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Physical properties of palm kernel olein-anhydrous milk fat mixtures transesterified using mycelium-bound lipase from *Rhizomucor miehei*

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Abstract

The transesterification activity of mycelium-bound lipase from *Rhizomucor miehei* on palm kernel olein:anhydrous milk fat (PKO:AMF) blends was investigated. Commercial immobilised *R. miehei* lipase preparation, Lipozyme IM60 (Novo Nordisk), was used as a comparison. Mixtures of PKO:AMF, at ratios of 100:0, 70:30, 60:40, 50:50 and 0:100, were transesterified using either enzyme in a solvent-free system. The triglyceride (TG) profile, slip melting point, solid fat content, melting thermogram and the polymorphic form of the unreacted and transesterified mixtures were evaluated. Results indicated that transesterification by either enzyme was able to produce an oil mixture with new TG profiles, generally lower slip melting points and solid fat contents. The melting thermograms from differential scanning calorimetry analysis indicated changes in the triglyceride's crystalline composition and an overall shift to lower melting TG. Although the catalytic activities were similar for both lipases, Lipozyme-catalysed mixtures produced higher degrees of transesterification (43–51%) than mycelium-bound lipase-catalysed (22–34%) mixtures. This study also demonstrated that the transesterified PKO:AMF mixture at 70:30 ratio completely melted at 25C, and this meets the melting criteria for fat used in ice cream formulation.

Keywords: Mycelium-bound; Lipase; *Rhizomucor miehei*; Transesterification; PKO; AMF

1. Introduction

Enzymatic transesterification (TE) has been widely used as a common fat modification process. It offers the possibility of manipulating the distribution and composition of fatty acids of triglycerides (TGs) to a more desirable physical and chemical property. In recent years, lipased-catalyzed TE has gained popularity over the traditional chemical TE process. This is mainly because lipases allow catalysis of TE to occur under mild conditions and with high specificity where lipases are capable of catalysing a more directed rearrangement of fatty acids in TG molecules (Huyghebaert, Verhaeghe & De Moor, 1994). Extracellular microbial lipases have been widely used as catalysts in the enzymatic TE reactions. A range of microbial lipases has been demonstrated to catalyse TE reaction to varying degrees with *Pseudomonas* and *Rhizomucor miehei* lipases amongst the most reactive lipases (Ghazali, Maisarah, Yusof & Yusoff, 1995a; Lai, Ghazali & Chong, 1998a).

Rearrangement of the fatty acids in TG molecules via the TE process can alter a number of physical properties of fats and oils (Laning, 1985). Generally, TE reaction results in lower slip melting points (SMPs), solid fat contents (SFCs) and change in the TG profile (Ghazali, Maisarah et al., 1995a; Hoffman, 1989; Zeitoun, Neff, List & Mounts, 1993). These have been demonstrated in the TE of palm sterin and sunflower oil mixture (Lai et al., 1998a), palm stearin and coconut oil mixture (Ghazali, Maisarah et al.) and palm stearin and palm kernel olein mixture (Lai, Ghazali & Chong, 1998b).

Mycelium-bound lipase (MBL), which is sometimes referred to as a naturally immobilised lipase (Long, Ghazali, Bucke, Ampou & Ariff, 1996a; Muzzarelli, 1980; Yamaguchi, Arai & Itoh, 1982), can be used directly as a source of lipase. This helps to eliminate the

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complex procedures of isolation, purification and immobilization of the enzyme which often result in problems of low yield and low activity. The use of MBL also offers added advantages such as thermal and pH stability, and increased stability in organic solvents (Patterson, Blain, Shaw, Todd & Bell, 1979). Thus, there is appreciable potential in the used of MBL in the fats and oils industries, namely in interesterification and TE reactions. Even though a portion of the bound lipase is loosely linked to the mycelium which may be lost during washing, the presence of a chelating agent, such as EDTA, was demonstrated to be able to enhance the production and maintain the stability of the extracted MBL (Long et al., 1996a). Other mycelium-bound enzymes especially proteases, are likely to pose denaturation problems only if they are co-extracted from the mycelium to form soluble enzymes.

In this paper, we report the physical properties of transesterified palm kernel olein:anhydrous milk fat (PKO:AMF) mixture catalysed by MBL from *R. miehei* and commercial immobilised *R. miehei* lipase (Lipozyme IM60) in a solvent-free system. The work is part of a study on the potential use of MBL *R. miehei* to produce a fat mixture which can be used in ice cream formulation.

2. Materials and methods

2.1. Materials

R. miehei (ATCC 26282) was purchased from American Type Culture Collection as a freeze-dried culture. Revived freeze-dried culture was maintained as spores in bacterial preservation beads (Protect, UK) and stored at −20°C (Long et al., 1996a). Lipozyme IM60 (*R. miehei* lipase) was obtained in the immobilised form (granule size: 0.2–0.6 mm, moisture content at 2–3%) from Novo Nordisk Industries (Copenhagen, Denmark). PKO and AMF were donated by Cargill Specialty Oils and Fats Sdn Bhd, Malaysia and purchased from Promac Sdn Bhd, Port Klang, Malaysia, respectively, while palm olein (PO) was obtained from a local supermarket. All other chemicals and reagents were either of analytical or high-performance liquid chromatography (HPLC) grade.

2.2. Preparation of spore suspension

A bead containing *R. miehei* was cultured on Dextrose-Agar (PDA) and incubated at 37°C for 7 days to obtain the vegetative spores. *R. miehei* spore suspension was prepared according to Long, Ghazali, Bucke, Ampon and Ariff (1996). The spores were harvested by washing the plate several times with sterile water to yield a final volume of 100 ml. Dilution in sterile water was carried out to obtain an average spore density of 10^5 spores/ml, determined using a hymocytometer. The spore suspension was then ultrasonicated for 2 min (Ultrasons-220v; Barcelona, Espana) to dissociate the spore agglomerates and to obtain a homogeneous spore suspension. The suspension was stored at 0–4°C for up to 30 days; storage beyond 30 days has shown a slight reduction in spore viability.

2.3. Preparation of mycelium-bound R. miehei lipase

MBL from *R. miehei*, which was used as the lipase source, was produced via submerged culture using conditions modified from Marek and Bednarski (1996). The medium contained (w/v) 0.2% $NaNO_3$, 0.05% $MgSO_4$, 0.05% KCl, 0.1% K_2HPO_4, 0.0001% $FeSO_4$, 0.1% D-glucose and 0.1% yeast extract. The medium pH was adjusted to 6.0 using 4 N HCl and it was sterilised at 121°C for 20 min. One millilitre of the *R. miehei* spore suspension was added to 100 ml liquid culture medium with an addition of 1% (w/v) sterilised PO. The culture was incubated at 37°C and shaken at 130 rpm in an incubator-shaker (Innova 4000, New Brunswick Scientific, USA) for 3 days. At the end of the cultivation period, mycelia were harvested using a Buchner funnel fitted with a Whatman No. 4 filter paper. The mycelia were then washed by filtration with 100 ml distilled water, followed by 100 ml hexane to remove oil residue. The mycelia were lyophilised using an Alpha 1-4 Christ LDC-1 freeze-dryer (B. Braun Gefriertrocknungsanlagen GmbH, Melsungen, Germany) at −43°C for 3 h and kept at 4°C prior to use. The dry mycelia were used as the source of MBL in the following experiments.

2.4. Transesterification

The reaction was carried out as previously reported (Ghazali, Maisarah et al., 1995; Ghazali, Hamidah & Che Man, 1995; Lai, Ghazali & Chong, 1998a–c) with slight modification in the amount of lipase added. Ten grams of PKO-AMF mixtures (at ratios 100:0, 70:30, 60:40, 50:50 and 0:100) in the liquid state were placed in a 50 ml conical flask and allowed to equilibrate to the reaction temperature (60°C) in an orbital shaker for 30 min. MBL (0.2 g) or Lipozyme IM60 (0.2 g) was added to the oil mixture and reacted at 200 rpm for 24 h. After the reaction, the reaction mixture was filtered using a Whatman No. 4 filter paper to remove the lipase from the oil mixture.

The TG compositions of reacted and unreacted PKO-AMF mixtures were analyzed by HPLC (Shimadzu Co., Kyoto, Japan) using a commercially packed RP-18 column (250×4 mm) with 5-μm particle size (E. Merck, Darmstadt, Germany; Swe, Che Man & Ghazali, 1994; Ghazali, Maisarah et al., 1995). Samples (0.1 g) were dissolved in 2 ml of chloroform in a HPLC vial and sealed with a crimp seal. Ten microlitres of the sample

were injected using an auto-injector and eluted with acetone/acetonitrile (60:40) at a flow rate of 1 ml/min at 30°C.

The percentage of TG remaining (%TGR) after 24 h reaction was calculated as the total TG concentration of the reacted sample compared to the unreacted sample (Ghazali, Maisarah et al., 1995). The degree of TE (%TE) is the change in percentages of peak areas of TG (after 5 min) and showed an increased after the course of reaction (TGI_t) with respect to the value at the start of the reaction (TGI_0) minus 100% (Ghazali, Hamidah & Che Man, 1995).

2.5. Hydrolytic activity

The amount of FFA liberated during the TE reaction was determined using the method modified from Cocks and van Rede (1966). At the end of the reaction, 25 ml of ethanol/acetone (1:1) was added to 1 g of the transesterified oil blend. The mixture was then titrated with 0.01 N NaOH to a phenolphthalein endpoint. The degree of hydrolysis is expressed as the percentage of FFA liberated and was corrected for the presence of the acids in the control (unreacted sample). Triplicate runs were carried out for each treatment.

2.6. Slip melting point (SMP)

SMP were determined according to AOCS Method Cc. 3.25(1a). Capillary tubes filled with 1 cm high column of fat were chilled at 10±1°C for 16 h before being immersed in a beaker of cold distilled water. The water bath was stirred and heated, and the temperature was recorded when the column of fat in the capillary tubes rose in the tube.

2.7. Solid fat content (SFC)

SFC for TE fat was determined with a Bruker wide-line pulse nuclear magnetic resonance spectrometer (Karlsruhe, Germany) using a direct measurement method. Each sample was tempered at 70°C for 30 min and then chilled at 0°C for 90 min; following chilling, the sample was kept at the desired temperature for 30 min before measurement was recorded. SFC was measured within the temperature range of 0–35°C (Ghazali, Maisarah et al., 1995; Lai et al., 1998a).

2.8. Thermal properties by differential scanning calorimetry (DSC) analysis

TE fat, weighing from 3–15 mg, was sealed in an aluminium pan and placed in the Perkin Elmer DSC-7 (Norwalk, CT) for DSC analysis. The parameters for analysis were adapted from Lai et al. (1998a) with slight modification to suit the melting range of the sample. The sample was heated to 70°C and tempered for 15 min to ensure all crystals were melted. The cooling rate was set at 10°C/min to cool the sample from 70°C to −30°C. Then, the sample was heated to 70°C at 5°C/min. An empty pan was used as the control pan for calibration. The heating thermograms were analyzed.

3. Results and discussion

The total TG concentration present in the lipase-reacted oil mixture in this study was calculated by subtracting the concentration of glycerides eluted before 5 min from the total concentration of all glycerides recorded on an HPLC chromatogram. This is to take into account of the triglycerides of PKO, which eluted as early as 5 min in the chromatogram.

The degree of TE varies greatly depending on the origins of the lipases used. Lai et al. (1998a) has demonstrated that the %TE varies from 4.1% (*R. niveus*) to 77.3% (*Pseudomonas*) when different lipases were used to catalyse the TE of palm stearin and sunflower oil. The rates of transesterification were also shown to differ greatly when different lipases were used (Ghazali, Hamidah & Che Man, 1995), in which case the highest rate of transesterification of palm olein was exhibited by *Pseudomonas* lipase, followed by *R. miehei* and *A. niger* lipases. In this study, %TE and the amount of FFA (μmol) liberated by the transesterified and unmodified PKO:AMF mixture are shown in Table 1. At different ratios of PKO:AMF, %TE were found to range from 22 to 34% when MBL was used, while, the commercial lipase, Lipozyme IM60, showed higher %TE which ranged from 43 to 51%. The %TE for *R. miehei* (Lipozyme IM60) demonstrated by Lai et al. was 32.7%. These results show that both MBL and Lipozyme IM60 catalysed TE reaction at all ratios of PKO:AMF.

As reported by Ghazali, Hamidah and Che Man (1995), an increase in the concentration of a specific TG present in the initial sample and/or the formation of a new TG shown in a HPLC chromatogram would indicate that TE had taken place. The changes in TG profile has been demonstrated by a number of researchers following a lipase-catalysed TE (Foglia, Petruso & Feairheller, 1993; Forssell et al., 1992; Ghazali, Hamidah & Che Man, 1995; Ghazali, Maisarah et al., 1995; Lai et al., 1998a,c). Changes in the TG profile of PKO:AMF (70:30) before and after TE reaction are shown in Fig. 1. The change in TG profile is less obvious when the amount of AMF used in the mixture is raised. As can be seen, the concentrations of two TG (LaPM and LaOM, where La, P, O and M represent lauric, palmitic, oleic and myristic acids, respectively) showed clear increases on transesterification. The increase indicates that TE

Table 1
Amount of FFA (in μmol) liberated and degree of transesterification (TE) of transesterified palm kernel olein:anhydrous milk fat (PKO:AMF) blends using mycelium-bound lipase (MBL) from *Rhizomucor meihei* and lipozyme IM60

PKO:AMF ratio	MBL *R. miehei*		Lipozyme IM60	
	μmol FFA[a]	Degree TE (%)[a]	μmol FFA[a]	Degree TE (%)[a]
100:0	189 ± 3.00	22 ± 2.50	207 ± 3.51	46 ± 3.37
70:30	190 ± 4.58	22 ± 1.00	206 ± 2.57	43 ± 4.36
60:40	130 ± 3.60	31 ± 2.64	205 ± 5.57	51 ± 3.21
50:50	174 ± 4.58	34 ± 2.84	186 ± 5.13	48 ± 5.57
0:100	165 ± 3.00	29 ± 2.08	208 ± 4.36	47 ± 4.93

[a] All values are means ($n = 3$).

has taken place and this occurred in samples catalysed by both MBL and Lipozyme IM60. The higher %TE achieved by Lipozyme IM60 (Table 1) corresponded to the relatively greater increase in peak areas (calculated as relative %TG in Table 2) shown by samples catalysed by lipozyme IM60.

Changes in the TG profiles of mixtures of oils after TE are usually accompanied by changes in the SMP and SFC of the oil blends (Laning, 1985; Foglia et al., 1993; Ghazali, Hamidah and Che Man, 1995; Lai et al., 1998a–c). The SMP Table and SFC -of the transesterified PKO:AMF mixtures are shown in Table 3 and Fig. 2, respectively. A slight reduction in the SMP was observed after TE with either MBL and Lipozyme IM60 as compared to the controls (Table 3). A reduction in SFC of PKO:AMF blends following TE was also demonstrated throughout the temperature range studied, i.e. 0–35°C. Fig. 1 indicates that, in all unmodified samples, the steepest drop in SFC was observed from 10–20°C. The drop was also evident in oil mixtures catalysed by both MBL and Lipozyme IM60 at various oil ratios. Generally, the SFC for transesterified oil mixture were lower than the control (Fig. 1), which correspond to the findings of Foglia et al. (1993), Ghazali, Hamidah and Che Man (1995b) and Lai et al. (1998a–c).

The sharpest drop in the SFC of mixtures transesterified with MBL was between 10 and 15°C. As the proportion of PKO increased, the drop in SFC became more prominent than when higher proportions of AMF were used, especially at temperatures ranging between 5 and 15°C. Since there is a close relationship between the amount of high melting fraction (HMF) and SFC (Timms, 1980), the reduction in SFC in the transesterified oil mixtures could be due to the rearrangement of fatty acids after TE to form a medium melting fraction (MMF) or low melting fraction (LMF). At 25°C, the PKO:AMF mixture at 70:30 was completely melted (SFC = 0%). This is suitable for the,fat phase used in ice cream formulations. All other mixtures have more than

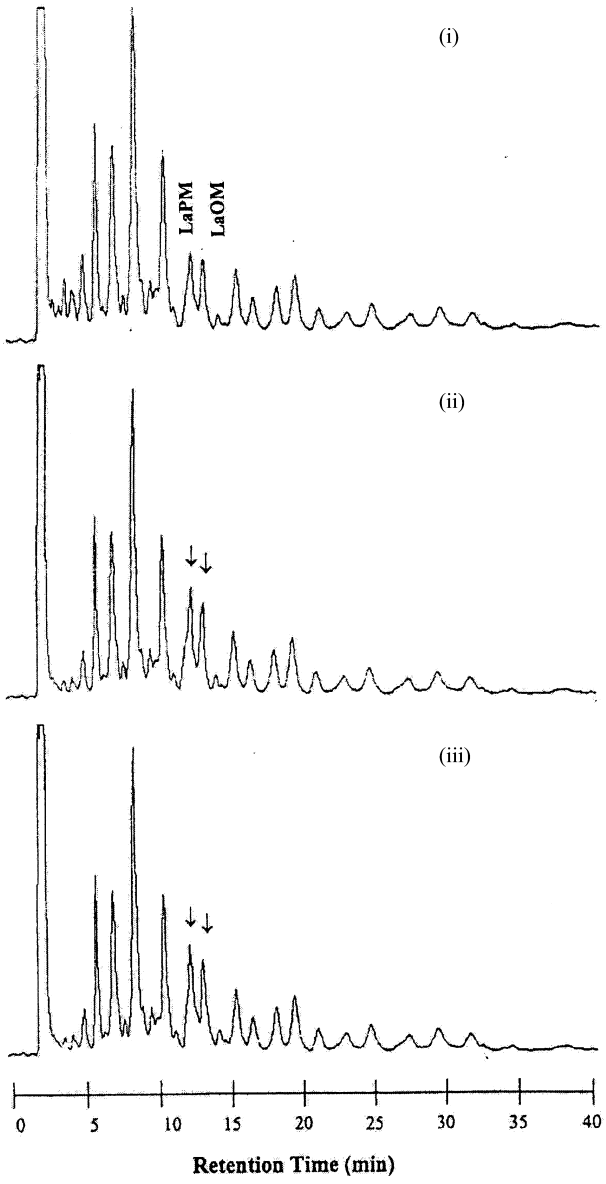

Fig. 1. Triglyceride profiles of palm kernel olein:anhydrous milk fat (PKO:AMF) mixture at 70:30 ratio before (a) and after TE with mycelium-bound lipases (b) and lipozyme IM60 (c). Triglycerides represented by arrows indicate increase in concentration.

Table 2
Relative % triglyceride (%G) for LaPM and LaOM of palm kernel olein:anhydrous milk fat (PKO:AMF) blends before (control) and after transesterification (TE) with mycelium-bound lipase (MBL) from *Rhizomucor miehei* and lipozyme IM60

% PKO:AMF (w/w)	Relative % TG					
	Control		MBL *R. miehei*		Lipozyme IM60	
	LaPM	LaOM	LaPM	LaOM	LaPM	LaOM
100:0	10.1	7.49	12.6	10.0	13.3	9.81
70:30	7.74	5.32	9.97	6.95	13.7	10.1
60:40	4.69	3.12	7.19	4.95	11.0	7.51
50:50	5.38	3.84	6.81	4.62	11.2	7.60
0:100	6.24	3.23	5.61	1.58	5.42	0.66

Table 3
Slip melting points of palm kernel olein:anhydrous milk fat (PKO:AMF) blends before (control) and after TE with mycelium-bound lipase (MBL) from *Rhizomucor miehei* and lipozyme IM60

% PKO:AMF (w/w)	Slip melting points (°C)[a]		
	Control	MBL *R. miehei*	Lipozyme IM60
100:0	23.0 ± 0.50	21.5 ± 0.50	21.5 ± 0.50
70:30	25.0 ± 0.29	23.0 ± 0.29	23.0 ± 0.29
60:40	26.0 ± 0.29	24.0 ± 0.50	24.5 ± 0.50
50:50	26.5 ± 0.00	25.5 ± 0.50	25.5 ± 0.87
0:100	32.0 ± 0.58	31.0 ± 0.29	30.0 ± 0.29

[a] All values are means ($n = 3$).

2% residual SFC at 25°C. As for the oil mixture catalysed by Lipozyme IM60, all transesterified blends exhibited lower SFC than that of the control mixture.

Fig. 3a–c shows the DSC thermograms for transesterified and unmodified mixtures of PKO:AMF at various ratios catalysed by MBL and Lipozyme IM60. In Fig. 3a, three endotherms, namely peaks A, B and C were observed in PKO before TE. Following TE, peak B became more prominent, and the overall thermogram shifted slightly to the left. The peak temperature for peak C has shifted from 21.5 to 17.5°C [Fig. 3a(i)] and 17.2°C [Fig. 3a(ii)], respectively, which indicated the formation of lower melting triglycerides. This observation

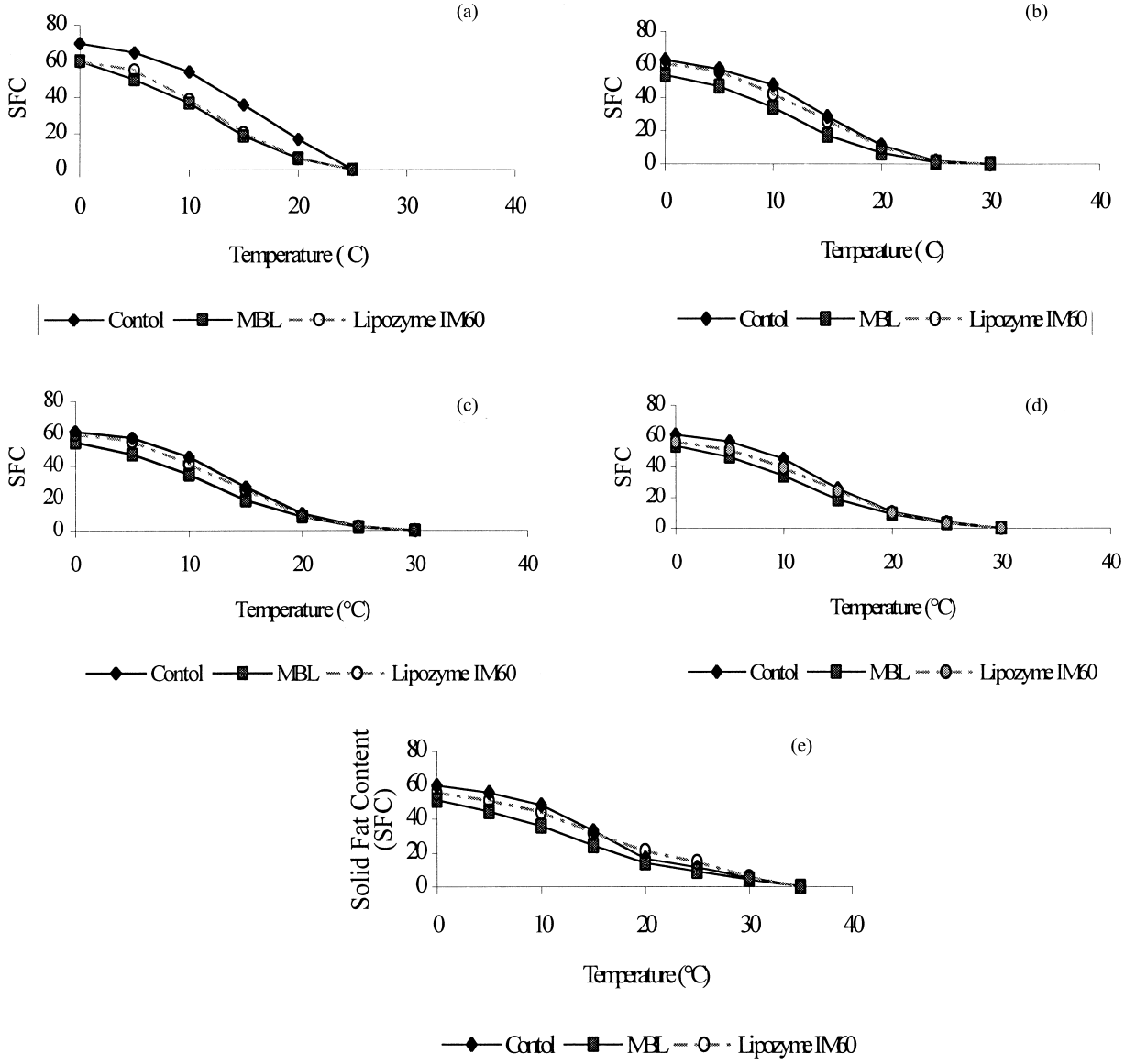

Fig. 2. Solid fat content (SFC) of palm kernel olein:anhydrous milk fat (PKO:AMF) mixture before (control) and after transesterification with mycelium-bound lipase and lipozyme IM60. [PKO:AMF (w/w); (a) 100:0, (b) 70:30, (c) 60:40, (d) 50:50, (e) 0:100.

is consistent with the slight lowering of SMP obtained for both transesterified PKO samples.

Fig. 3b shows the DSC thermograms for PKO:AMF mixtures at 70:30. The thermogram show four endotherms (peak A, B, C and D) and one exotherm (X_A) at approximately −23°C in all the unmodified oil mixture (controls). Following TE, a new endotherm, E, appeared [Fig. 3b(iii)]. Generally, the exotherm, X_A has become less prominent, while endotherm E became more prominent. As discussed previously by Lai et al.

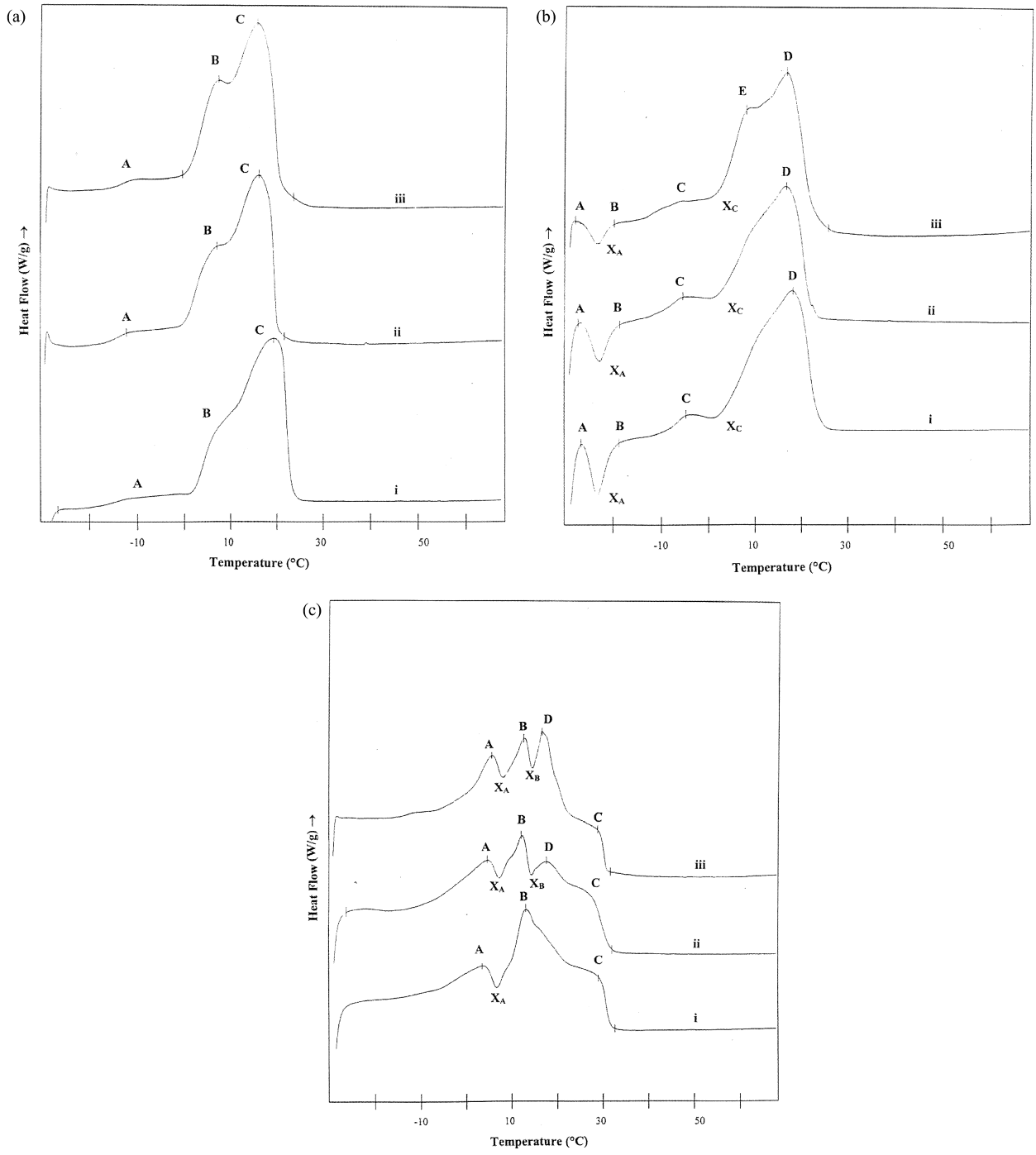

Fig. 3. Differential scanning calorimetry (DSC) heating thermograms of palm kernel olein (i) before and (ii) after TE with MBL and (iii) lipozyme IM60. Pretreatment: cooled from 70°C to −30°C at 10°C/min. Samples were heated from −30°C to 70°C at 5°C/min. (b) DSC heating thermograms of palm kernel olein:anhydrous milk fat (PKO:AMF) mixture at 70:30 (i) before and (ii) after TE with MBL and (iii) lipozyme IM60. Pretreatment: cooled from 70°C to −30°C at 10°C/min. Samples were heated from −30°C to 70°C at 5°C/min. (c) DSC heating thermograms of anhydrous milk fat (i) before and (ii) after TE with MBL and (iii) lipozyme IM60. Pretreatment: cooled from 70°C to −30°C at 10°C/min. Samples were heated from −30°C to 70°C at 5°C/min.

(1998a,b), the shallowing of exotherm, X_A and lowering of endotherm A may be explained by the transformation of low melting crystalline material, which is responsible for peak A, into other forms of triglycerides through simple arrangement of fatty acids. As in PKO samples, all transesterified PKO:AMF thermograms indicated a slight left shift on the overall peaks. This evidence suggests that the overall triglyceride composition after TE by both MBL and Lipozyme IM60 gives slightly lower melting properties. These observations were also supported by the slight lowering of SMP for all the transesterified blends. The shifting of the DSC thermograms to a lower melting region, accompanied by the lowering of SMP, was also reported by Lai et al. (1998a–c).

DSC scans for AMF are shown in Fig. 3c. The control sample of AMF has three endotherms (peaks A, B and C) and one exotherm, X_A, which are typical in the melting curve of milk fat (Deffense, 1993; Timms, 1980). Milk fat has been classified into three fractions, namely HMF, MMF and LMF (Deffense). The three endotherms correspond to the respective fractions, where LMF, MMF and HMF melt at −25–10°C, 10–19°C, and 19–34°C, respectively (Deffense). Exotherm, X_A, indicated that the LMF crystals had probably undergone transformation to form MMF crystals. After TE by MBL and Lipozyme IM60, a new peak (D) and a new exotherm, X_B were observed. The rearrangement of fatty acids, via TE, resulted in the melting of crystal structure responsible for peak B and transformed into a crystal structure with higher melting TG in peak D. Lowering of peak C and a left shift of peak C indicate a decrease of HMF in the transesterified milk fat. This observation was also demonstrated by Oba and Witholt (1994). The TE by both MBL and Lipozyme have resulted in the rearrangement of fatty acids, which lead to the variation in the DSC thermograms.

In the study of the polymorphic patterns by Woodrow and deMan (1968), bands at specific short spacing in an X-ray diffraction (X′RD) were identified as different forms of polymorphic crystals. Three basic polymorphic structures have been identified, namely alpha (α), beta (β) and beta-prime (β′) forms. In the X′RD of milk fat, two strong bands at short spacings of 4.17 Å and 3.78 Å indicated the presence of the β′ form while a spacing at 4.64 Å represents the presence of β form as has been previously demonstrated (Woodrow & deMan). The polymorphic structures of the crystals in different PKO:AMF blends before and after transesterification are shown in Table 4. Generally, all mixtures showed weak to medium diffraction at short spacing, 4.41, 4.24, 4.10 and 3.8 Å, which indicated that the dominant crystals were of β′ form. The exception is milk fat (AMF control), which has additional diffraction at the short spacing of 4.64 Å, indicating a mixture of β and β′ crystals. This observation agrees with Timms' (1980) findings, which demonstrated that milk fat is predominantly β′ crystals with little of the β form.

Table 4
Polymorphic forms of PKO:AMF blends before (control) and after transesterification with mycelium-bound lipase (MBL) from *Rhizomucor miehei* and lipozyme IM60

% PKO:AMF (w/w)	Polymorphic forms		
	Control	MBL *R. miehei*	Lipozyme
100:0	β′	β′	β′
70:30	β′	β′	β′
60:40	β′	β′	β′
50:50	β′	β′	β′
0:100	β′ ⩾ β	β′	β′

Results obtained in this study indicate that lipase-catalysed TE between PKO and AMF can produce a novel fat which has the potential to be used in ice cream formulation. The transesterified PKO:AMF (70:30) had lower melting point and SFC than the unmodified sample, which allows the mixture to melt completely at 25°C. This melting characteristic is desirable for the fat phase used for ice cream production to ensure a complete melt when the ice cream is consumed. This study also showed that MBL is capable of catalysing a TE reaction similarly to Lipozyme IM60.

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