Acidolysis of Several Vegetable Oils by Mycelium-Bound Lipase of *Aspergillus flavus* Link

K. Long[a], H.M. Ghazali[a,*], A. Ariff[a], and C. Bucke[b]

[a]Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor D.E., 43400 Malaysia, and [b]School of Biological and Health Sciences, University of Westminster, WIM 8JS London, United Kingdom

ABSTRACT: The ability of mycelium-bound lipase of a locally isolated *Aspergillus flavus* to modify the triglyceride structure of vegetables oils was studied. The catalysis involved the acidolysis of vegetable oils, such as palm olein, coconut oil, cottonseed oil, rapeseed oil, corn oil and soybean oil, with selected fatty acids (FA). The reactions were followed against time, and the percentages of FA incorporated were determined by gas chromatography. Percentage of FA incorporated after 20-h reaction was in the range of 13 to 18%. Reaction between cottonseed oil with lauric acid gave the highest percentage of incorporation (18%), followed by soybean oil with lauric acid (16%) and coconut oil with oleic acid (16%). The results indicated that the hydrolytic affinity of *A. flavus* lipase demonstrates an acyl group specificity toward short-chain FA (C_8–C_{10}). Changes in triglyceride profiles of each oil were also monitored by reverse-phase high-pressure liquid chromatography. In all products, there were increases in the concentrations of several existing triglycerides and formation of new triglycerides. The melting points of all acidolyzed vegetable oils were determined by differential scanning calorimetry, and significant changes in melting profiles were noted.
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KEY WORDS: Acidolysis, *Aspergillus flavus*, fatty acid incorporation, melting point, mycelium-bound lipase, triglyceride profiles.

In recent years, the use of lipases as biocatalysts for interesterification has become of great industrial interest for the production of useful triglyceride (TG) mixtures. This is mainly because of the specificity of these enzymes with respect to glyceride positions and fatty acid types. The high temperatures (220–250°C) used in current chemical technologies for glycerolysis and hydrolysis of fats and oils cause discoloration and some degradation of fatty acids (FA) (1,2). This causes a loss of product quality and yield, particularly for highly unsaturated and hydroxyl FA. Such disadvantages may be overcome by using lipases, which react under mild conditions and allow production of desired products without leaving harmful residues. However, in spite of their apparent superiority, the commercial exploitation of lipases in this area is disappointing at present.

*To whom correspondence should be addressed.
E-mail: hasanah@fsb.upm.edu.my.

The major obstacle to the use of microbial lipases for the manufacture of bulk products is cost of the enzymes (3,4). Accordingly, research has been developed along many different fronts with the aim of reducing the operational cost of enzymatic processes. One of the most promising techniques that allow the enzyme to be used directly without isolation, purification and immobilization is as naturally mycelium-bound lipase. Although this technique offers such great advantages, a survey of the literature showed that little work has been carried out on mycelium-bound lipase compared with man-made immobilized lipases. Furthermore, studies with mycelium-bound lipase as a catalyst in synthetic reactions were limited to only a few microorganisms, e.g., *Rhizopus arrhizus* (5–10), *R. chinesis* (11,12), *Aspergillus niger* (13), and *Penicillium cyclopium* (10).

In an effort to find alternative sources of mycelium-bound lipase, we isolated a local strain of *A. flavus* (14) that was subsequently shown to produce such a lipase during the growth of the fungus (15). Despite a vast amount of information on *A. flavus* and its ability to hydrolyze and utilize oils (16–20), there is little information on the properties of its lipase and application in synthesis reactions. Our studies thus concentrate on the ability of the mycelium-bound lipase of *A. flavus* to catalyze the modification of the FA composition of vegetable oils through acidolysis. In this paper, the incorporation of various FA into vegetable oils was investigated. Changes in TG profiles of each vegetable oil and melting points after modification are presented.

EXPERIMENTAL PROCEDURES

Materials. Soybean oil, corn oil, and palm olein were purchased from a local supermarket. Coconut oil was purchased from Lee Oil Mills Sdn. Bhd. Kelang (Selangor, Malaysia). Refined, bleached, and deodorized rapeseed and cottonseed oils were kindly donated by Lam Soon (M) Bhd. Petaling Jaya (Selangor, Malaysia). Reference triglycerides, diglycerides, and FA (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO).

Source of lipase. The source of lipase was from an organism that was locally isolated from copra meal and identified as *A. flavus* Link (IMI 361648) by the International Mycological Institute, United Kingdom.

Mycelium-bound lipase preparation. The liquid culture conditions used for growing *A. flavus* were as described by Long *et al.* (15). The medium contained (wt/vol) 2% yeast extract, 2% palm olein, 0.3% $(NH_4)_2SO_4$, 0.02% $CaCl_2$, 0.2% KH_2PO_4, 0.1% glucose, and 0.037% EDTA-Na. The medium was adjusted to pH 6.0 with 0.1 M NaOH and sterilized at 121˚C for 15 min. Spore suspension (4 mL) with an average of 10^7 spores/mL, obtained from 7-day-old culture of *A. flavus,* was added into a 1-L flask that contained 200 mL medium. Cultivation was carried out at 30˚C and 120 rev/min (20-mm displacement) for 72 h. Mycelia were harvested and washed with distilled water, followed by defatting with 100 mL *n*-hexane (15). The defatted mycelia were then freeze-dried for 3 h, homogenized in a blender for 90 s, and stored in a desiccator with dehydrated silica gel before use.

Acidolysis reaction. The acidolysis reaction was initiated by adding 0.2 g dried, homogenized mycelium-bound lipase into a 100-mL flask that contained 3.0 g vegetable oil and 1.0 g lauric acid (unless otherwise stated), dissolved in 30 mL *n*-hexane. The reaction mixture was then agitated in an orbital shaker at 200 rpm and 40˚C. Each reaction was carried out in duplicate. At various times during incubation, 8 mL of the reaction mixture was withdrawn for analysis. The percentages of lauric acid incorporated into the oils were followed by gas chromatography after FA were removed from reaction mixtures. The TG profiles of reacted samples after 20-h reaction were analyzed by reverse-phase high-performance liquid chromatography (HPLC) without removal of FA.

Removal of free FA from reaction mixtures. Free FA were removed by the method described by Foglia *et al*. (21) with slight modification. Samples (6 mL) were placed in 250-mL conical flasks, and 20 mL of acetone/ethanol (1:1, vol/vol) was added. The mixture was titrated with 0.1 M NaOH to a phenolphthalein endpoint. The titrated samples were diluted with water (5 mL) and transferred into a 100-mL separating funnel. After shaking and standing for several minutes, the bottom aqueous layer was discarded, and the top layer, which was the organic phase, and which contained mono-, di- and

TABLE 1
Changes in the Fatty Acid Composition of Acidolyzed Vegetable Oils

	Fatty acid (%)										
Substrate	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	SFA[a]	USFA[b]
Soybean oil + lauric acid											
0 h	—	—	—	—	11.32	3.88	20.03	59.22	5.55	15.20	84.80
4 h	—	—	4.15	—	11.04	3.66	18.53	57.31	5.30	18.85	81.15
10 h	—	—	7.43	—	9.69	3.34	19.27	55.37	4.90	20.46	79.54
20 h	—	—	16.14	—	9.40	3.10	17.22	49.74	4.40	28.64	71.36
Cottonseed oil + lauric acid											
0 h	—	—	—	0.97	25.06	2.99	21.73	49.25	—	29.02	70.98
4 h	—	—	3.36	0.80	24.45	2.40	21.55	47.44	—	31.01	68.99
10 h	—	—	9.45	0.98	24.90	3.05	21.30	40.32	—	38.38	61.62
20 h	—	—	18.29	0.97	24.89	2.87	21.90	31.08	—	47.02	52.98
Rapeseed oil + lauric acid											
0 h	—	—	—	—	4.95	1.54	63.52	20.93	9.06	6.49	93.51
4 h	—	—	3.18	—	4.47	1.59	61.64	20.35	8.77	9.24	90.76
10 h	—	—	6.94	—	4.23	1.41	58.91	20.03	8.48	12.58	87.42
20 h	—	—	13.55	—	4.08	1.26	54.90	18.46	7.75	18.89	81.11
Corn oil + lauric acid											
0 h	—	—	—	—	11.16	2.76	32.73	52.53	0.80	13.92	86.06
4 h	—	—	3.84	—	10.78	2.62	31.25	50.72	0.79	17.24	82.76
10 h	—	—	7.69	—	9.68	2.41	30.06	49.59	0.66	19.78	80.31
20 h	—	—	14.23	—	9.31	2.20	28.34	45.24	0.68	25.74	74.26
Palm olein + lauric acid											
0 h	—	—	0.66	1.05	37.02	3.83	46.54	10.90	—	42.56	57.44
4 h	—	—	5.00	1.14	36.02	3.48	43.80	10.56	—	45.64	54.36
10 h	—	—	8.54	1.07	33.93	3.41	42.66	10.39	—	46.95	53.05
20 h	—	—	13.83	1.03	31.15	3.15	40.99	9.85	—	49.16	50.83
Coconut oil + oleic acid											
0 h	9.89	6.91	47.01	17.47	8.70	2.42	6.44	1.16	—	92.40	7.60
4 h	6.61	6.30	47.53	17.13	8.60	2.04	10.54	1.25	—	88.21	11.79
10 h	4.84	4.56	44.19	16.80	8.53	2.53	17.26	1.29	—	81.45	18.55
20 h	4.38	4.35	42.81	15.56	7.30	2.16	22.18	1.26	—	76.56	23.44
Coconut oil + palmitic acid											
0 h	9.33	7.31	47.03	17.97	8.51	2.38	6.26	1.21	—	92.53	7.47
4 h	8.33	6.15	46.50	17.69	12.20	1.77	6.14	1.22	—	92.64	7.36
10 h	6.01	5.18	45.53	16.23	17.36	2.08	6.19	1.42	—	92.39	7.61
20 h	4.28	4.25	42.83	15.29	23.74	2.21	6.10	1.30	—	92.60	7.40

[a]SFA, saturated fatty acid.
[b]USFA, unsaturated fatty acid; —, not detected. The values shown are the means of two replications and have been corrected to 100% and are relative to the total concentration of fatty acids in the reaction mixture. Each sample was analyzed after the removal of free fatty acid.

triglycerides, was collected. The organic phase was transferred into a McCartney bottle and dried overnight in an oven at 60°C. The absence of FA was confirmed by titration with 0.05 M NaOH and also by thin-layer chromatography with a solvent system of petroleum ether, diethyl ether, and formic acid (210:90:0.4), and viewed in iodine vapor.

Determination of FA incorporated. The FA composition of unreacted and enzyme-reacted oils was determined after conversion to fatty acid methyl esters (FAME) by sodium methoxide in anhydrous methanol (22). FAME were analyzed by injection of 0.3 µL of a FA-free sample into a gas chromatograph, GC-17AC (Shimadzu Corporation, Kyoto, Japan), equipped with a flame-ionization detector. A polar capillary column BPX70 (0.32-mm internal diameter, 30-m length and 0.25-µm film thickness; SGE Australia Pty. Ltd., Ringwood, Australia) was used to separate the FAME. The flow rate of hydrogen carrier gas was 50 mL/min, and the injector and detector temperatures were maintained at 240°C, while the column temperature was at 180°C. When analyzing FA composition of coconut oil, the temperature of the injector and detector was reduced to 220°C, and the column temperature to 150°C. A split injection ratio (1:68) was used. The FA composition was expressed in relative percentages.

Analysis of TG. The TG compositions of oils with and without enzyme catalysis after 20 h were analyzed by nonaqueous reverse-phase HPLC in a Shimadzu liquid chromatograph LC-10AD and SLC-10A, equipped with an auto-injector and a Shimadzu C-R4AX integrator. A commercially packed RP-18 column (250 × 4 mm) with 5-µm particle size (E. Merck, Darmstadt, Germany), which was placed in an oven at 29°C, was used to separate the TG. TG was eluted from the column with an acetone/acetonitrile (63.5:36.5) mixture at a flow rate of 1 mL/min (23) and detected with a refractive-index detector (RID-6A; Shimadzu Corporation). The injection volume was 10 µL per injection.

Determination of melting point. Melting points of reaction products and controls were analyzed by a differential scanning calorimeter (DSC 7; Perkin-Elmer, Norwalk, CT). The instrument was calibrated with indium and *n*-decane. Samples, from which FA had been removed, were weighed into aluminum pans (ranging from *ca.* 9 to 11 mg), and lids were crimped into place. The sample and reference (empty) pan were placed in the calorimeter at room temperature, while the cell block of the DSC was cooling to −55°C and flushed with nitrogen. Samples were subjected to the following temperature program: −55°C isotherm for 10 min and heating from−55 to 50°C. For coconut oil and palm olein, the heating temperature program was adjusted to range from −50 to 75°C at 10°C/min. The melting points of vegetable oil, expressed as X_1 (start melting), X_2 (totally melted) and peak temperature, are reported based on the means of triplicate runs. The values for X_1 and X_2 were obtained from the intersection of the tangents to the slope against the base line.

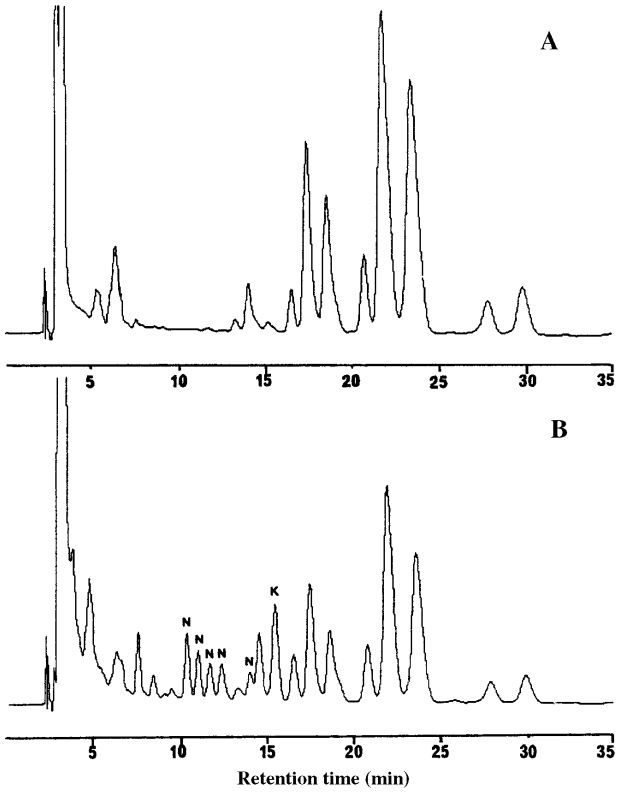

FIG. 1. Triglyceride profiles of palm olein reacted with lauric acid: (A) control, (B) acidolyzed product. N—new peak; K—peak area increased.

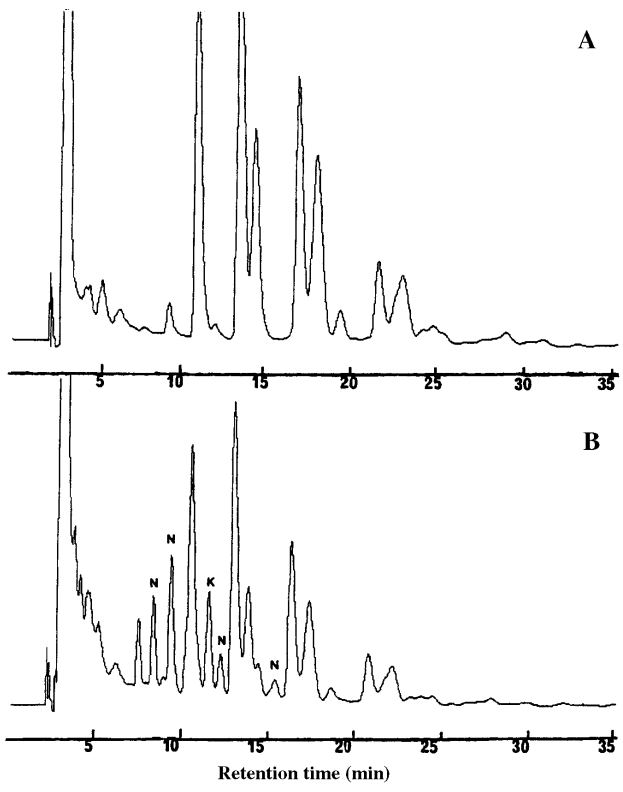

FIG. 2. Triglyceride profiles of corn oil reacted with lauric acid: (A) control, (B) acidolyzed product. N—new peak; K—peak area increased.

TABLE 2
Melting Properties of Acidolyzed Compared with Nonacidolyzed Vegetable Oils

Vegetable oil	Temperature (°C)				
	X_1	X_2	Peak$_1$	Peak$_2$	Peak$_3$
Soybean oil	−30.80	−1.40	−25.40[b]	−8.80	—
Acidolyzed soybean oil[a]	−32.80	16.00	−20.00[b]	1.20	10.40
Cottonseed oil	−24.00	1.20	−7.00	—	
Acidolyzed cottonseed oil[a]	−26.80	18.20	−14.80[b]	13.20	—
Rapeseed oil	−34.80	−9.20	−22.80[b]	−16.60	—
Acidolyzed rapeseed oil[a]	−37.20	16.00	−21.60[b]	−5.20	8.40
Corn oil	−28.60	0.80	−23.80[b]	−17.20	—
Acidolyzed corn oil[a]	−29.20	18.60	−21.00[b]	−10.60	2.20
Palm olein	−25.00	15.67	4.83[b]	7.67	—
Acidolyzed palm olein[a]	−28.33	21.00	−17.50	4.83	9.50[b]
Coconut oil	7.68	31.03	24.67[b]	—	—
Acidolyzed coconut oil[c]	−8.18	26.67	−0.83	22.00[b]	—
Coconut oil	7.17	30.17	24.33[b]	—	—
Acidolyzed coconut oil[d]	−7.67	44.33	26.00[b]	37.50	—

[a]With lauric acid.
[b]Major peak; X_1 start melting, X_2 totally melted.
[c]With oleic acid.
[d]With palmitic acid.

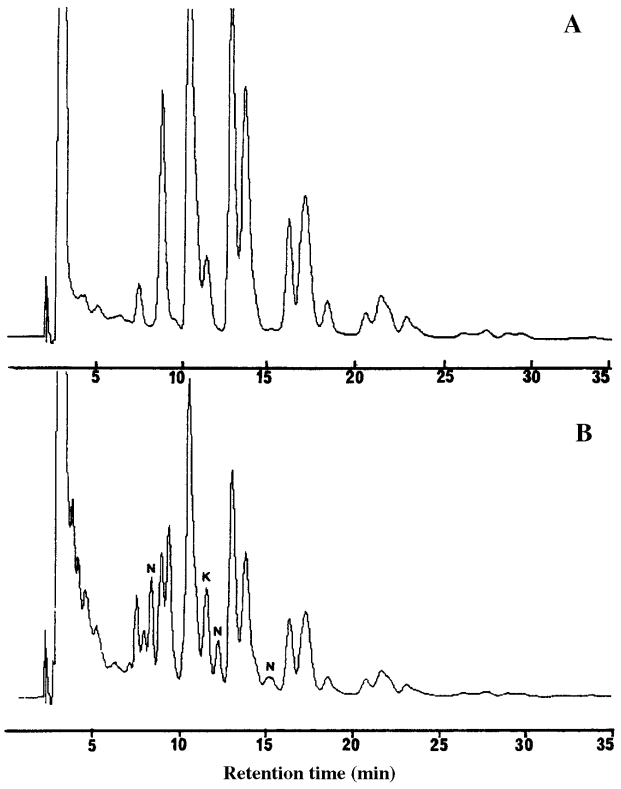

FIG. 3. Triglyceride profiles of soybean oil reacted with lauric acid: (A) control, (B) acidolyzed product N—new peak; K—peak area increased.

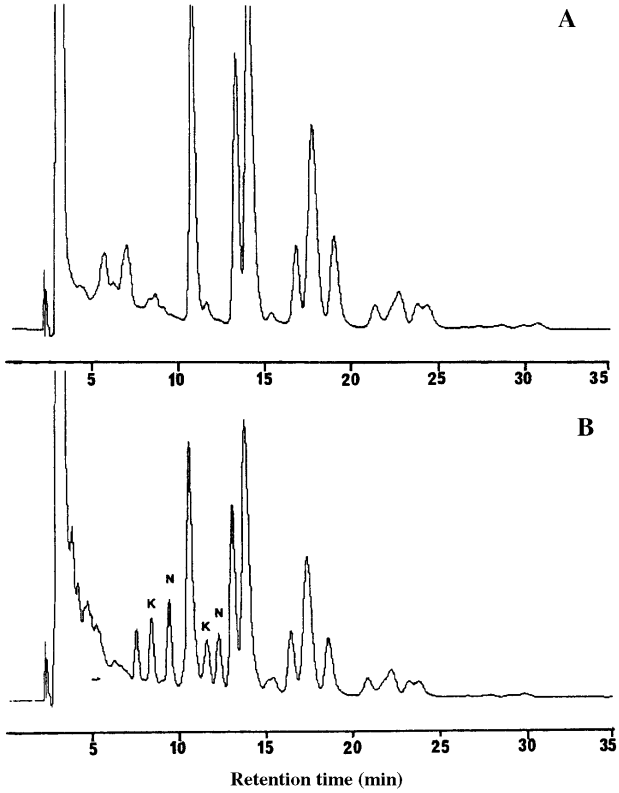

FIG. 4. Triglyceride profiles of cottonseed oil reacted with lauric acid: (A) control, (B) acidolyzed product. N—new peak; K—peak area increased.

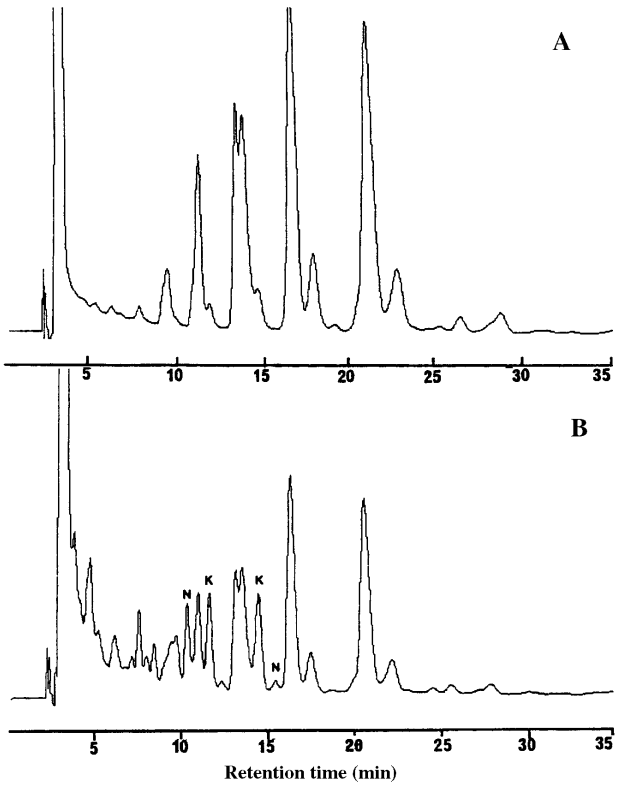

FIG. 5. Triglyceride profiles of rapeseed oil reacted with lauric acid: (A) control, (B) acidolyzed product. N—new peak; K—peak area increased.

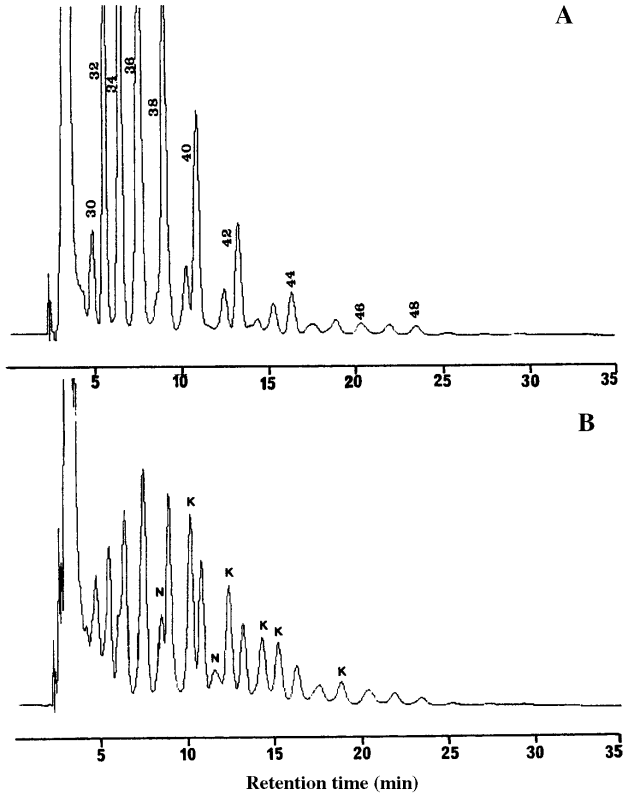

FIG. 6. Triglyceride profiles of coconut oil reacted with oleic acid: (A) control, (B) acidolyzed product. N—new peak; K—peak area increased.

RESULTS AND DISCUSSION

The ability of mycelium-bound lipase from a local strain of *A. flavus* to carry out acidolysis reactions was examined by reacting an oil and an FA in the presence of the enzyme. The percentages of FA incorporated during acidolysis reactions were followed as a function of time and are shown in Table 1. The reaction resulted in changes in the FA composition of each vegetable oil and makes the reaction products rather novel. The highest incorporation of FA occurred in the acidolysis of cottonseed with lauric acid (18% relative concentration), which after 20 h of reaction became the fourth major FA component of the reaction product. Lauric acid was not detected in the initial cottonseed oil. The successful incorporation of lauric acid by mycelium-bound lipase of *A. flavus* was also observed with rapeseed oil and corn oil, where 13 and 14% lauric acid were incorporated after 20 h reaction, respectively. After the same period, the relative concentration of lauric acid in palm olein increased by nearly 21 times. The changes in FA composition of coconut oil after reaction with oleic and palmitic acid were also noted after the acidolysis process.

Incorporation of exogenous FA has been reported previously by Gioielli *et al*. (24) who showed that the use of Lipozyme (1,3-specific lipase of *Rhizomucor miehei*) resulted in a 12% incorporation of palmitic acid into babassu fat (at a molar concentration 0.5:1). However, the incorporation did not alter the melting and softening points as compared to the unreacted fat. Incorporation of oleic acid into vegetable oils was also reported by Sridhar *et al*. (25), with the aim of reducing oxidative deterioration caused by high amounts of polyunsaturated fatty acids (PUFA) in oils such as peanut, sunflower, safflower, soybean, and linseed. They reported between 18 to 32% incorporation of oleic acid into the oils by using Lipozyme as the biocatalyst. The ability of the *Rhizopus delemar* lipase to catalyze acidolysis between olive oil and stearic acid was studied by Macrae (26). The percentage of stearic acid incorporated was 13.6%, with most of incorporation taking place in the 1- and 3-positions of the triacylglycerol. However, a lower percentage (0.9%) of incorporation of stearic acid into olive oil was reported when *Geotrichum candidum* lipase was used as the catalyst (26). The successful incorporation of stearic acid into palm oil fraction has been reported by many researchers (27–29). Reactions that involved 1,3-specific lipases were reported to give rise to formation of cocoa butter-like TG in the FA glyceride portion, namely distearoyl-oleoyl-glycerol, palmitoyl-oleoyl-stearoyl-glycerol, and dipalmitoyl-oleoyl-glycerol.

An examination of the FA composition of the products obtained after reaction of coconut oil with oleic or palmitic after 20 h (Table 1) revealed that the relative percentages of caprylic acid (C_8) and capric acid (C_{10}) were much less than

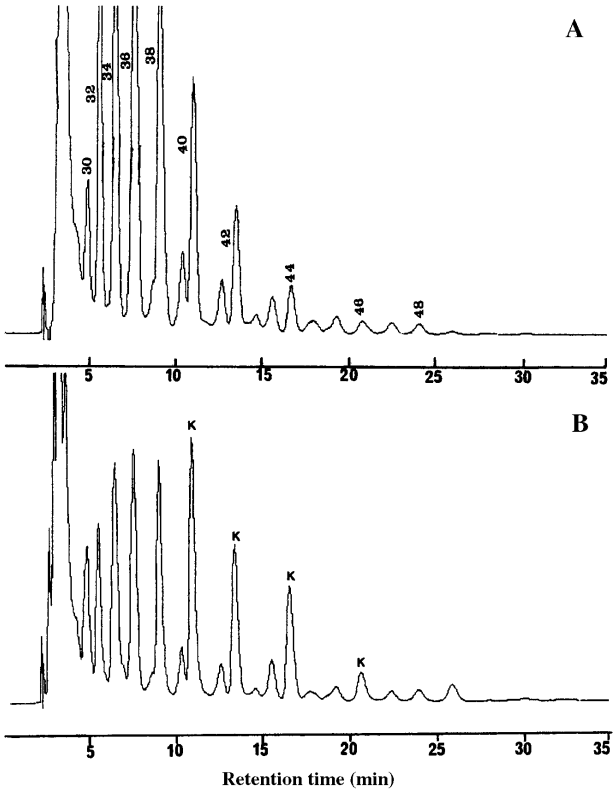

FIG. 7. Triglyceride profiles of coconut oil reacted with palmitic acid: (A) control, (B) acidolyzed product. N—new peak; K—peak area increased.

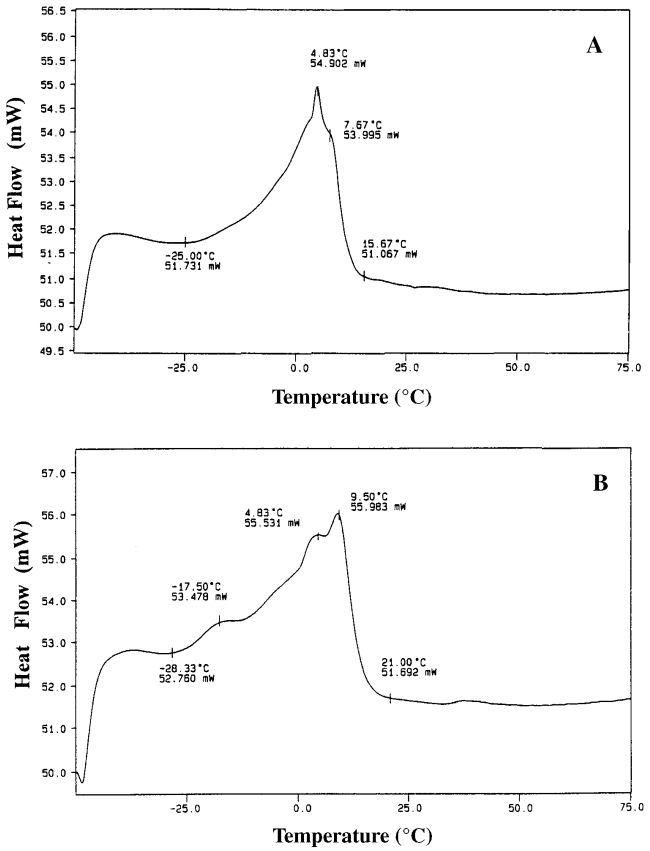

FIG. 8. Differential scanning calorimetry heating thermograms of: (A) palm olein, (B) acidolyzed palm olein with lauric acid.

those of the medium- and long-chain and unsaturated FA, suggesting that, under the microaqueous condition, *A. flavus* lipase might have preference for the hydrolysis of shorter-chain FA. Also, the relative concentrations of unsaturated FA in coconut oil reacted with oleic acid increased by 3.1 times, largely due to a marked increase in the FA in the final product and decreases in saturated FA. However, reaction with palmitic acid caused no significant change in the ratio of saturated to unsaturated FA (Table 1). The increase in palmitic acid is mainly offset by decreases in the relative concentration of saturated FA rather than the unsaturated FA, whose levels were relatively unaffected. The ratio of saturated and unsaturated FA of acidolyzed cottonseed oil with lauric acid was similar to palm olein (Table 1). However, the increase in relative concentration of lauric acid in the acidolyzed oil was caused by reduction in the levels of unsaturated FA.

Successful acidolysis of vegetables oils with FA by mycelium-bound lipase of *A. flavus* is also shown by changes in the TG profiles of the original oil (Figs. 1–7). Reactions resulted in either the formation of new TG or increases in the concentrations of several existing TG or both. A case in point is the reaction of palm olein with lauric acid, which resulted in the formation of five new TG peaks (Fig. 1). Four new TG peaks were obtained after acidolysis of corn oil with lauric acid (Fig. 2), three from soybean oil with lauric acid (Fig. 3), and two each from cottonseed oil (Fig. 4), rapeseed oil (Fig. 5) and coconut oil (Fig. 6), which were all reacted with lauric acid, except for the latter which was with oleic acid. However, no new peak was observed in the reaction of coconut oil with palmitic acid (Fig. 7). In this study, all peaks that appeared after 8 min on the HPLC chromatograms are regarded as TG peaks, based on the retention times of several standard diglycerides and TG. Results are averages of three determinations, although it is possible that peaks before 8 min could be due to TG with shorter-chain FA. Times of several diglycerides [1,3-dilaurin, 1,2-(2,3) dipalmitin, 1,3-distearin, and 1,3-diolein] are 3.88, 6.72, 7.50, and 5.98 min, respectively, and of TG (tricaprin, trilaurin, trimyristin, tripalmitin, triolein, and trilinolein) are 4.77, 7.48, 13.19, 25.38, 20.21, and 6.38 min, respectively. The retention times of the standards were determined by using the same analytical conditions as for the samples, and the time for 1,3-distearin (7.5 min), which corresponded to the longest retention time for the diglycerides group, was used as a retention time boundary for diglycerides and TG.

In addition to the formation of new peaks, there were also increases in the concentrations of several TG peaks. In the reaction between coconut oil and palmitic acid, the concentrations of four peaks with carbon numbers (total acyl carbon atoms) C_{42}, C_{44}, C_{46}, and C_{48} increased by 39, 127, 43, and

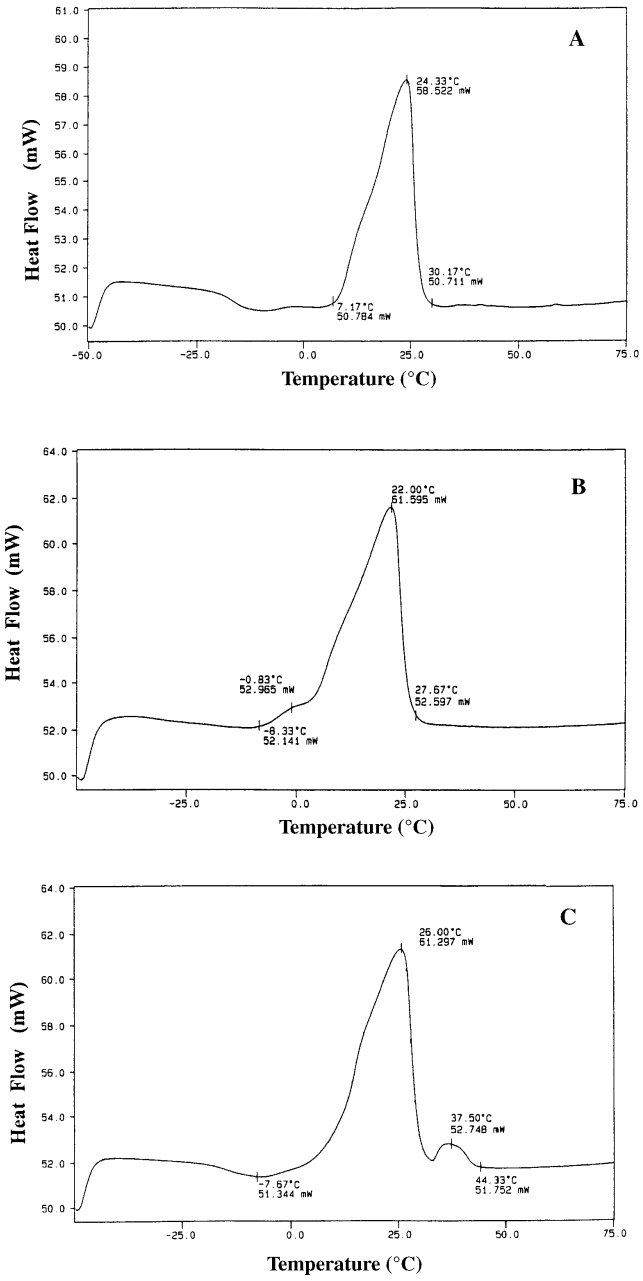

FIG. 9. Differential scanning calorimetry heating thermograms: (A) coconut oil, (B) acidolyzed coconut oil with oleic acid, (C) coconut oil with palmitic acid.

64%, respectively (Fig. 7), after 20-h reaction. The total acyl carbon atoms of these peaks were determined based on a study reported by Herslof *et al.* (30). The formation or increase in concentration of a TG after the reaction of coconut oil with oleic acid between C_{36} and C_{38} (new peak), C_{40} and C_{42} (new peak), and C_{38} and C_{44} (peak area increased) could be due to TG with greater degrees of unsaturation. Herslof *et al.* (30) have shown that the peak that forms between C_{38} and C_{40} was a monounsaturated TG ($C_{40:1}$).

The physical properties of oil, especially the melting points, are important criteria for the marketing strategy in the oils and fats industry. The melting points of acidolyzed products were compared with the controls (Table 2) after removal of free FA. As shown in Table 2, there were changes in the melting properties of oils after acidolysis. The X_1 value of each acidolyzed oil decreased, while the X_2 value increased, consequently exhibiting a broader melting peak (e.g., Fig. 8). The decrease in X_1 value of acidolyzed vegetable oil is probably due to the increase in the amount of unsaturated TG or diglycerides, as reported by Cebula and Smith (31), or monoglycerides (32). On the other hand, the increase in X_2 values could be due to increases in levels of saturated TG (31,33). Generally, the heating thermograms of acidolyzed vegetable oils consisted of two to three merging peaks (e.g., Fig. 8). For acidolyzed coconut oil with palmitic acid, an extra distinct peak (peak temperature 37.5°C) was obtained after the major peak (Fig. 9). The secondary peak could be due to the melting characteristic of saturated TG (C_{40} to C_{48}, Fig. 7), which generally increased in concentration after acidolysis.

We conclude that the mycelium-bound lipase of *A. flavus* successfully modified all oils tested by incorporating exogenous FA into the original TG structure. The results indicate potential use of the bound lipase in catalytic modification of oils and fats. The reduction in the amount of unsaturated FA with incorporation of lauric acid clearly implies that this lipase has the ability to improve oxidative stability of vegetable oils.

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