Enzymatic Transesterification of Triolein and Stearic Acid and Solid Fat Content of Their Products

Vimon Seriburi and Casimir C. Akoh*

Department of Food Science and Technology, Food Science Building, The University of Georgia, Athens, Georgia 30602-7610

ABSTRACT: Two systems were investigated and compared as models for making margarine-type fats. Two immobilized lipases, IM60 from *Rhizomucor miehei* and SP435 from *Candida antarctica*, were used to catalyze the transesterification of triolein with stearic acid and stearic acid methyl ester, respectively, in *n*-hexane. The optimal reaction temperature for both enzymes was 55°C at a mole ratio of triolein to acyl donor of 1:2. Equilibria were reached at 18 h for IM60 and 24 h for SP435. Analysis of the overall yield and incorporation of fatty acid at the *sn*-2 position indicated that the triacylglycerol products contained 38.4 and 16.2% 18:0 for acidolysis and 34.2 and 11.3% for interesterification reactions, respectively, at the 2-position. With SP435, the softest fat was produced after 18 h of incubation, and the hardest after 30 h. For IM60 system, 18 h of incubation gave the most plastic fat.

JAOCS 75, 511–516 (1998).

KEY WORDS: Acidolysis, *Candida antarctica*, interesterification, *Rhizomucor miehei*, solid fat content, stearic acid, stearic acid methyl ester, transesterification, triolein.

Enzymatic interesterification of fats and oils is at the experimental stage in efforts to replace the hydrogenation process for making margarine-type fats and butters (1). Hydrogenation reduces double bonds to single bonds, thereby increasing the melting point of the fat. However, this method results in the formation of geometric isomers, i.e., *trans* rather than *cis* configuration. Several reports have been published on the effect of *trans* fatty acid (FA) consumption. Epidemiologic evidence has linked *trans* FA consumption to high blood total cholesterol and low-density lipoprotein cholesterol levels and to increased incidence of death related to coronary heart disease (2).

Lipases from *Rhizomucor miehei* (IM60) and *Candida antarctica* (SP435) can be used for acylglycerol modifications to achieve desirable physical properties of fats for margarine or specialty fats (3–5). This can be done through positional and specific FA exchange. Essential and other desirable FA have been used as substrates in enzymatic modification of fats and oils for better nutritional products (3,4). Reports indicate that enzymatic interesterification enables higher unsaturation at *sn*-2 position (6) where the FA are absorbed most readily (7,8).

The use of high- and low-melting fats has been investigated to improve the spreadability of butter (9). Hernqvist *et al.* (10) studied the polymorphic behavior of complex triacylglycerols that are formed by interesterification of simple triacylglycerols, such as triolein, trielaidin, and tristearin. These triacylglycerols were chosen as model systems for vegetable oils with possible application in the manufacture of margarine. D'Souza *et al.* (11) compared the chemical and physical characteristics of the high-melting acylglycerols extracted from soft margarines and used the data to explain the differences observed in polymorphic behavior.

IM60, a 1,3-specific lipase, has been shown to work best with free FA (12,13), and SP435, which is a nonspecific lipase under some conditions, to perform best with FA esters in transesterification reactions (14,15). Therefore, in this paper, two systems were chosen and compared: transesterification of triolein and stearic acid (acidolysis) with IM60, and interesterification with stearic acid methyl ester with SP435. Oleic acid is commonly found in several vegetable oils, such as canola, peanut, soybean, and sunflower. Stearic acid is present in saturated fats, such as lard, beef tallow and cocoa butter (1). The stearic acid methyl ester model system can act as a general and useful model for the interesterification of complex nonhomogeneous triacylglycerols.

MATERIALS AND METHODS

Rhizomucor miehei (IM60) and *C. antarctica* (SP435) lipases were provided by Novo Nordisk Biochem North America Inc. (Franklinton, NC). Porcine pancreatic lipase (Type II, crude), triolein (99% pure), stearic acid (18:0, 99% pure), and stearic acid methyl ester (99% pure) were purchased from Sigma Chemical Company (St. Louis, MO). All organic solvents were obtained from Fisher Scientific (Norcross, GA).

Enzymatic transesterification. For general synthesis of modified triolein, 88.54 mg of triolein was mixed with stearic acid or stearic acid methyl ester at a mole ratio of triolein (TAG/FA or methyl ester) of 1:1 and immobilized lipase, IM60 and SP435 (10% combined weight of substrates), respectively, in 5 mL hexane. The mixture was incubated in an orbital shaking waterbath at 200 rpm for 24 h at 55°C. Mo-

*To whom correspondence should be addressed at Department of Food Science and Technology, Food Science Building, Rm. 211, The University of Georgia, Athens, GA 30602-7610. E-mail: cmscakoh@uga.cc.uga.edu.

lecular sieves of 4 Å were added after 2 h. All reactions were conducted in duplicate, and average values are reported.

Analytical procedure. The products of transesterification were passed through an anhydrous sodium sulfate column to remove enzymes. Solvent was evaporated under nitrogen, and 50 µL of the remaining material was analyzed by thin-layer chromatography (TLC) on a silica gel G plate (Fisher Scientific), developed with petroleum ether/ethyl ether/acetic acid (90:10:1, vol/vol/vol). The bands were visualized under ultraviolet radiation after spraying with 0.2% 2,7-dichlorofluorescein in methanol. Bands corresponding to triacylglycerols were scraped, methylated with 3 mL of 6% HCl in methanol at 75°C for 2 h, extracted with hexane (2 mL) and 0.1 M KCl solution (1 mL), centrifuged (1000 rpm, 3 min), and concentrated under nitrogen.

FA compositional analysis. FA were analyzed with an HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA), equipped with a DB-225 fused-silica capillary column (30 × 0.25 mm i.d.) (J&W Scientific, Folsom, CA) and flame-ionization detector and operated in a splitless mode. The injector and detector temperatures were 250 and 260°C, respectively. The column temperature was held at 215°C for 10 min, and total helium carrier gas flow was 23 mL/min. The relative content of fatty acid methyl esters as mole percentage was quantitated by an on-line computer with heptadecanoic acid as an internal standard.

Hydrolysis by pancreatic lipase. Determination of the *sn*-2 positional distribution of FA in triacylglycerols obtained after TLC was conducted by the method of Luddy *et al.* (16). Briefly, 1 mg of triacylglycerol was mixed with 1 mL of 1 M Tris-HCl buffer (pH 7.6), 0.25 mL of 0.05% bile salts, 0.1 mL of 2.2% $CaCl_2$, and 1 mg pancreatic lipase. The mixture was incubated in a waterbath at 37°C for 3 min, vortexed vigorously (1 min), centrifuged (1900 rpm, 3 min), extracted with 3 mL diethyl ether (twice), and dried by passing through a 3-cm packed sodium sulfate column. TLC analysis was done on silica gel G, and the developing solvent system was hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). The band corresponding to 2-monoacylglycerol was scraped, methylated, and analyzed by gas chromatography.

Melting characteristics. Differential scanning calorimetry on a Perkin-Elmer (Perkin-Elmer Corporation, Norwalk, CT) Model DSC7, connected to an IBM computer, was used to determine melting profile and percentage solid fat content (SFC) of the triacylglycerols. The samples obtained after TLC were sealed in aluminum, with an empty pan serving as a reference. Heating thermograms were obtained by holding the samples at –25°C for 10 min and heating at 5°C per min to a final temperature of 40°C. Liquid nitrogen (–196°C) was the coolant.

RESULTS AND DISCUSSION

The enzymatic modification of triolein was examined by using three different parameters (temperature, mole ratio of substrates, and incubation time) with two lipases, SP435 and IM60. The initial conditions used in the temperature study were an equimolar mixture of triolein and stearic acid (18:0) or stearic acid methyl ester, with 24 h of incubation time. Figure 1 shows the yield of 18:0 incorporation by interesterification catalyzed by SP435, and acidolysis catalyzed by IM60, at various temperatures. Temperature played a more important role in the SP435 reaction with the stearic acid methyl ester system than in the IM60 system with 18:0. The change in temperature did not dramatically alter catalytic activity of the IM60/18:0 system. Previous experiments showed that IM60 has the ability to withstand high temperature without a great loss of catalytic activity (17). The activities of both SP435 and IM60 lipases increased with temperature and reached an optimum at 55°C. SP435 lipase showed a decrease in yield at 65°C, indicating either a loss of enzyme activity or product hydrolysis. From 35 to 65°C, the 18:0 incorporation at the *sn*-2 position by both enzymes increased. SP435 produced a slightly higher incorporation than IM60. A possible explanation is the positional nonspecificity of SP435 compared to the *sn*-1,3-specific IM60 lipase. Based on the temperature study, we chose 55°C for both enzyme systems for the mole ratio study.

Maximal incorporation of stearic acid was reached at a mole ratio of triolein to 18:0 or 18:0 methyl ester of 2. Incorporation of 18:0 increased from 20.1 to 34.2% for SP435 and 23.3 to 38.4% for IM60 lipase as the ratio of triolein to 18:0 or 18:0 methyl ester was increased from the equimolar mixture (Fig. 2). The percentage of 18:0 incorporation by acidolysis was higher than that by interesterification. Maximal 18:0 incorporation at the *sn*-2 position for both SP435 and IM60 was obtained at the mole ratio of triolein to 18:0 or 18:0 methyl ester of 2.

Novo Nordisk Biochem North America, Inc. (18) reported that IM60 (or Lipozyme IM) is particularly useful in catalyz-

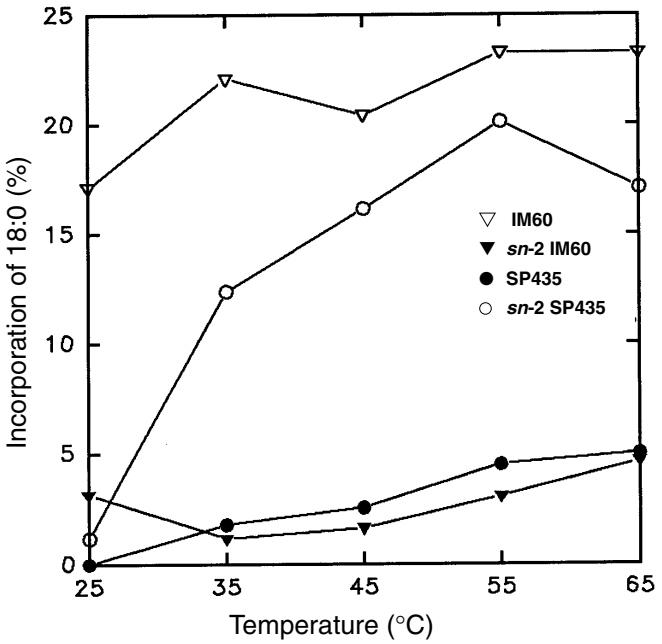

FIG. 1. Incorporation of 18:0 into triolein by IM60 and 18:0 or 18:0 methyl ester by SP435 lipase. Reactions were incubated at temperatures from 25 to 65°C for 24 h with an equimolar mixture of triolein and 18:0 or 18:0 methyl ester.

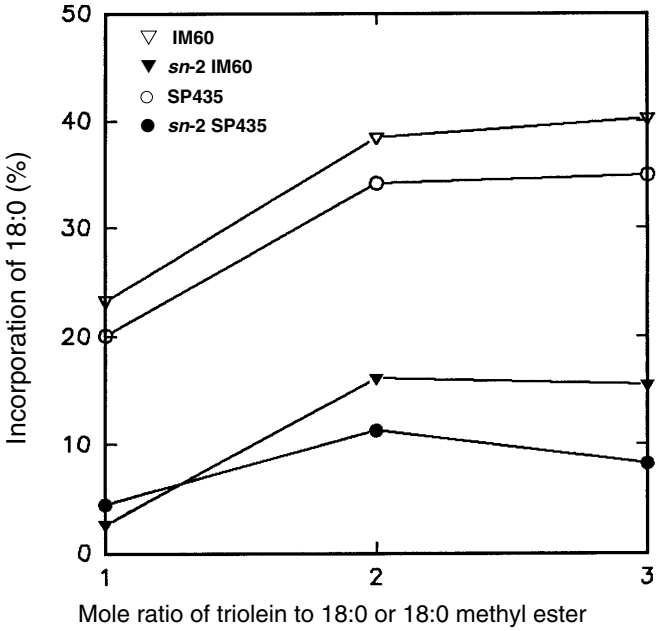

FIG. 2. Incorporation of 18:0 into triolein by IM60 and stearic acid or 18:0 methyl ester by SP435 lipase incubated at 55°C for 24 h with various mole ratios of triolein to 18:0 or 18:0 methyl ester.

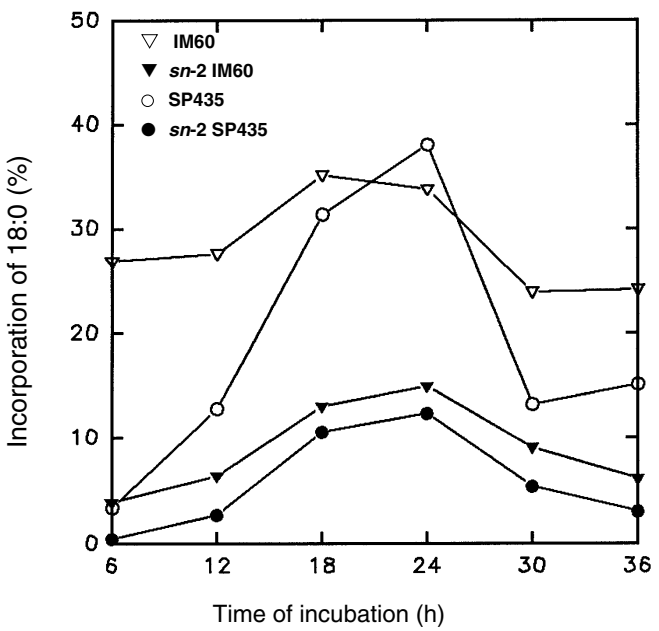

FIG. 3. Incorporation of 18:0 into triolein with IM60 and stearic acid or 18:0 methyl ester with SP435, incubated at 55°C at a 1:2 mole ratio of triolein to 18:0 or 18:0 methyl ester after various incubation times.

ing interesterification reactions that involve the 1- and 3-position ester bonds in triacylglycerols. It has been used as a 1,3-specific lipase in interesterification of triolein and stearic acid by Cho and Rhee (19). Both intra- and intermolecular acyl residue exchanges occur in the reaction until an equilibrium is reached (20). Even in pancreatic lipase hydrolysis, a lipase that has been known as 1,3-specific showed a low level of acyl migration occurring during the reaction (16). Despite this problem, pancreatic lipase is still a common enzyme used in determining the distribution of FA at the *sn*-2 position of triacylglycerols (21). Therefore, in this reaction, the incorporation of 18:0 at the *sn*-2 position by IM60 can be explained by acyl migration occurring after the catalytic reaction at either the *sn*-1 or *sn*-3 position. This acyl exchange would be an advantage to a reaction if high incorporation of new FA is desired. Our result showed that, as the mole ratio of triolein to 18:0 was increased from 1 to 2, the incorporation of 18:0 at the *sn*-2 position by IM60 increased from 2.6 to 15.5%.

The positional specificity of SP435 (or Novozyme® 435) depends on the reactants. In some reactions, SP435 shows a 1,3-positional specificity, whereas in other reactions, the lipase functions as a nonpositional-specific lipase (18). Our results gave a lower yield of 18:0 at the *sn*-2 position for SP435 than for IM60. As the mole ratio of triolein to 18:0 was increased from 1 to 2, the incorporation of 18:0 at the *sn*-2 position, catalyzed by SP435, increased from 4.5 to 11.3%. One could argue that SP435 behaved as a nonspecific lipase in this reaction. Whether this is due to actual hydrolysis at the *sn*-2 position or to FA migration to either *sn*-1 or *sn*-3 position is still not clear. A clear difference between the two enzyme systems is shown in Figure 3 where the reactions were incubated for various times. Both had their maximal 18:0 incorporation between 18 and 24 h of incubation. The highest incorporation and content of 18:0 at the *sn*-2 position by IM60 were 35.1% (at 18 h) and 14.9% (at 24 h), respectively, while with SP435, they were 38.0 and 12.3% at 24 h. It is possible that the transesterification reaction may have attained equilibrium between those hours, and therefore, prolonged reaction time beyond 18 h (for acidolysis by IM60) and 24 h (for interesterification by SP435) did not improve 18:0 incorporation. Therefore, the optimal reaction temperature for both enzymes was 55°C at a mole ratio of triolein to 18:0 or 18:0 methyl ester of 1:2. Equilibria were reached at 18 h for IM60 and 24 h for SP435. Cho and Rhee (19) reported that the interesterification activity of an equimolar mixture of stearic acid and triolein reached an equilibrium after 2 h and was almost constant above 37°C when catalyzed by *M. miehei* lipase immobilized on Florisil with 3-aminopropionic acid as a spacer.

There are factors that determine the range within which a fat could be considered plastic. Such factors include the percentage of solids in a fat and the characteristic continuous variation that occurs with change in temperature. The *Handbook of Soy Oil Processing and Utilization* (22) indicated that the spreadability of margarine at refrigerator temperatures was related to its content of solid fats at 2 to 10°C. Also, the solid content at 25°C influences plasticity at room temperature, whereas the solid content at 33 and 38°C largely influences mouthfeel. DeMan (23) noted that desirable spreadability occurs within a range of roughly 15 to 35% solids. This is called the plastic range of fats.

Average percentage solid fat contents (% SFC) of the product as a function of temperature, mole ratio, and time course study are shown in Figures 4, 5, and 6, respectively, where A represents the graph with products of interesterification by

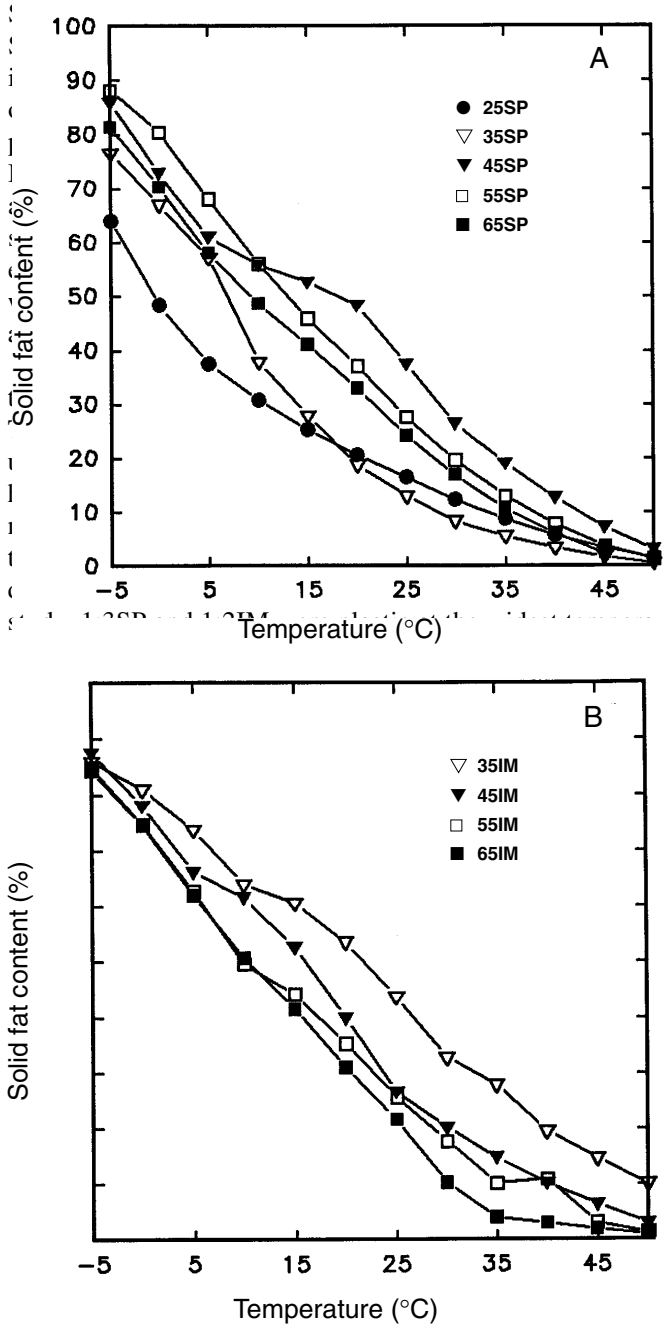

FIG. 4. Percentage solid fat content (SFC) of products of interesterification by SP435 (A) and acidolysis by IM60 (B) incubated at various reaction temperatures. The key represents the temperature of incubation with the indicated enzyme system. For example, 25SP stands for a sample interesterified at 25°C with SP435. IM stands for IM60. See Figure 1 for reaction conditions.

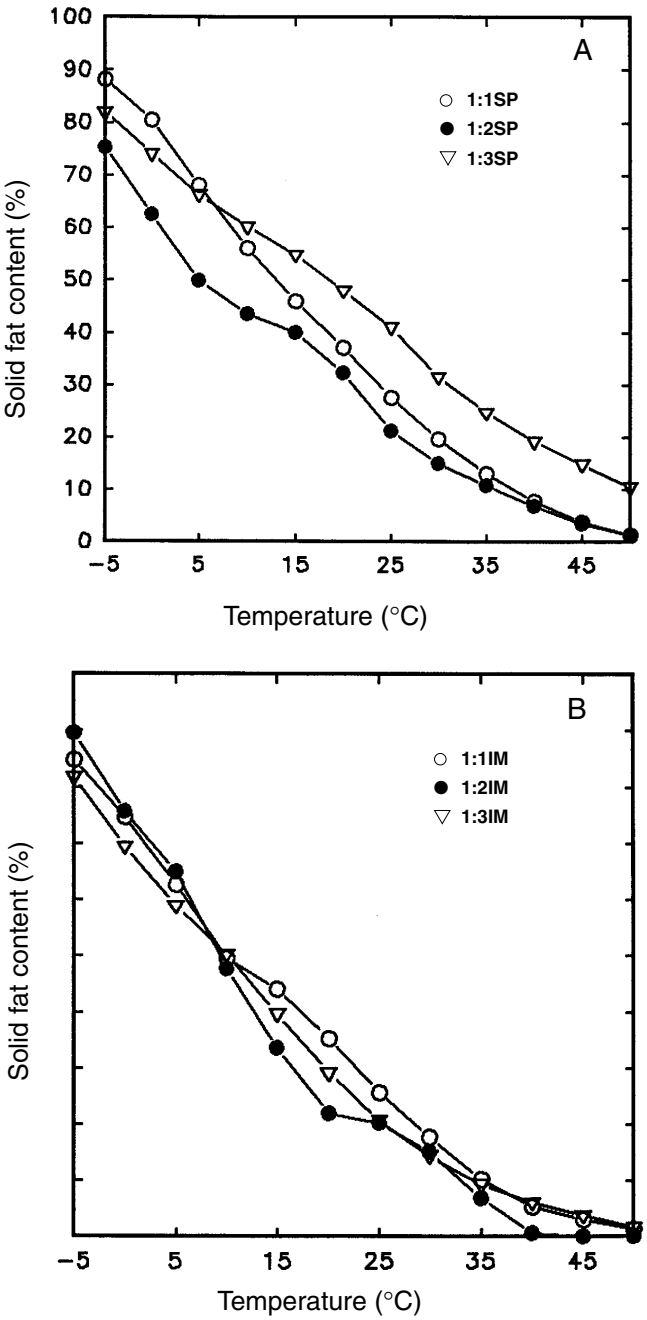

FIG. 5. Percentage solid fat content of products of interesterification by SP435 (A) and acidolysis (B) by IM60 at 55°C and various mole ratios. The keys represent the mole ratios of triolein to 18:0 (for B) or to 18:0 methyl ester (for A) with the indicated enzyme systems. For example, 1:1SP stands for a sample interesterified at 1 mole part of triolein to 1 mole part of 18:0 methyl ester with SP435. IM stands for IM60. See Figure 2 for reaction conditions.

ture range. Also, 18hSP and 12hIM were able to maintain their plasticity over the widest temperature range in the time course study. At 50°C, the SFC of 18hIM was 16.1% (Fig. 6B). It is estimated that the SFC would reach 15% at roughly 52°C. That means that the temperature range would be about 13.5°C. At optimal assay conditions, 24hSP maintained its plasticity over a wider and lower temperature range than 18hIM. Thus, enzymatic transesterification is an option for producing plastic fats.

ACKNOWLEDGMENT

Contributed by the College of Agricultural and Environmental Sciences, The University of Georgia. Research supported by a Food-PAC grant and Food Science Research.

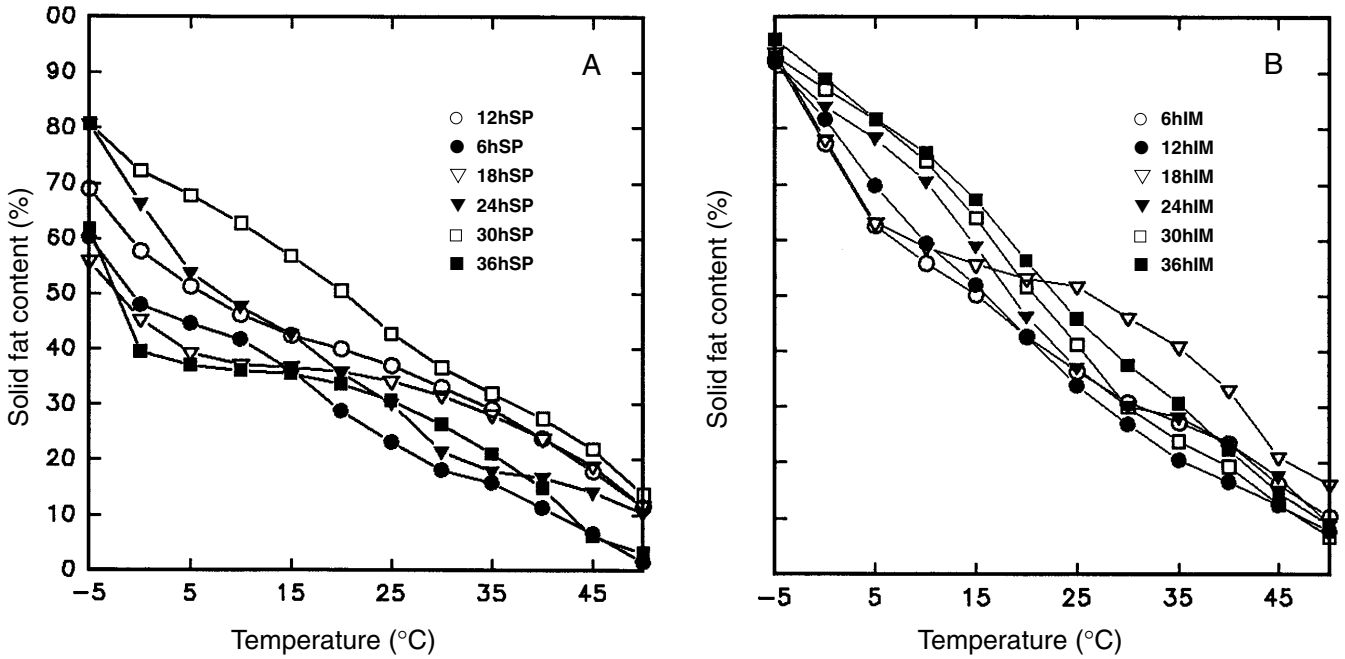

FIG. 6. Percentage solid fat content of products of interesterification by SP435 (A) and acidolysis by IM60 (B) at 55°C at a 1:2 mole ratio of triolein to 18:0 or 18:0 methyl ester after various incubation times. The legends represent the incubation times with the indicated enzyme systems. For example, 6hSP stands for a sample interesterified for 6 h with SP435. IM stands for IM60. See Figure 3 for reaction conditions.

REFERENCES

1. Mansma, C.C., and D.M. Ney, Interrelationship of Stearic Acid Content and Triacylglycerol Composition of Lard, Beef Tallow and Cocoa Butter in Rats, *Lipids 28*:539–547 (1993).
2. Kris-Etherton, P.M., and R.J. Nicolosi, in Trans *Fatty Acids and Coronary Heart Disease Risk*, edited by P.M. Kris-Etherton and R.J. Nicolosi, ILSI Press, Washington, D.C., 1995, p. 17.
3. Foglia, T.A., K. Petruso, and S.H. Feairheller, Enzymatic Interesterification of Tallow-Sunflower Oil Mixtures, *J. Am. Oil Chem. Soc. 70*:281–285 (1993).
4. Forssell, P., R. Kervinen, M. Lappi, P. Linko, T. Suortti, and K. Poutanen, Effect of Enzymatic Interesterification on the Melting Point of Tallow-Rapeseed Oil (LEAR) Mixture, *Ibid. 69*:126–129 (1992).
5. Ghazali, H.M., S. Hamidah, and Y.B. Che Man, Enzymatic Transesterification of Palm Olein with Nonspecific and 1,3-Specific Lipases, *Ibid. 72*:633–639 (1995).
6. Ray, S., and D.K. Bhattacharyya, Comparative Nutritional Study of Enzymatically and Chemically Interesterified Palm Oil Products, *Ibid. 72*:327–330 (1995).
7. Lee, K.T., and C.C. Akoh, Immobilized Lipase-Catalyzed Production of Structured Lipids with Eicosapentaenoic Acid at Specific Positions, *Ibid. 73*:611–615 (1996).
8. Lien, E.L., The Role of Fatty Acid Composition and Positional

TABLE 1
Temperatures (°C) at Which the Solid Fat Contents (SFC) of the Esterified Products Are at 15 and 35% Solids, and Their Temperature Ranges

Sample[a]	Temperature at 15% SFC	Temperature at 35% SFC	Temperature range[b] (°C)	Sample[a]	Temperature 15% SFC	Temperature 35% SFC	Temperature range[b] (°C)
25SP	27	7	20	25IM	n.d.	n.d.	n.d.
35SP	23	12	11	35IM	43	29.5	13.5
45SP	28	26	12	45IM	44	22.5	21.5
55SP	33.5	21	12.5	55IM	31	20.5	10.5
65SP	31	19.5	11.5	65IM	27.5	19	8.5
1:1SP	34	21	13	1:1IM	31	20	11
1:2SP	29.5	19	10.5	1:2IM	30	14.5	15.5
1:3SP	44.5	28.5	16	1:3IM	30	17.5	12.5
6hSP	47.5	38.5	9	6hIM	44	26	18
12hSP	35.5	15.5	20	12hIM	41.5	24	17.5
18hSP	48	25.5	22.5	18hIM	52[c]	38.5	13.5[d]
24hSP	42	20.5	21.5	24hIM	43	26.5	16.5
30hSP	49	33	16	30hIM	44	27.5	16.5
36hSP	39.5	15.5	24	36hIM	44.5	33	11.5

[a]See Figures 4,5, and 6 for legends; n.d. = not determined.
[b]Temperature range = difference between the temperature at 15% SFC and 35% SFC.
[c]Temperature of 18hIM at 15% SFC estimated from Figure 6B.
[d]Temperature range of 18hIM calculated from the estimated temperature in footnote *c* of this table.

Distribution in Fat Adsorption in Infants, *J. Pediatr. 125*:S62–68 (1994).
9. Kaylegian, K.E., and R.C. Lindsay, Performance of Selected Milk Fat Fractions in Cold-Spreadable Butter, *J. Dairy Sci. 75*:3307–3317 (1992).
10. Hernqvist, L., B. Herslof, and M. Herslof, Polymorphism of Interesterified Triglycerides and Triglycerides Mixtures, *Fette Seifen Anstrichm. 86*:393–397 (1984).
11. D'Souza, V., J.M. deMan, and L. deMan, Chemical and Physical Properties of the Solid Fats in Commercial Soft Margarines, *J. Am. Oil Chem. Soc. 69*:1198–1205 (1992).
12. Huang, K.H., C.C. Akoh, and M.C. Erickson, Enzymatic Modification of Melon Seed Oil: Incorporation of Eicosapentaenoic Acid, *J. Agric. Food Chem. 42*:2646–2648 (1994).
13. Akoh, C.C., and K.H. Huang, Enzymatic Synthesis of Structured Lipids: Transesterification of Triolein and Caprylic Acid, *J. Food Lipids 2*:219–230 (1995).
14. Huang, K.H., and C.C. Akoh, Lipase-Catalyzed Incorporation of n-3 Polyunsaturated Fatty Acids into Vegetable Oils, *J. Am. Oil Chem. Soc. 71*:1277–1280 (1996).
15. Huang, K.H., and C.C. Akoh, Enzymatic Synthesis of Structured Lipids: Transesterification of Triolein and Caprylic Acid Ethyl Ester, *Ibid. 73*:245–250 (1996).
16. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman, and R.W. Riemenschneider, Pancreatic Lipase Hydrolysis of Triglycerides by a Semimicro Technique, *Ibid. 41*:693–696 (1963).
17. Moussata, C.O., and C.C. Akoh, Lipase-Catalyzed Incorporation of Oleic Acid into Melon Seed Oil, *Ibid*. *74*:177–179 (1997).
18. Novo Nordisk Industri, *Product Information*, B 665a-GB 200 (1992).
19. Cho, S.W., and J.S. Rhee, Immobilization of Lipase for Effective Interesterification of Fats and Oils in Organic Solvent, *Biotechnol. Bioeng. 41*:204–210 (1993).
20. Belitz, H.-D., and W. Grosch, Lipids, in *Food Chemistry,* translated by D. Hadziyev, Springer-Verlag, New York, 1987, p. 140.
21. Newar, W.W., Lipids, in *Food Chemistry,* 3rd edn., edited by O.R. Fennema, Marcel Dekker, Inc., New York, 1996, p. 235.
22. Brekke, L.O., Food Uses of Soybean Oil, in *Handbook of Soy Oil Processing*, edited by D.R. Erickson, E.H. Pryde, O.L. Brekke, T.L. Mounts, and R.A. Falb, American Soybean Association and AOCS, 1980, p. 396.
23. DeMan, J.M., Fats and Oils: Chemistry, Physics and Applications, in *Encyclopedia of Food Science and Technology*, edited by Y.H. Hui, Wiley, New York, 1992, p. 824.

[Received February 3, 1997; accepted September 17, 1997]