given in Table 1 are the data obtained for the interesterification of high-oleic sunflower oil glycerides by the two 1,3-selective lipases. In both instances, hydrolysis decreased to about 8%; however the S/U ratio of the FFA doubled from that of the intact sunflower oil, .15 *vs.* .08.

Direct separation of the partially hydrolyzed fats into glyceride subclasses was achieved by normal-phase HPLC, as shown in Figure 3. Changes in the triglyceride molecular species for interesterified fats were followed by reverse-phase HPLC after removal of FFA (Fig. 4). The molecular species separation of tallow before and after interesterification with Lipozyme are shown in panels B and C of Figure 4. In agreement with the GLC data on released fatty acids, the major differences between these two traces occur in the saturate-containing glyceride peaks.

Data for the interesterification of solid fats with liquid oils by the three supported enzymes are also given in Table 1. The data in this table list the extent of hydrolysis along with the S/U ratio of the released fatty acids for equal-weight mixtures of tallow and sunflower oil, tallow and soy oil, and finally for butterfat and sunflower oil. As observed, the percent hydrolysis values for the mixtures are comparable to those observed for tallow alone. On the other hand, the S/U ratios for the interesterified fat mixtures varied depending upon the initial fat mixture and

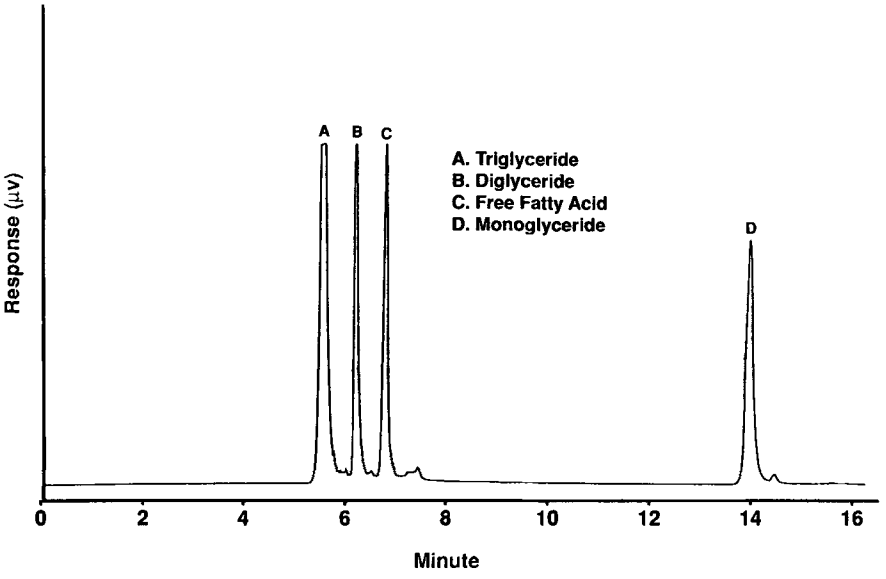

FIG. 3. Normal-phase high-performance liquid chromatographic separation of triglycerides, diglycerides, monoglycerides and free fatty acids. For conditions see Materials and Methods section.

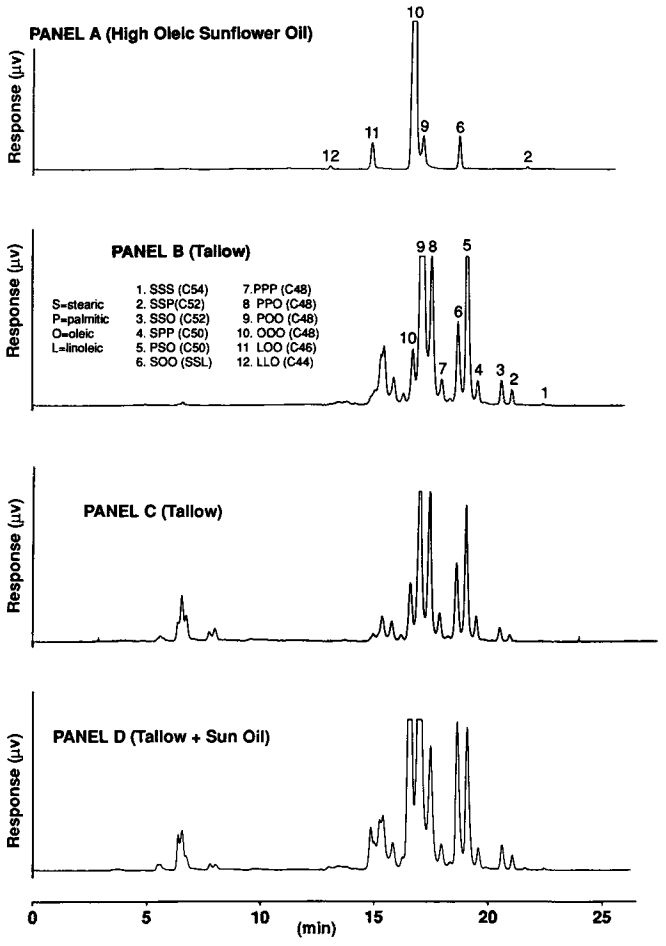

FIG. 4. Reverse-phase high-performance liquid chromatographic separation of triglycerides before and after interesterification with enzyme for 5 h at 60°C. For conditions see Materials and Methods section. Designations of glycerides and fatty acids are shown in Panel B. Panel A—sunflower oil; Panel B—tallow; Panel C—tallow interesterified with lipozyme; Panel D—tallow and sunflower oil interesterified with lipozyme. Panel E—tallow interesterified with *R. delemar*.

type of lipase used. For example, use of *R. delemar* lipase with tallow/sunflower oil increased this ratio to .94, demonstrating its preference for the fatty acids at the 1,3 positions of tallow (13), which are principally occupied by stearate and palmitate. On the other hand, with the *G. candidum* enzyme, this ratio was 0.3, which again is an expression of this lipase's preference for unsaturated over saturated acyl residues in the glyceride mixes. The HPLC TG molecular species separation of the interesterified tallow and sunflower oil product with Lipozyme is shown in Figure 4. This chromatogram is representative of the changes observed for the other interesterified glycerides mixes given in Table 1. As shown for the tallow-sunflower oil chromatogram, there is an increase in the unsaturated fatty acid-containing glycerides, peaks 6, 9,10 and 11, over the saturate-containing glycerides, peaks 5 and 8.

Table 2 gives the melting points and percent solids for the fats and oils used in this work and compares them with those obtained for interesterified tallow and the interesterified mixes listed in Table 1. For comparative purposes, selected data from Table 2 are presented also in Figure 5, which shows the solid fat content as a function of temperature. The data in Table 2 are given for the three types of immobilized lipases used in the study and compare the interesterified results with those obtained upon physically mixing the fat and oil. For tallow alone, treatment with the two 1,3-selective lipases caused a 2-3°C decrease in the melting point. This result is comparable with that reported by Forssell *et al.* (10) for the interesterification of tallow with the Lipozyme preparation. For the interesterification of tallow and sunflower oil with each of the lipases, the melting point change (3-4°C) was of the same magnitude as for the physically blended mix. However, the solid fat content of the interesterified mixture showed significantly lower values, especially at the lower temperature range. A similar result was obtained for the tallow-soy oil interesterified mixes, in that melting points of the products decreased on the same order of magnitude as for the blended fat and oil. The more significant changes occurred in the solid fat content of the tallow-soy interesterified glycerides. Larger decreases in

TABLE 2

Melting Points and Solid Fat Index Values of Interesterified Glycerides

Glyceride(s)	Melting point °C	% Solid fat at °C					
		−10	0	10	20	30	40
Tallow	43.6	88.0	69.6	51.2	46.5	23.6	3.8
Sun oil	1.0	81.7	0.1	—	—	—	—
Soy oil	2.7	6.9	0.4	—	—	—	—
Butter fat	41.2	97.1	84.8	59.5	36.9	14.5	0.4
Tallow (L)[a,b]	41.6	80.2	65.0	50.0	49.0	25.1	2.6
Tallow (RD)	40.4	80.5	61.4	54.4	58.7	20.7	0.2
Tallow and sun (mix)[c]	42.0	80.8	39.7	26.6	18.0	8.0	0.2
Tallow and sun (RD)	40.2	46.7	10.7	2.4	18.9	11.6	0.2
Tallow and sun (GC)	41.3	47.1	17.1	9.3	18.6	18.1	0.2
Tallow and sun (L)	39.8	66.0	35.1	18.5	29.7	18.0	—
Tallow and soy (mix)	30.0	42.1	20.0	8.2	2.5	—	—
Tallow and soy (RD)	39.5	47.0	37.9	18.6	10.9	2.9	—
Tallow and soy (L)	40.3	46.0	36.1	14.2	8.9	1.8	0.2
Butter and sun (mix)	36.9	70.7	49.3	26.7	10.5	2.1	—
Butter and sun (L)	37.7	63.0	43.2	18.5	6.8	0.4	—

[a]L = *M. miehei* on anion exchange resin; RD = *R. delemar* on Celite; GC = *G. candidum* on Accurel EP-100.
[b]Interesterification reactions conducted at 60°C for 5 h.
[c]Mix = 1:1 weight mixture of fat and oil.

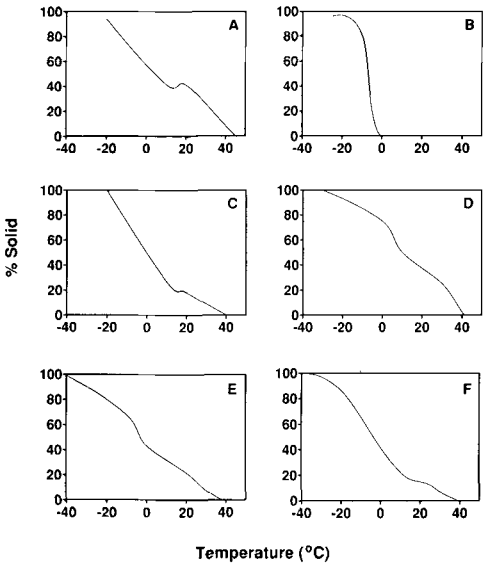

FIG. 5. Percent solids curves for selected fats and oils as determined by differential scanning calorimetry conditions given in text. Panel A: tallow; Panel B: sunflower oil; Panel C: 1:1 wt mix of tallow/sunflower oil; Panel D: tallow interesterified by Lipozyme; Panel E: 1:1 wt mix of tallow/sunflower oil interesterified by Lipozyme; and Panel F: tallow interesterified by *R. delemar* on Celite.

melting points were obtained for the interesterified products by increasing the amount of enzyme used (data not shown), but this increased the hydrolysis amount to high levels (20–25%). The use of larger amounts of dried enzyme preparations to increase melting point depressions was not fruitful because it required reaction times exceeding 24 h to attain the same levels of change reported in Table 2. The final entry in this Table gives the results obtained for the interesterification of butterfat-sunflower oil. Again, melting point depression is not indicative of the induced changes in glyceride composition. For this type of information, it is best to look at percent solid fat content over a wider temperature range.

The results obtained in this study show that alterations in the glyceride composition of fats and oils can be induced in a somewhat controlled manner by the proper choice of enzyme type under specifically controlled reaction conditions. While the interesterification reactions described herein may not be optimal for a given application, they nevertheless demonstrate, along with the work of others, that enzyme-catalyzed interesterification of fats and oils is a viable alternative to physical blending or chemical interesterification. Moreover, with the proper choice of lipase, it is possible to control the position-selective or fatty acyl-selective exchange of fatty acids within a glyceride structure. This is in stark contrast to chemical reactions that are completely random in nature.

REFERENCES

1. Mukherjee, K.D., *Fette Wissenschaft Technol. 94*:542 (1992).
2. Kalo, P., H. Huotari and M. Antila, *Fat Sci. Technol. 91*:276 (1989).
3. Lazar, Von G., *Fette Seifen Anstrichm. 87*:394 (1985).
4. Elliott, J.M., and K.L. Parkin, *J. Am. Oil Chem. Soc. 68*:171 (1991).
5. Hansen, T.T., and P. Eigtved, in *Proceedings of World Conference on Emerging Technologies in the Fats and Oils Industry*, edited by A. R. Baldwin, American Oil Chemists' Society, 1986, p. 365.
6. Eigtved, P., Eur. Patent EP 140542 (1985).
7. Chang, M.F., G. Abraham and V.T. John, *J. Am. Oil Chem. Soc. 67*:831 (1990).
8. Bloomer, S., P. Adlercreutz and B. Mattiasson, *Ibid. 67*:519 (1990).
9. Ergan, F., M. Trani and G. Andre, *Ibid. 68*:412 (1991).
10. Forssell, P., R. Kervinen, M. Lappi, P. Linko, T. Suortti and K. Poutanen, *Ibid. 69*:126 (1992).
11. Marks, T.A., J.G. Quinn, J. Sampugna and R.G. Jensen, *Lipids 3*:143 (1968).
12. Jensen, R.G., D. Rubano Galluzzo and V.J. Bush, *Biocatalysis 3*:307 (1990).
13. Sonnet, P.E., and J.A. Gazzillo, *J. Am. Oil Chem. Soc. 68*:11 (1991).
14. Marmer, W.N., and R.J. Maxwell, *Lipids 16*:365 (1981).
15. *Official and Tentative Methods of the American Oil Chemists' Society*, 3rd edn., edited by R.O. Walker, American Oil Chemists' Society, Champaign, 1980.

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