Effect of Enzymatic Transesterification on the Melting Points of Palm Stearin–Sunflower Oil Mixtures

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ABSTRACT: Transesterification with lipases may be used to convert mixtures of fats to plastic fats, making them more suitable for use in edible products. In our study, 1,3-specific (*Aspergillus niger, Mucor javanicus, Rhizomucor miehei, Rhizopus javanicus,* and *Rhizopus niveus*) and nonspecific (*Pseudomonas* sp. and *Candida rugosa*) lipases were used to transesterify mixtures of palm stearin and sunflower oil (PS–SO) at a 40:60 ratio in a solvent-free medium. The transesterified mixtures of PS–SO were analyzed for their percentage free fatty acids (FFA), degree and rate of transesterification, solid fat content, slip melting point (SMP), and melting characteristics by differential scanning calorimetry. Results indicated that *Pseudomonas* sp. lipase produced the highest degree (77.3%) and rate (50.0 h^{-1}) of transesterification, followed by *R. miehei* lipase at 32.7% and 27.1 h^{-1}, respectively. The highest percentage FFA liberated was also in the reaction mixtures catalyzed by *Pseudomonas* sp. (2.5%) lipase and *R. miehei* (2.4%). *Pseudomonas*-catalyzed mixtures produced the biggest drop in SMP (13.5°C) and showed complete melting at below body temperature. All results indicated conversion of the PS–SO mixtures to a more fluid product. The findings also suggest that the specificity of lipases may not play a significant role in lowering the melting point of the PS–SO mixtures.
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KEY WORDS: Lipases, melting properties, palm stearin, sunflower oil, transesterification.

Palm stearin (PS), the more solid fraction obtained by fractionation of palm oil after crystallization at a controlled temperature, is a useful source of fully natural hard component for products such as shortening, pastry, margarine, and other edible fats. PS consists of a high proportion of palmitic acid (47–74%) and oleic acid (16–37%), with a slip melting point (SMP) ranging between 44–56°C (1). The production of edible fats, however, requires fat blends that are able to impart plasticity and body to the end product. This necessitates the enrichment of polyunsaturated oils, such as soybean oil and sunflower oil (SO), into PS, which basically lacks the ability to impart the required plasticity to the end product and must therefore be modified. Transesterification reactions between solid fats and liquid oils were traditionally carried out with chemical catalysts (2). Enzymes allow catalysis at mild conditions and reduce deterioration in quality and characteristics of the oils. Enzymes are biodegradable and therefore reduce environmental loading.

Positional specificity allows lipases to distinguish between the *sn*-1 and/or *sn*-3 position on the triglyceride (TG) molecules. Such fatty acid rearrangements can alter the physical properties, such as the melting point of the oil. The current interest in lipase-catalyzed transesterification of fats and oils stems from the need for liquid oil. Lipase-catalyzed transesterification has been carried out with palm olein (3,4), tallow and SO (5), tallow and rapeseed oil (6), and PS–coconut oil mixtures (7). After treatment with enzymatic methods, the prepared fats do not contain *trans* fatty acids, which have been implicated in adverse effects on the high-density lipoprotein levels in blood (8).

In this study, we report on the efficacy of various immobilized 1,3-specific and nonspecific lipases in improving the melting properties of PS–SO (40:60) mixtures in a solvent-free system. The work was carried out in an effort to provide transesterified blends of glycerides that have a wider range of thermal and melting properties than the starting fat and oil used.

MATERIALS AND METHODS

Materials. Refined, bleached, and deodorized hard PS (SMP 54.5°C) and SO were obtained from Ngo Chew Hong Oils and Fats Pte. Ltd. (Semenyih, Malaysia) and a local supermarket (Malaysia), respectively. The fatty acid composition of the PS, as analyzed by gas chromatography after conversion to fatty acid methyl esters (FAME) (9), was $C_{12:0}$ (0.24%), $C_{14:0}$ (1.46%), $C_{16:0}$ (62.0%), $C_{16:1}$ (0.07%), $C_{18:0}$ (4.8%), $C_{18:1}$ (25.0%), $C_{18:2}$ (5.6%), $C_{18:3}$ (0.4%), and $C_{20:0}$ (0.43%). The fat and oil were stored at 0–4°C. Prior to use, the PS was melted at 60°C in the oven. Celite, used as a carrier for the lipases, was purchased from BDH Ltd., (Poole, England). Amano Pharmaceutical Co. (Nagoya, Japan) donated the *Aspergillus niger, Mucor javanicus, Rhizopus javanicus, Rhizopus niveus, Candida rugosa,* and *Pseudomonas* sp. lipases (all in powder form). *Rhizomucor miehei* lipase (Lipozyme 1M60) was obtained in the immobilized form (moisture content: 2–3%) from Novo Nordisk Ind. (Copenhagen, Denmark). All

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other chemicals used were of analytical or high-performance liquid chromatography (HPLC) grade.

Immobilization of lipase. Lipase powder (0.1 g) was dissolved in 100 µL of cold deionized water, followed by mixing with 0.25 g of Celite (7). The preparation was lyophilized for 4h at −43°C with an Alpha 1-4 Christ LDC-1 (B. Braun Gefriertrocknungsanlagen GmbH, Melsungen, Germany) freeze dryer prior to the transesterification process. The effect of water content on transesterification and hydrolytic activities was studied by using immobilized lipase preparations that were lyophilized for up to 8 h. Duplicate preparations were used for each time of lyophilization. Both activities were then assayed as described below.

Transesterification. Transesterification was carried out as previously reported (7). Prior to transesterification, all lipases, except *R. miehei* lipase, were first immobilized (7). Ten grams of PS–SO (40:60) mixture was reacted with 0.1 g equivalent of immobilized lipase at 60°C, 200 rpm for 8 h for all lipases, except for *R. miehei* lipase, which was reacted for 6 h. After the transesterification reaction, 1 mL of the reaction mixture was withdrawn, and the TG composition was determined by HPLC (Shimadzu Co., Kyoto, Japan) with a commercially packed RP-18 column (250 × 4 mm) with 5-µm particle size (E. Merck, Darmstadt, Germany). TG were eluted with acetone/acetonitrile (60:40) at 1 mL/min flow rate. Percentage TG remaining (%TGR) is the total concentration of TG (eluted after 12 min) after reaction, compared to the unreacted mixture (7). The degree of transesterification is defined as the change in peak area (arbitrary units) of TG that increased in percentage value at reaction time t [TGI_t] with respect to the value at the start of the reaction [TGI_0] minus 100%. The rate of transesterification (X) was calculated according to Ghazali *et al*. (7):

$$X\left(\mathrm{h}^{-1}\right) = \frac{\text{initial rate (\%/h)}}{\text{enzyme activity (\%)}} \qquad [1]$$

where initial rate is ([TGI_t] − [TGI_0])/t in the linear range of reaction, and enzyme activity is the actual activity of the lyophilized immobilized enzyme used. The enzyme activity is expressed as percentage of TG hydrolyzed (total TG minus remaining TG). The activity was measured under the same reaction conditions described earlier for the transesterification reaction because the rate of TG hydrolyzed was linear for all enzymes up to 3 h of reaction.

Hydrolytic activity. The amount of free fatty acids (FFA) present was determined according to the method of Cocks and van Rede (9). At the end of the incubation period, 100 mL of ethanol/diethyl ether (1:1) was added to 4 g of the sample, and the mixture was titrated with 0.05 N NaOH to a phenolphthalein end-point. The degree of hydrolysis is expressed as percentage of FFA liberated and was corrected for the presence of acids in the controls. Duplicate runs were carried out for each sample.

Solid fat content (SFC). A Bruker wideline pulse nuclear magnetic resonance spectrometer (Karlsruhe, Germany) was employed for the SFC measurements by using the direct measurement procedure. Nine tubes were used for each sample. Each sample was tempered at 70°C for 30 min, followed by chilling at 0°C for 90 min, and then kept at the desired temperature for 30 min prior to measurement. Melting, chilling, and holding of the samples were carried out in pre-equilibrated thermostated baths. SFC was measured within the temperature range of 5–40°C.

Thermal properties by differential scanning calorimetry (DSC) analysis. The instrument used was a Perkin Elmer DSC-7 (Norwalk, CT). Samples, weighing 3–15 mg and sealed in aluminum pans, were heated to 70°C for 15 min to ensure that no residual nuclei remained. The samples were then cooled from melt (70°C) at 80°C/min to −30°C and held for 1 min before being heated to 70°C again at 10°C/min for the melting thermograms.

SMP. This was determined by the method described in AOCS Method Cc. 3.25 (10).

RESULTS AND DISCUSSION

In this study, the transesterification process was conducted in a solvent-free system where the temperature of reaction was maintained at 60°C so that the substrates used remained in the liquid state. The melting point of the PS used here was high (54.5°C); thus, it was necessary to perform the reaction at a minimum temperature of 60°C. Cho and Rhee (11) studied the temperature effect (30–60°C) on the interesterification of fats and oils with immobilized *A. niger*, *R. javanicus*, *R. niveus,* and *R. miehei* lipases, and reported that the interesterification activity of the immobilized lipases did not change much at temperatures above 37°C. At 30°C, the interesterification activity was somewhat reduced, and between 37–60°C, the interesterification activity was almost constant. In the enzymes' specifications, given by the manufacturer, the optimal temperatures for nonimmobilized *M. javanicus, Pseudomonas,* and *C. rugosa* lipases used in this work were 30–45, 40–65, and 30–50°C, respectively. Because immobilization is known to

TABLE 1
Degree of Hydrolysis (% FFA), Degree and Rate of Transesterification, and Enzyme Activity of Palm Stearin/Sunflower Oil (40:60) Mixtures After Use of Different Lipases

Source of lipases	FFA (%)	Degree of transesterification (%)	Rate of transesterification (h^{-1})	Activity (% TG hydrolyzed)
A. niger[a]	1.41	23.8	11.3	13.5
M. javanicus[a]	0.14	11.2	8.8	7.0
R. miehei[a]	2.40	32.7	27.1	19.0
R. javanicus[a]	0.45	10.8	8.2	9.0
R. niveus[a]	0.85	4.1	6.0	6.0
Pseudomonas[b]	2.51	77.3	50.0	15.0
C. rugosa[b]	1.97	6.2	9.5	9.5

[a]1,3-Specific lipase.
[b]Nonspecific lipase. Abbreviations: FFA, free fatty acids; TG, triglycerides; *A. niger, Aspergillus niger; M. javanicus, Mucor javanicus; R. miehei, Rhizomucor miehei; R. javanicus and R. niveus, Rhizopus javanicus* and *niveus; C. rugosa, Candida rugosa.*

often enhance the thermal stability of lipases, it was assumed that the reaction temperature used in this study would not drastically lower the activity of the lipases.

Table 1 shows the degree of hydrolysis (% FFA), degree of transesterification, rate of transesterification, and enzyme activity of different lipases for the transesterification of PS–SO (40:60) mixtures. Based on the degree and rate of transesterification, *Pseudomonas* lipase had the highest degree of transesterification (77.3%), followed by *R. miehei* lipase (32.7%), and the rates of transesterification were 50.0 h^{-1} and 27.1 h^{-1}, respectively. The numerical values in Table 1 show that the degree of transesterification by *Pseudomonas* lipase was approximately 18.9 times higher than that by *R. niveus* lipase. Interestingly, the highest percentage FFA liberated was also in the reaction mixtures catalyzed by lipases from *Pseudomonas* (2.5%) and *R. miehei* (2.4%). As both lipases differ in terms of specificity, this result may indicate that specificity of the lipases may not play a big role in the changes that take place in the TG molecules that cause the increase in the degree of transesterification. This result is similar to the findings of Ghazali *et al*. (7) and Li and Ward (12) who reported no clear correlation between positional specificity and the degree of synthesis. According to Li and Ward (12), lipases from *Pseudomonas* sp. (PS-30) and *R. miehei* (1M-60) showed the highest degree of synthesis of glycerides, compared with other commercially available lipases investigated under the same conditions. Ghazali *et al.* (7), in their transesterification work on palm olein, also reported that *Pseudomonas* lipase showed the greatest degree and fastest rate of change, followed by lipases from *R. miehei* (1M-20) and *A. niger*. There seems to be no clear correlation between the enzymes' positional specificity and the formed products. The degrees and rates of transesterification for mold lipases, such as *R. javanicus* (10.8% and 8.2 h^{-1}, respectively), *R. niveus* (4.1% and 6.0 h^{-1}, respectively), and *C. rugosa* (6.2% and 9.5 h^{-1}, respectively), were relatively low.

Table 2 shows the SMP and SFC values of the transesterified PS–SO (40:60) mixtures after using the different lipases. The SMP of the mixtures were generally lower after transesterification. Mixtures of PS–SO (40:60) catalyzed by *Pseudomonas* sp. and *R. miehei* lipases showed the biggest drop in SMP. *Pseudomonas* lipase reduced the SMP of the PS–SO (40:60) mixtures by 13.5°C. Figure 1 shows the TG profile of the PS–SO (40:60) mixtures before (Fig. 1A) and after (Fig. 1B–H) transesterification. In terms of relative concentration, several TG were found to increase, while others decreased as indicated by the reduction in peak areas. The reduction of TG could be due to the cleavage of fatty acids at the glycerol backbone either randomly or at *sn*-1 and/or *sn*-3 positions, indicating that hydrolysis does take place. FFA released into the mixture will be reattached randomly or specifically to the free positions on the glycerol backbone to form new TG to cause an increase in existing TG in the mixture. Changes in the TG profile are often accompanied by changes in the physical characteristics, such as the SMP and SFC of the mixture. Although the TG profile of the reaction mixture (Fig. 1B–H) after transesterification with different lipases looks similar, the degrees and rates of transesterification catalyzed by different lipases differ (Table 1).

Transesterification was also shown to reduce SFC consistently for all lipases used throughout the temperature ranges investigated. When compared to the control, the addition of a liquid oil (SO) to increase the polyunsaturated fatty acid content in the mixture resulted in more than 50% reduction in SFC for the *Pseudomonas*-catalyzed mixtures throughout the temperature range. The SFC of the PS–SO (40:60) mixtures catalyzed by *Pseudomonas* was reduced to 0°C at 30°C, indicating rearrangement of the fatty acids on the resulting TG, and causing a lowering of their melting points. A soft mixture, such as the *Pseudomonas* lipase-catalyzed mixture, may find possible usage as tub margarine or polyunsaturated margarine. The rather low SFC of the mixture at low temperatures indicates that the produced margarine would be spreadable at refrigerator temperature. In margarine production, the melting point of margarine has an organoleptic significance. The margarine must melt readily in the mouth with a minimum of waxiness or greasiness. Thus, the melting point of the margarine must be below body temperature (37°C). The

TABLE 2
Slip Melting Point (SMP) and Solid Fat Content (SFC) of Transesterified Palm Stearin/Sunflower Oil (40:60) Mixtures

	Control	*A.niger*	*M. javanicus*	*R. miehei*	*R. javanicus*	*R. niveus*	*Pseudomonas*	*C. rugosa*
SFC (°C)								
5	33.1	31.8	32.3	28.4	25.1	32.4	14.7	31.4
10	30.4	27.5	28.8	24.1	22.8	28.8	11.3	27.8
15	27.3	24.1	26.0	20.2	20.3	25.3	8.6	24.7
20	22.5	19.9	21.9	15.7	16.3	20.9	5.5	20.1
25	19.7	17.0	18.9	13.2	14.0	18.3	2.9	18.3
30	16.4	13.6	15.6	10.5	11.3	15.4	0.0	15.2
35	13.3	11.4	12.7	8.6	9.5	12.2	0.0	12.5
37	12.7	10.8	11.8	7.1	8.7	11.4	0.0	11.2
40	11.0	9.0	10.6	5.5	7.4	10.0	0.0	9.8
SMP (°C)	48.5	47.0	48.0	45.5	47.0	47.5	35.0	46.5

[a]After use of *A. niger, M. javanicus, R. miehei, R. javanicus, R. niveus, Pseudomonas* sp., and *C. rugosa* lipases. For other abbreviations see Table 1.

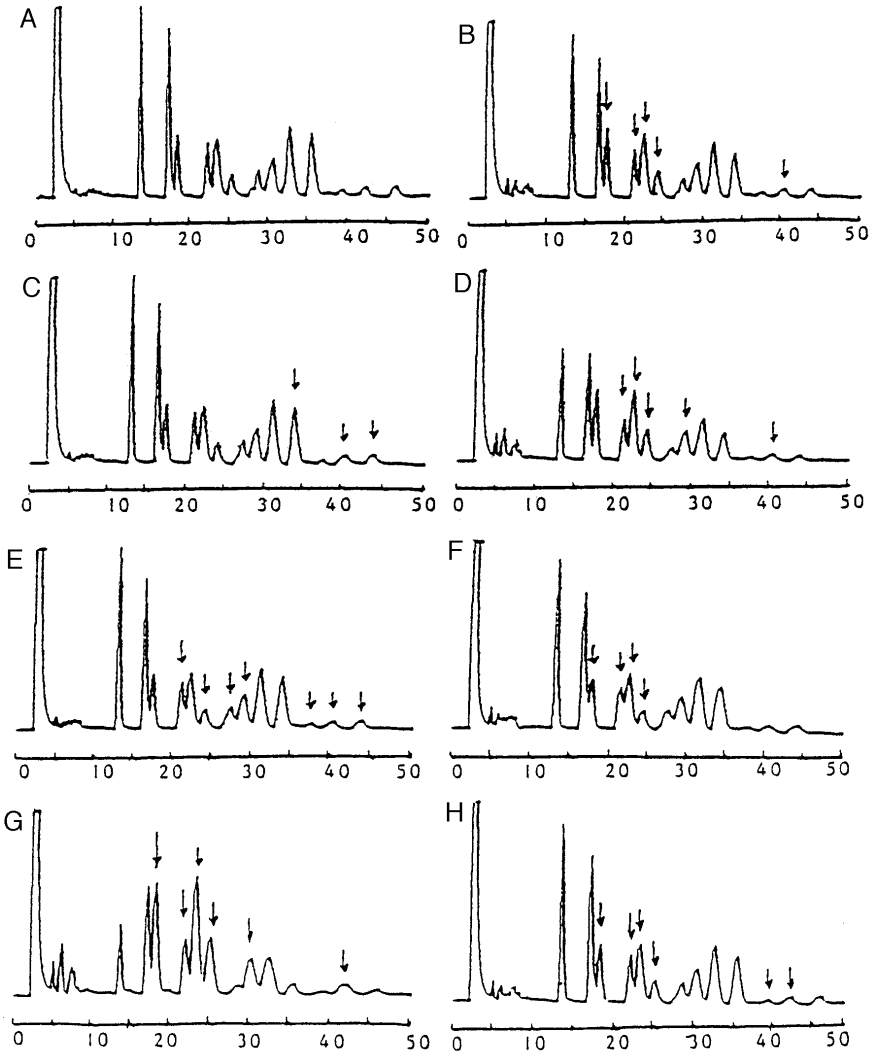

FIG. 1. Triglyceride profiles of palm stearin/sunflower oil (40:60) mixtures before (A) and after transesterification with lipases from *Aspergillus niger* (B), *Mucor javanicus* (C), *Rhizomucor miehei* (D), *Rhizopus javanicus* (E), *Rhizopus niveus* (F), *Pseudomonas* sp. (G), and *Candida rugosa* (H). Triglycerides represented by arrows indicate increases in concentrations.

Pseudomonas lipase-catalyzed mixture melted completely below 37°C (Table 2) and hence would give good oral meltdown (13).

Figure 2 shows the melting thermograms of PS–SO (40:60) mixtures before (Fig. 2A) and after (Fig. 2B–H) being catalyzed with different lipases. In the control mixture (Fig. 2A), three endotherms, A, B and C, and one exotherm, X_C, were noticeable. Taking into consideration the SMP obtained after transesterification by the lipases, the thermograms could be differentiated into three groups. Group 1 comprises the mixtures catalyzed by lipases from *A. niger, M. javanicus, R. javanicus, R. niveus,* and *C. rugosa* (Fig. 2C, E, F, and H). These mixtures have SMP ranging between 46.5–48.0°C (Table 2), which explains the close similarity of the DSC melting thermograms to that of the control mixture (Fig. 2A), which has an SMP of 48.5°C. Group 2 comprises the *R. miehei* lipase-catalyzed mixture, which has an SMP of 45.5°C. For this mixture, the exotherm X_C has almost disappeared, while endotherm C is reduced significantly in size. Because endotherm C is due to the higher-melting TG, a decrease in its value indicates lowering of the SMP. The appearance of peak D at a melting temperature higher than that for peak B suggests that the crystalline form of TG, responsible for peak B, underwent simple rearrangement into forms which gave rise to peak D. The *Pseudomonas* lipase-catalyzed mixture is also the only representative for the third group and has an SMP of 35.0°C. From Figure 2G, it is evident that a new exotherm, X_E, and a minor peak E appeared, while exotherm X_C has become indistinguishable. Endotherm C has completely disappeared, probably because the crystals responsible for the peak have melted in the liquid phase of the mixture as a consequence of random fatty acid rearrangement and acyl exchange of the higher-melting TG to form lower- and middle-melting TG species.

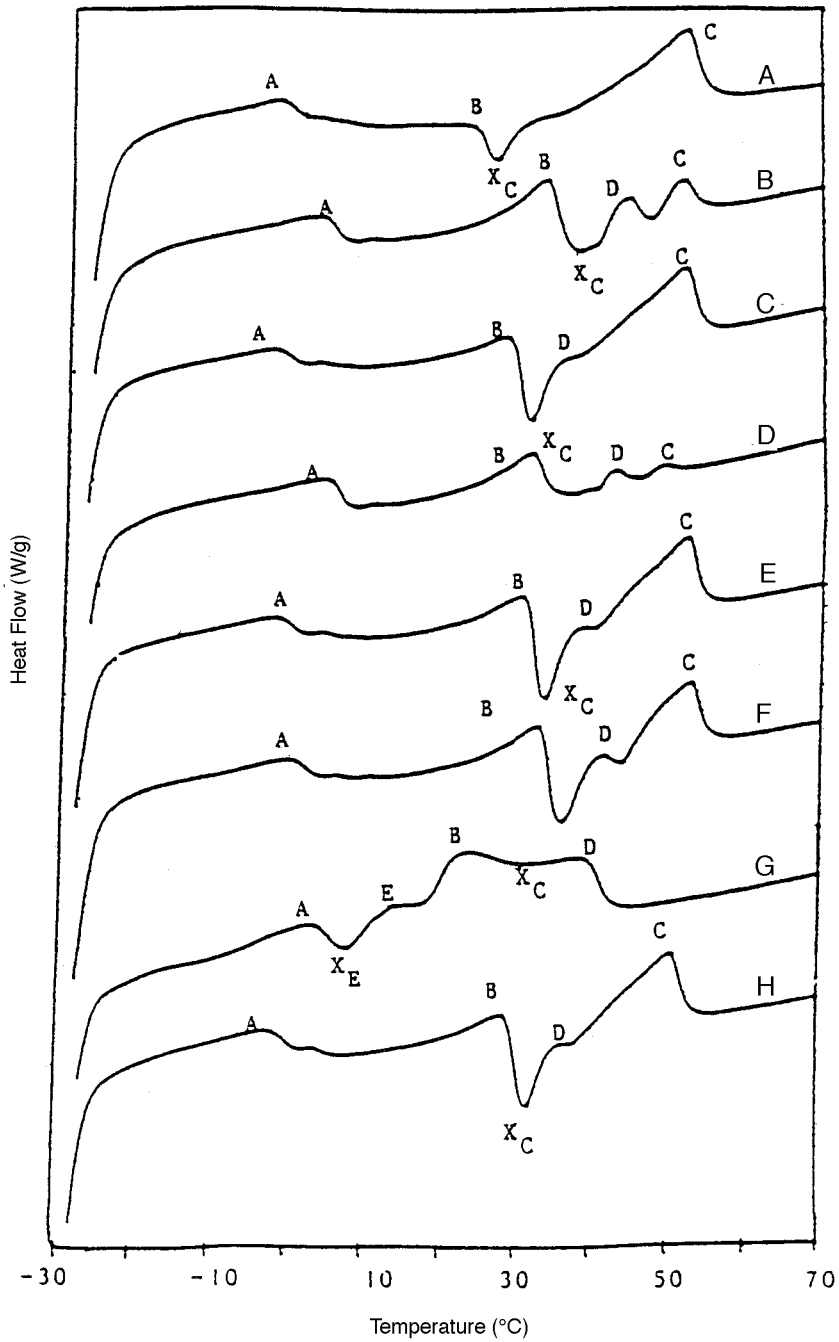

FIG. 2. Differential scanning calorimetry heating thermograms of palm stearin/sunflower oil (40:60) mixtures before (A) and after transesterification with lipases from *A. niger* (B), *M. javanicus* (C), *R. miehei* (D), *R. javanicus* (E), *R. niveus* (F), *Pseudomonas* sp. (G), and *C. rugosa* (H) at heating rates of 10°C/min. Pretreatment: cooled from +70 to −50°C at 80°C/min. Heating program was started after 1 min at −50°C. For abbreviations see Figure 1.

From our study of the physical properties of the PS–SO mixtures (40:60), it would appear that lipases from *Pseudomonas* and *R. miehei* would be the most noteworthy for the preparation of a more fluid product. Both of these lipases produced large reductions in SMP and had the highest degrees and rates of transesterification. Results of SFC and DSC also helped to justify this selection for our future work in the formulation of softer products.

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