Alteration of Steryl Ester Content and Positional Distribution of Fatty Acids in Triacylglycerols by Chemical and Enzymatic Interesterification of Plant Oils[1]

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ABSTRACT: Steryl ester content of refined and interesterified corn, soybean, and rapeseed oils has been measured *via* clean-up on a short silica gel column, followed by high performance liquid chromatography with evaporative light-scattering mass detector. Chemical interesterification, catalyzed by sodium methoxide, led to random positional distribution of fatty acids in triacylglycerols and some increase in the steryl ester content of all three oils. Enzymatic interesterification, catalyzed by the immobilized lipase from *Rhizomucor miehei* (Lipozyme), resulted in a distinct reduction in steryl ester content, but essentially no alteration in positional distribution of fatty acids in triacylglycerols occurred. Formation of steryl esters during chemical and enzymatic interesterification was also examined by radioactive tracer technique with [4–^{14}C]β-sitosterol added as marker to refined rapeseed oil and measurement of the radioactive steryl esters formed. Chemical interesterification of rapeseed oil resulted in moderate formation (10% of total radioactivity) of radioactive β-sitosteryl esters. Enzymatic interesterification of the oil, catalyzed by Lipozyme, led to little formation of radioactive β-sitosteryl esters, whereas with the lipase from *Candida cylindracea* high proportions (>90% of total radioactivity) of ^{14}C-labeled β-sitosteryl esters were formed.
JAOCS 74, 93–96 (1997).

KEY WORDS: *Candida cylindracea* lipase, chemical interesterification, enzymatic interesterification, positional distribution of fatty acids, radioactive tracer technique, *Rhizomucor miehei* lipase, β-sitosterol, steryl esters.

Sterols occur in plant oils partly as unesterified sterols and partly as steryl esters of fatty acids, in addition to steryl glycosides and esterified steryl glycosides (1,2). We have recently developed a method for quantitative determination of steryl esters in oils by clean-up on a short silica gel column, followed by high-performance liquid chromatography (HPLC) with detection by means of an evaporative light-scattering mass detector (Ferrari, R., E. Schulte, and K.D. Mukherjee, manuscript in preparation). The above method was used to monitor changes in steryl esters in plant oils at different stages of industrial refining (Ferrari, R., E. Schulte, and K.D. Mukherjee, manuscript in preparation). Here we report the alterations in steryl ester content of plant oils by interesterification, e.g., chemical interesterification (randomization) catalyzed by sodium methoxide, and enzymatic interesterification catalyzed by lipase from *Rhizomucor miehei* (Lipozyme). Moreover, we report the formation of steryl esters during chemical interesterification and enzymatic interesterification of rapeseed oil, catalyzed by Lipozyme and the lipase from *Candida cylindracea*, as monitored by a radioactive tracer technique. [4–^{14}C]β-Sitosterol was added as marker to the oil, and the formation of radioactive steryl esters during interesterification was followed.

EXPERIMENTAL PROCEDURES

Materials. Refined corn oil was a product of Rafinacoes de milho Brasil Ltda. (São Paulo, Brazil), and soybean and rapeseed oils were provided by Cocamar Ltda. (Maringá, Brazil). Immobilized lipase from *R. miehei* (Lipozyme IM20, 25 batch interesterification units/g) was a generous gift of Novo Nordisk (Bagsvaerd, Denmark). Powdered lipase preparation from *C. cylindracea* (syn. *C. rugosa*) with an activity of 850 U/mg was purchased from Sigma (Deisenhofen, Germany). β-Sitosterol and [4–^{14}C]β-sitosterol (56 mCi/mmol) were products of Sigma and Amersham (Braunschweig, Germany), respectively. β-Sitosteryl oleate was prepared according to Gupta *et al.* (3). All reagents and adsorbents were of analytical grade and purchased from E. Merck (Darmstadt, Germany).

Chemical interesterification. Refined oil (10 g) and 15 mg sodium methoxide were placed in a round-bottom flask, and the mixture was stirred magnetically at 110°C for 30 min under vacuum generated by a water jet aspirator. The reaction products were cooled to room temperature, dissolved in hexane, and washed repeatedly with an aqueous 1% (wt/vol) sodium chloride solution to remove the soaps completely. The hexane solution of the interesterified product was dried over sodium sulfate and stored refrigerated under nitrogen.

[1]Part of doctoral thesis of Roseli Ap. Ferrari to be submitted to Faculdade de Engenharia de Alimentos, Universidade de Campinas, Campinas, Brazil.

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In a similar manner, refined oil (1 g) was interesterified in the presence of 2 μCi [4–^{14}C]β-sitosterol (specific activity 56 mCi/mmol) and 20 mg β-sitosterol with 1.5 mg sodium methoxide as catalyst, and the reaction product was worked up as described above.

Enzymatic interesterification. Refined oil (10 g) and 500 mg Lipozyme were weighed into a round-bottom flask, and the mixture was stirred magnetically at 40°C for various periods. The reaction products were dissolved in hexane, and the enzyme preparation was separated by centrifugation.

In a similar manner, refined oil (1 g) was interesterified in the presence of 2 μCi [4–^{14}C]β-sitosterol (specific activity 56 mCi/mmol) and 20 mg β-sitosterol with 100 mg of either Lipozyme or lipase powder preparation from *C. cylindracea* as catalyst, and the reaction product was worked up as described above.

Determination of steryl esters. Steryl esters were determined after separation of this fraction *via* clean-up on a short silica gel column manuscript (Ferrari, R., E. Schulte, and K.D. Mukherjee, manuscript in preparation). Essentially, the oil sample (500 mg), dissolved in 2 mL petroleum ether (boiling point 40–60°C), was chromatographed on 5 g Silica Gel (70–230 mesh ASTM, Merck No. 7734, adjusted to 5% water content) in a glass column (10-mm i.d.) by eluting successively with 22 mL petroleum ether, 15 mL petroleum ether/dichloromethane (85:15, vol/vol), and 20 mL petroleum ether/dichloromethane (85:15, vol/vol). The last fraction was collected, concentrated to a small volume, transferred to a pasteur pipette that contained 0.6 mg of the above type of Silica Gel, and eluted with 10 mL petroleum ether/dichloromethane (85:15, vol/vol). The resulting eluate containing the steryl esters was evaporated to dryness and dissolved in 500 μL dichloromethane. The total steryl esters were measured by HPLC on four 125 × 4 mm Lichrospher 100 RP-18 (5μm) columns (E. Merck) with acetonitrile/dichloromethane/methanol (1:1:1, vol/vol/vol), 1 mL/min, for elution and an evaporative light-scattering detector (ACS model 750/14 Mass Detector; Applied Chromatography Systems, Macclesfield, United Kingdom) operated at 50°C and air pressure of 1.38×10^4 Pa for quantitation. The detector was calibrated with β-sitosteryl oleate. The amount of steryl esters was determined from the total area of the steryl ester peaks.

Positional distribution of fatty acids in triacylglycerols. Samples of triacylglycerols were subjected to hydrolysis by pancreatic lipase (Pancreatin; Merck), and the *sn*-2-acylglycerols formed were isolated by thin-layer chromatography (4). The fatty acid composition of triacylglycerols and *sn*-2-acylglycerols formed by lipolysis was determined by treatment with trimethyl sulfonium hydroxide (5), followed by gas chromatography of the methyl esters in a Varian model 3700 instrument (Varian Analytical Instruments, Darmstadt, Germany) equipped with flame-ionization detectors. The separations were carried out on a DB-23 column, 25 m × 0.25 mm, film thickness 0.25 μm (J&W, Asschem, Bad Homburg, Germany), which was programmed from 150 to 230°C at 5°C/min with H_2 at 3.5×10^4 Pa inlet pressure as carrier gas. Other conditions were: injector 280°C, detector 250°C, and split ratio 1:15.

Analysis of radioactive substances. The products formed by interesterification in the presence of [4–^{14}C]β-sitosterol were fractionated by thin-layer chromatography on Silica Gel H with hexane/diethyl ether/acetic acid (85:15:1, vol/vol/vol) as developing solvent. The chromatograms were scanned by a Berthold Radio-TLC Scanner LB 2722-21 (Laboratorium Prof. Dr. Berthold GmbH & Co., Wildbad, Germany) to determine the relative proportions of radioactivity in sterols and steryl esters. Alternatively, the thin-layer chromatograms were briefly exposed to iodine vapor to stain the lipid fractions, the regions containing sterols and steryl esters were scraped, and the radioactivity present in these fractions was measured in a LKB Rackbeta Liquid Scintillation Counter (Pharmacia, Freiburg, Germany) with Aquasol 2 [DuPont de Nemours (Deutschland) GmbH, Bad Homburg, Germany] as scintillation cocktail.

RESULTS AND DISCUSSION

Interesterification of plant oils, catalyzed by alkali or methoxy or ethoxy derivatives of alkaline metals, is an established industrial process for the structural modification of the constituent triacylglycerols, aimed at altering the physical properties of such oils (6). Lately, numerous applications of the enzymatic interesterification of oils, catalyzed by triacylglycerol lipases, have been described for the production of specialty fats (7,8). Little is known so far on the effect of chemical and enzymatic interesterification on the sterol ester content and composition of plant oils.

In the present study, refined corn, soybean, and rapeseed oils were subjected to chemical interesterification, catalyzed by sodium methoxide, and enzymatic interesterification, catalyzed by an immobilized lipase preparation (Lipozyme) from *R. miehei*. The resulting products were analyzed *via* selective lipolysis catalyzed by pancreatic lipase to determine the acyl moieties at the *sn*-2 position of the triacylglycerols. The results, given in Tables 1–3, show that chemical interesterification led, as to be expected, to randomization of the acyl moieties, which is evident from the similarity in the com-

TABLE 1
Positional Distribution (%) of Fatty Acids in Refined Corn Oil Before and after Chemical and Enzymatic Interesterification

		Position *sn*-2		
			After interesterification	
Fatty acid[a]	Total triglycerides	Before interesterification	Chemical	Enzymatic
16:0	12.5	1.5	13.0	4.3
18:0	2.3	2.3	2.9	1.5
18:1	34.8	34.0	34.3	34.3
18:2	47.7	61.5	48.4	52.3
18:3	0.9	0.8	0.8	4.9

[a]Fatty acids are designated as number of carbon atoms:number of *cis* olefinic bonds.

TABLE 2
Positional Distribution (%) of Fatty Acids in Refined Soybean Oil Before and after Chemical and Enzymatic Intersterification

		Position *sn*-2		
			After interesterification	
Fatty acid[a]	Total triglycerides	Before interesterification	Chemical	Enzymatic
16:0	11.1	1.9	11.1	5.1
18:0	3.5	0.9	4.1	0.9
18:1	24.7	24.3	25.9	24.9
18:2	51.7	66.7	52.5	64.0
18:3	5.9	5.5	6.3	5.0

[a]Fatty acids are designated as number of carbon atoms:number of *cis* olefinic bonds.

position of total acyl moieties of the triacylglycerols and those at the *sn*-2 position after chemical interesterification.

Enzymatic interesterification, catalyzed by Lipozyme, yielded products in which the composition of the acyl moieties at the *sn*-2 position was similar to that before interesterification (Tables 1–3). These results show that, during enzymatic interesterification, little acyl exchange occurred between the *sn*-2 position and the *sn*-1,3 positions, as is to be expected from the known strong *sn*-1,3 positional specificity of Lipozyme (9). The similarity between the composition of the fatty acids esterified at the *sn*-2 position before and after interesterification could also imply that little interesterification had taken place. However, earlier studies in our laboratory on interesterification reactions of triacylglycerols with fatty acids, methyl esters, long-chain alcohols, and triacylglycerols with Lipozyme, under conditions used in the present study, have shown conclusively that extensive interesterification occurs (9,10).

The steryl ester contents of corn, soybean, and rapeseed oil before and after chemical and Lipozyme-catalyzed interesterification were determined *via* clean-up on a short silica gel column, followed by HPLC. The results, given in Table 4, show that chemical interesterification led to some increase in the steryl ester content of all three oils, whereas enzymatic interesterification resulted in a distinct reduction in steryl ester content. It appears from these data that moderate esterification of sterols occurs during chemical interesterification, whereas some cleavage of steryl esters occurs during enzymatic interesterification, catalyzed by the *sn*-1,3-specific Lipozyme. Hydrolysis of the secondary esters of sterols by the *sn*-1,3-specific Lipozyme is surprising, because this lipase is likely to hydrolyze only primary esters. It is conceivable that the observed hydrolysis was partly due to the catalytic action of the enzyme support. Some measurable action of Lipozyme on secondary esters has also been observed by others (Haas, M.J., personal communiction). Since little or no such activity was observed in the interesterification experiments (Table 5), it is possible that the selectivity of the enzyme is less in the hydrolytic direction (Table 4) than in the esterification direction.

TABLE 3
Positional Distribution (%) of Fatty Acids in Refined Rapeseed Oil Before and after Chemical and Enzymatic Intersterification

		Position *sn*-2		
			After interesterification	
Fatty acid[a]	Total triglycerides	Before interesterification	Chemical	Enzymatic
16:0	4.5	1.01	6.1	1.1
18:0	2.4	1.4	3.2	n.d.
18:1	64.1	57.3	63.7	58.2
18:2	17.6	28.4	17.7	29.1
18:3	7.2	11.8	7.6	11.6

[a]Fatty acids are designated as number of carbon atoms:number of *cis* olefinic bonds.

TABLE 4
Changes in Steryl Ester Content of Plant Oils after Interesterification Catalyzed by Sodium Methoxide or Lipozyme

Interesterification catalyst (Time h)	Steryl esters (mg/100 g oil)		
	Corn oil	Soybean oil	Rapeseed oil
None	911	114	735
Sodium methoxide (0.5)	1094	353	996
Lipozyme (1)	774	18	352
Lipozyme (24)	677	39	482

Radiochemical tracer techniques have been used earlier to study lipase-catalyzed reactions (10–12). In the present study, the formation of steryl esters during chemical and enzymatic interesterification was examined by using [4–^{14}C]β-sitosterol as radioactive marker, added to refined rapeseed oil before interesterification, and measuring the radioactive steryl esters formed. The results given in Table 5 show that chemical interesterification of rapeseed oil resulted in slight formation (10% of total radioactivity) of radioactive β-sitosteryl esters, whereas enzymatic interesterification of the oil, catalyzed by Lipozyme, led to very little formation of radioactive β-sitosteryl esters. These results agree with those given in Table 4.

Formation of radioactive steryl esters from [4–^{14}C]β-sitosterol was also studied with a nonspecific lipase preparation

TABLE 5
Formation of Radioactive β-Sitosteryl Esters of Fatty Acids After Interesterification of Rapeseed Oil, Catalyzed by Sodium Methoxide or Lipases in the Presence of [4–^{14}C]β-Sitosterol

Interesterification catalyst (Time h)	Distribution of radioactivity (%)		
	Steryl esters	Sterols	Others
Control[a] (0.5)	3	97	Traces
Sodium methoxide (0.5)	11	86	3
Control[a] (2)	<1	96	3
Control[a] (6)	<1	96	3
Control[a] (24)	<1	96	3
Lipozyme (2)	<1	98	1
Lipozyme (6)	<1	97	3
Lipozyme (24)	<1	98	1
Candida cylindracea (2)	62	35	3
C. cylindracea (6)	80	15	5
C. cylindracea (24)	91	5	4

[a]Without interesterification catalyst.

from *C. cylindracea* as catalyst for interesterification of rapeseed oil. With the lipase from *C. cylindracea* as biocatalyst high proportions (> 90% of total radioactivity) of ^{14}C-labeled β-sitosteryl esters were formed in the course of time (Table 5). Obviously, randomization of the triacylglycerols caused by both chemical interesterification, catalyzed by sodium methoxide, and enzymatic interesterification, catalyzed by the nonspecific lipase from *C. cylindracea,* results in measurable formation of steryl esters, whereas little steryl ester is formed by enzymatic interesterification catalyzed by the *sn*-1,3-specific Lipozyme (Table 5). During enzymatic interesterification of butterfat, catalyzed by a nonspecific lipase from *Pseudomonas fluorescens*, extensive formation of cholesteryl esters was also observed (13).

It appears from the present findings, taken together with the earlier observation on butterfat (13), that both chemical and enzymatic interesterification catalysts that cause randomization of triacylglycerols also favor the formation of steryl esters during interesterification. The simple and rapid radiochemical technique described here should be useful for screening lipases with regard to their abilities to esterify sterols.

REFERENCES

1. Mudd, J.B., Sterol Interconversions, in *Biochemistry of Plants*, edited by P.K. Stumpf and E.E. Conn, Academic Press, New York, 1980, Vol. 4, pp. 509–534.
2. Eichenberger, W., Steryl Glycosides and Acylated Steryl Glycosides, in *Lipids and Lipid Polymers from Higher Plants*, edited by M. Tevini and H.K. Lichtenthaler, Springer-Verlag, Berlin, 1977, pp. 169–182.
3. Gupta, C.M., R. Radhakrishnan, and H.G. Khorana, Glycerophospholipid Synthesis: Improved General Method and New Analogs Containing Photoactivable Groups, *Proc. Natl. Acad. Sci. USA 74*:4315–4319 (1977).
4. Christie, W.W., *Lipid Analysis*, 2nd edn., Pergamon Press, Oxford, 1980, pp.156–157.
5. Schulte, E., Gas Chromatography of Acylglycerols and Fatty Acids with Capillary Columns, in *CRC Handbook of Chromatography: Analysis of Lipids*, edited by K.D. Mukherjee and N. Weber, CRC Press Inc., Boca Raton, 1993, pp. 139–148.
6. Rozendaal, A., Interesterification and Fractionation, in *Proceedings of the World Conference on Oilseed Technology and Utilization*, edited by T.H. Applewhite, American Oil Chemists' Society, Champaign, 1992, pp. 180–185.
7. Macrae, A.R., Lipase-Catalyzed Interesterification of Oils and Fats, *J. Am. Oil Chem. Soc. 60*:243A–246A (1983).
8. Mukherjee, K.D., Lipase-Catalyzed Reactions for Modification of Fats and Other Lipids, *Biocatalysis 3*:277–293 (1990).
9. Schuch, R., and K.D. Mukherjee, Interesterification of Lipids by an *sn*-1,3-Specific Lipase, in *Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry*, edited by T.H. Applewhite, American Oil Chemists' Society, Champaign, 1988, pp. 328–329.
10. Schuch, R., and K.D. Mukherjee, Radiochemical Methods for Studying Lipase-Catalyzed Interesterification of Lipids, *Z. Naturforsch. 42c*:1285–1290 (1987).
11. Schuch, R., and K.D. Mukherjee, Rapid Radio Thin-Layer Chromatography for Assay of Lipase-Catalyzed Esterification and Interesterification Reactions, *J. Chromatogr. 450*:448–451 (1988).
12. Schuch, R., and K.D. Mukherjee, Lipase-Catalyzed Reactions of Fatty Acids with Glycerol and Acylglycerols, *Appl. Microbiol. Biotechnol. 30*:332–336 (1989).
13. Kalo, P., J. Rinne, H. Huotari, and M. Antila, Changes in the Contents of Cholesterol and Cholesteryl Esters Occurring During Lipase-Catalysed Interesterification, *Fat Sci. Technol. 95*:58–62 (1993).

[Received November 13, 1995; accepted September 18, 1996]