Lipase-Catalyzed Transesterification of Trilinolein or Trilinolenin with Selected Phenolic Acids

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ABSTRACT: The enzymatic transesterification of selected phenolic acids with TAG, including trilinolein (TLA) and trilinolenin (TLNA), was investigated in an organic solvent medium. Maximal bioconversion of 66% was obtained with a dihydrocaffeic acid (DHCA) to TLA ratio of 1:2 after 5 d of reaction. Similarly, the highest bioconversion of 62% was obtained with a DHCA to TLNA ratio of 1:2, but after 12 d of reaction. However, a ratio of 1:4 DHCA/TLA decreased the bioconversion to 53%. Transesterification reactions of ferulic acid with both TAG, using a ratio of 1:2, resulted in low bioconversion of 16 and 14% with TLA and TLNA, respectively. The overall results indicated that bioconversion of phenolic MAG was higher than that of phenolic DAG. The structures of mono- and dilinoleyl dihydrocaffeate as well as those of mono- and dilinolenyl dihydrocaffeate were confirmed by LC-MS analyses. The phenolic lipids demonstrated moderate radical-scavenging activity.

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KEY WORDS: Lipase, organic solvent media, phenolic acids, transesterification, trilinolein, trilinolenin.

The numerous health benefits of PUFA, such as linoleic (LA: n-6) and linolenic (LNA: n-3) acids, and their TAG have resulted in a growing interest in their use as functional foods and nutraceutical supplements. Although LA and LNA are implicated in vital biological processes, such as the prevention of cardiovascular diseases (1), they are susceptible to rapid oxidation (2), which could result in lowering their quality and nutritional value.

Most phenolic acids are known to be potent antioxidants (3) and are also reported to provide protective effects as anti-inflammatory, anticarcinogenic, and antimutagenic compounds (4,5). Phenolic acids are, however, less effective as antioxidants in fats and oils as a result of their low solubility in hydrophobic media (6).

The incorporation of phenolic acids in TAG may result in novel structured phenolic lipids, with combined potential health benefits and functional properties. Only a few reports have appeared in the literature regarding the biosynthesis of structured TAG containing a phenolic acid moiety (7,8).

The present work is part of on-going research in our laboratory (8–10) aimed at the development of a biosynthetic process for the production of biomolecules of high nutritional and functional properties. The specific objective of the research was to investigate the biosynthesis of phenolic lipids through the lipase-catalyzed transesterification reaction of selected phenolic acids, including dihydrocaffeic acid (DHCA) and ferulic acid (FA), with trilinolein (TLA) and trilinolenin (TLNA). The effects of reaction time and substrate molar ratio on the transesterification reactions were investigated. In addition, the structural characterization of end products was performed and the radical-scavenging properties were determined.

MATERIALS AND METHODS

Materials. Immobilized lipase from *Candida antarctica* (Novozym 435, CAL-B, with an activity of 10,000 propyl laurate units/g solid enzyme) was obtained from Novozymes (Bagsværd, Denmark). DHCA, FA, and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) were purchased from Sigma Chemical Co. (St. Louis, MO). TLA, TLNA, and their FFA as well as MAG and DAG were obtained from Nu-Chek-Prep (Elysian, MN). Organic solvents of analytical and HPLC grades were purchased from Fisher Scientific (Fair Lawn, NJ).

Transesterification reactions of phenolic acids with trilinolein and trilinolenin. Lipase-catalyzed transesterification reactions were carried out in 50-mL Erlenmeyer flasks, using the method developed previously in our laboratory (10). The transesterification reaction of DHCA with TLA, at a final concentration of 5 mM, was carried out in 9 mL of a homogeneous organic solvent mixture of hexane and 2-butanone (75:25, vol/vol). Stock solutions of DHCA and TLA were prepared, prior to their addition into the reaction flask, in 2-butanone and hexane, respectively. The enzymatic reaction was initiated by the addition of 25 mg of the solid enzyme. Control experiments, without enzyme, were carried out in tandem in these trials. The enzymatic mixtures were incubated at 55°C in an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ), with continuous shaking (150 rpm). All the assays were run in duplicate. The enzymatic reaction was halted by termination of contact with the immobilized lipase by decantation of the organic solvent off of the immobilized lipase. The effect of different molar ratios of DHCA to TLA (1:1, 1:2, 1:4, and 2:1) on the transesterification reactions was investigated. In addition, the transesterification reaction of DHCA and FA with TLA or TLNA, respectively, was carried out with a substrate molar ratio of 1:2.

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Characterization of enzymatic reaction components. The transesterification reaction was monitored by HPLC analysis, according to the modified method of Andrikopoulos *et al.* (11). Samples of reaction mixtures were dried at room temperature under vacuum, using an Automatic Environmental Speed Vac System (Savant Instruments Inc., Holbrook, NY). The dried samples were dissolved in 4.5 mL of isopropanol prior to HPLC analysis. The separation of reaction components was performed on a Zorbax SB-C18 reversed-phase column (250 × 4.6 mm, 5 µm; Agilent, Wilmington, DE) using a Beckman Gold system (Model 126; Beckman Instruments Inc., San Ramon, CA) equipped with an autosampler (Model 507), a UV diode-array detector (Model 168), and computerized data handling and integration analysis. Elution of the 20-µL injected sample was carried out with a gradient system using an acetonitrile/methanol mixture (7:5, vol/vol) as solvent A and isopropanol as solvent B; the elution was initiated by a flow of 100% solvent A for 10 min, followed by a 20-min linear gradient to 100% solvent B, which was maintained for an additional 5 min. The flow rate was 1 mL/min, and detection was performed at 215 and 280 nm. The bioconversion was calculated on the basis of the total peak area of phenolic lipids, monitored at 280 nm, at a defined reaction time divided by the area of phenolic acid of the blank, multiplied by 100. The enzymatic activity was calculated from the slope, obtained by polynomial regression of the initial part of the plot of residual phenolic acid concentration vs. reaction time. The unit of enzymatic activity was defined as µmol of esterified phenolic acid per g of solid immobilized lipase per min.

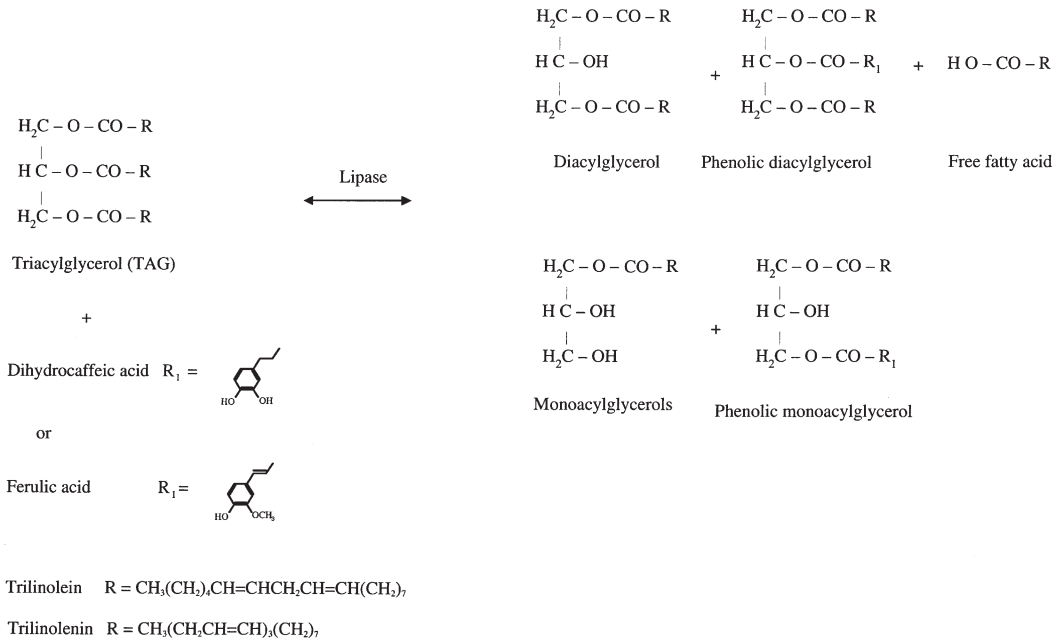
The characterization of reaction components of the lipase-catalyzed transesterification reactions was also performed by HPLC interfaced to atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). The APCI-MS system (ThermoFinnigan, San Jose, CA) was equipped with the Zorbax SB-C18 reversed-phase column (Agilent) as well as a Surveyor liquid chromatography pump, an autosampler, and an Xcalibur® system control software (version 1.3) for data acquisition and processing. The mass spectrometer was operated in positive ion mode with a collision energy source of 15 V. The ion spray and capillary voltage were set at 4.0 kV and 15.6 V, respectively.

Determination of radical-scavenging activity of phenolic lipids. The scavenging activity of phenolic lipids on the free radical DPPH$^{\bullet}$ was determined according to a modification of the method of Silva *et al.* (5). In a 1-mL spectrophotometric cuvette, the sample was mixed with 10^{-4} M ethanolic DPPH$^{\bullet}$ solution. DPPH$^{\bullet}$ reduction was monitored spectrometrically at 517 nm against a blank assay without DPPH$^{\bullet}$, over a 20 min incubation period, using a Beckman spectrophotometer (Model 650; Beckman Instruments, Inc., Fullerton, CA). Control reactions, containing only DPPH$^{\bullet}$, were carried out in tandem with these trials. All assays were carried out in duplicate. The percentage of residual DPPH$^{\bullet}$, at any given time (min), was determined as the absorbance of DPPH$^{\bullet}$ divided by that of the control trial multiplied by 100.

RESULTS AND DISCUSSION

The mixture of hexane/2-butanone (75:25, vol/vol), which was determined to be an appropriate organic solvent reaction medium for the lipase-catalyzed biosynthesis of phenolic lipids (10), was used for the transesterification reactions of selected phenolic acids with TLA and TLNA (Scheme 1).

Effect of substrate molar ratios on the transesterification reaction. The effect of different substrate molar ratios on the time



SCHEME 1

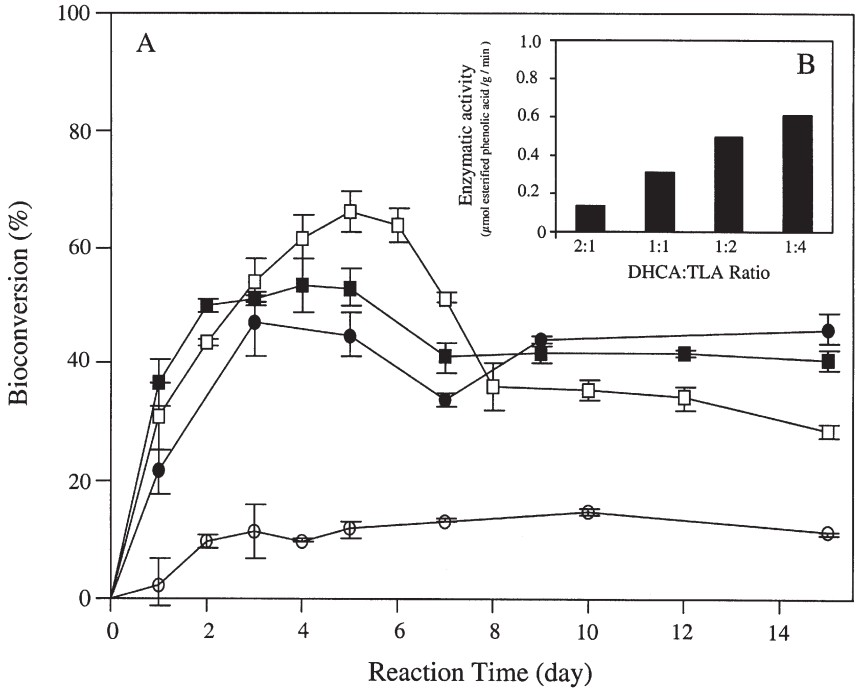


FIG. 1. Effect of dihydrocaffeic acid (DHCA) and trilinolein (TLA) ratios of 2:1 (○), 1:1 (●), 1:2 (□), and 1:4 (■) on the bioconversion (A), and on the enzymatic activity (B) of lipase-catalyzed reaction in a mixture of hexane/2-butanone (75:25, vol/vol).

course of the transesterification reaction of DHCA with TLA is shown in Figure 1. Decreasing the molar ratio of DHCA to TLA from 1:1 to 1:4 resulted in a concomitant increase in enzymatic activity (Fig. 1B). When using a 1:2 ratio of DHCA to TLA, the highest bioconversion of phenolic lipids of 66% (Fig. 1A) was obtained after 5 d of reaction, with an enzymatic activity of 0.50 μmol esterified phenolic acid/g solid enzyme/min (Fig. 1B). Although the highest enzymatic activity of 0.61 μmol esterified phenolic acid/g solid enzyme/min was obtained with the DHCA to TLA ratio of 1:4 (Fig. 1B), the bioconversion of 53% was lower than that (66%) obtained with the ratio of 1:2 (Fig. 1A). The limited increase in the bioconversion with a DHCA to TLA ratio of 1:4 may be due to the reaction having reached its equilibrium and/or to enzyme denaturation (12). The results (Fig. 1A) also show that there was a decrease in the bioconversion of phenolic lipids after its maximum just before the reaction reached equilibrium; this phenomenon may be due to a shift in the thermodynamic reaction toward hydrolysis of phenolic lipids as a result of the increase in the concentration of the polar hydrolytic products (8).

Figure 1 also shows that the presence of an excess of phenolic acid substrate, at the DHCA to TLA ratio of 2:1, resulted in a much lower enzymatic activity (0.14 μmol esterified phenolic acid/g solid enzyme/min) and bioconversion (15%). The inhibitory effect of an excess of DHCA could be due to an increase in its concentration in the enzyme microenvironment, which may result in high interaction between the substrate and the enzyme. Yesiloglu (13) reported that a high ethanol concentration in the ethanolysis of sunflower oil resulted in low lipase activity and yield.

HPLC and LC-MS characterization of enzymatic reaction components. Figure 2 shows a typical HPLC chromatogram of reaction components of lipase-catalyzed transesterification reactions of DHCA with TLA and TLNA, monitored at 215 and 280 nm. Figures 2A and 2B demonstrate the analysis of the reaction components of the transesterification reactions of DHCA with TLA. Peak #1, which absorbed at both wavelengths (215 and 280 nm), and peak #7, absorbing only at 215 nm, were characterized as the substrates DHCA and TLA, respectively, with reference to standards. Peaks #2 and 5 were characterized as the two main end products of the transesterification reactions of DHCA with TLA; they absorbed at both 215 and 280 nm, with a UV-spectral scanning close to that of DHCA. However, the hydrolytic by-products of TLA were demonstrated as peaks #3 and 6 for mono- and dilinolein, respectively. Peak #4, which also absorbed at both wavelengths, could be a side-reaction product. Figures 2A′ and 2B′ represent the analysis of reaction components of the transesterification reactions of DHCA with TLNA. Similarly, peaks #1′ and 7′ represent the substrates DHCA and TLNA, respectively; the main phenolic lipid products were characterized as peaks #2′ and 5′. The hydrolytic products of TLNA were represented by peaks #3′ and 6′ for mono- and dilinolenin, respectively. Peak #8′ could be a possible oxidation product of TLNA, since TLNA is a more unsaturated lipid and hence is more prone to oxidation than TLA.

Further analysis of potential end products of the lipase-catalyzed transesterification reactions of DHCA with TLA and TLNA was performed by APCI-MS (Fig. 3).The fragmentation pattern of peak #2 (Fig. 2A) showed abundant fragmented ions with *m/z* of 337 and 501 as well as a molecular ion of *m/z*

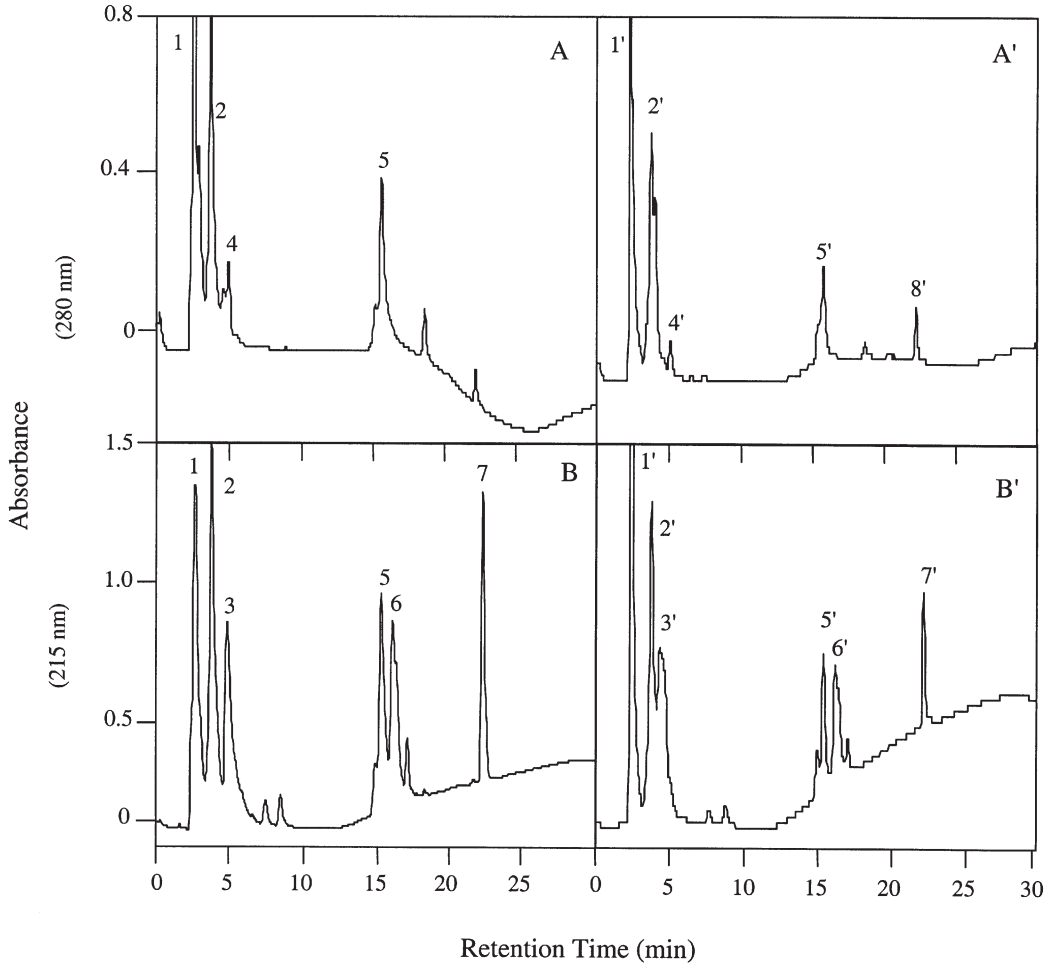


FIG. 2. HPLC chromatograms of the samples of the enzymatic transesterification reactions of DHCA with TLA (A and B) and trilinolenin (TLNA) (A′ and B′) monitored at 280 and 215 nm. Peak numbers were identified as follows: dihydrocaffeic acid, #1 and 1′; TLA, #7; TLNA, #7′; phenolic MAG, #2 and 2′; phenolic DAG, #5 and 5′; MAG, #3 and 3′; DAG, #6 and 6′; side reaction product, #4; and TLNA oxidation product, #8′.

518 corresponding to monolinoleyl dihydrocaffeate with a M.W. of 518 g/mol (Fig. 3A). Fragmentation of peak #2′ (Fig. 2A′) resulted in ion fragments of *m/z* 335 and 500 with a molecular ion of *m/z* 517, characteristic of monolinolenyl dihydrocaffeate with a M.W. of 517 g/mol (Fig. 3A′). Similarly, APCI-MS analysis of peak #5 (Fig. 2A) produced ions of *m/z* 379 and 660, which correspond to the structural pattern characteristic of dilinoleyl dihydrocaffeate with a M.W. of 782 g/mol (Fig. 3B). The ions with *m/z* 596 and 500 as well as the molecular ion with *m/z* 782 (Fig. 3B′), obtained from ion fragmentation of peak #5′ (Fig. 2A′), indicate the formation of dilinolenyl dihydrocaffeate with a M.W. of 781 g/mol.

Effect of substrate type on the transesterification reaction. Figure 4 shows the progress curves of the hydrolysis products as well as those of phenolic lipids over a 13-d period of lipase-catalyzed transesterification reactions of DHCA with TLA or TLNA, using a molar ratio of 1:2. The results (Fig. 4A) show that the hydrolysis of TLA (50%) was rapidly achieved within the first 2 d of the reaction, which was followed by a slower rate of hydrolysis reaching 75 and 90% after 6 and 8 d, respectively. In parallel, the biosynthesis of total dihydrocaffeoylated linoleyl lipids increased steadily, reaching a maximal bioconversion of 66% after 5 d of reaction before its decrease to 38% after 8 d. The decrease in bioconversion of dihydrocaffeoylated linoleyl lipids, with a concomitant maximal hydrolysis of TLA, could confirm the shift of the equilibrium reaction toward hydrolysis rather than biosynthesis. The results (Fig. 4A) also indicate that the bioconversion of mono- and dilinoleyl dihydrocaffeate showed similar trends over the time course of the reaction; however, the extent of bioconversion was higher for monolinoleyl dihydrocaffeate than for dilinoleyl dihydrocaffeate, with a maximum value of 48 and 22%, respectively, after 5 d of reaction.

Figure 4B shows a rapid initial hydrolysis of TLNA (55%) within the first 2 d, followed by a steady increase to reach 70% after 4 d of reaction; this maximum hydrolysis degree of TLNA was lower than that of TLA (90%). The results also indicate a rapid production of dihydrocaffeoylated linolenyl lipids (35%)

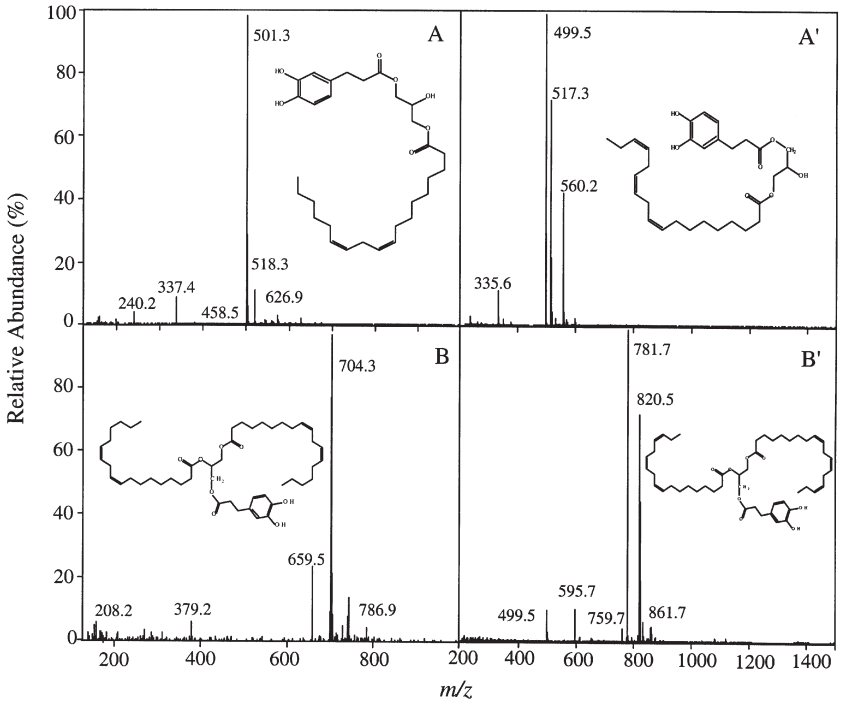


FIG. 3. Atmospheric pressure chemical ionization MS analysis of monolinoleyl dihydrocaffeate (A), dilinoleyl dihydrocaffeate (B), monolinolenyl dihydrocaffeate (A′), and dilinolenyl dihydrocaffeate (B′).

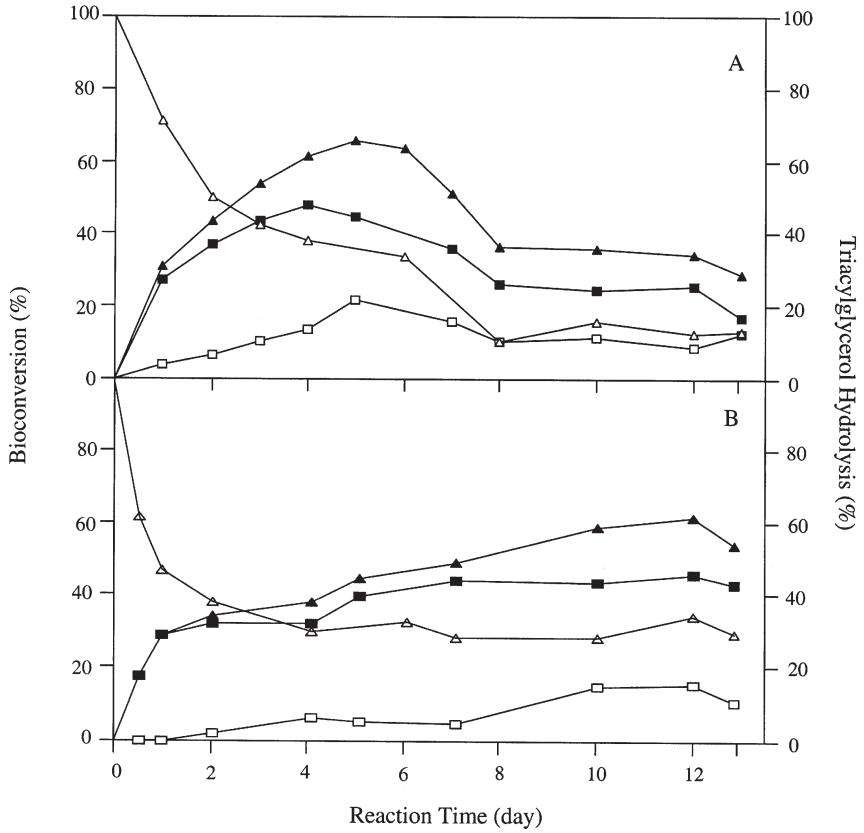


FIG. 4. Progress curves of the biosynthesis of phenolic MAG (■), phenolic DAG (□), and total phenolic lipids (▲) as well as the hydrolysis of TAG (△) during the lipase-catalyzed transesterification of dihydrocaffeic acid with TLA (A) and TLNA (B).

TABLE 1
Effects of Nature of Phenolic Acids and TAG on the Enzyme Activity and Bioconversion of Lipase-Catalyzed Transesterification in Hexane/2-Butanone Mixture (75:25, vol/vol)

	Trilinolein		Trilinolenin	
Phenolic acid	Enzyme activity[a]	Bioconversion[b]	Enzyme activity[a]	Bioconversion[b]
Dihydrocaffeic acid	0.50	66.0 (2.7)[c]	0.52	62.0 (1.9)[c]
Ferulic acid	0.04	15.6 (3.7)[c]	0.03	14.0 (5.8)[c]

[a]Enzymatic activity was defined as μmol esterified phenolic acid/g solid enzyme/min reaction time.
[b]Bioconversion (%) was calculated on the basis of the total peak area of phenolic lipids, monitored at 280 nm, divided by the area of phenolic acid of the blank, multiplied by 100.
[c]Relative SD was calculated from the SD of duplicate samples divided by their mean, multiplied by 100.

during the first day of reaction, followed by a steady increase in the yield reaching its maximum of 62% after 12 d. The biosynthesis of dihydrocaffeoylated linolenyl lipids did not show, over the time course of reaction, the same pattern as that of dihydrocaffeoylated linoleyl lipids (Fig. 4A); these findings may be explained by the limited increase in the hydrolysis products of TLNA after 4 d of reaction, which could favor the reverse biosynthesis reaction. In addition, the results (Fig. 4B) show that the bioconversion of monolinolenyl dihydrocaffeate (46%) was higher than that of dilinolenyl dihydrocaffeate (16%). The overall results (Fig. 4, Table 1) show that the enzymatic activity (0.50–0.52 mmol esterified phenolic acid/g solid enzyme/min) and the maximum bioconversion of phenolic lipids (62–66%) were similar for both transesterification reactions of DHCA with TLA and TLNA.

In using the same reaction conditions, the enzymatic activity and bioconversion for the transesterification reactions of FA with TLA and TLNA were lower than those obtained with DHCA (Table 1). The highest total bioconversion of feruloylated linoleyl and linolenyl lipids was only 16 and 14%, respectively. The affinity of Novozym 435 is therefore greater for DHCA than for FA. The presence of both the methoxyl substituent and the double bond on the side chain of the aromatic ring of FA could explain why its affinity is lowest for the transesterification reactions with TAG (7,14,15).

Free radical-scavenging activity of DHCA and its acylglycerol esters. The ability of dihydrocaffeoylated lipids to scavenge the stable free radical DPPH$^{\bullet}$ was investigated (Fig. 5).The steady state of the radical scavenging reaction was reached within 20 min. The radical-scavenging activity was 84, 42, 16, and 16% for DHCA, for α-tocopherol, and for the dihydrocaffeoylated linoleyl and linolenyl lipids, respectively. These findings indicate that the structured phenolic lipids, formed by the transesterification reactions of DHCA with selected TAG, have certain antioxidant potency. The decrease in the antiradical effect of DHCA following the esterification of its carboxyl group could be due to its conformational changes (5). DHCA has a single bond on its side chain, which allows its rotation around this bond; however, this rotation may have been restricted to a certain degree by the attachment of a lipid moiety on the carbonyl group of the DHCA. Similarly, Sabally *et al*. (10,16) reported that the structural modification of the carboxyl group of DHCA by esterification with linoleyl or linolenyl alcohols affected its radical-scavenging activity; however, the radical-scavenging activity of linoleyl and linolenyl dihydrocaffeate was higher than that of dihydrocaffeoylated lipids, indicating that the size and nature of the attached lipid has a significant effect on the antioxidant efficacy of phenolic lipids. The fatty alcohols, being less bulky than TAG, could have conferred less constraint on changes or rotation around the single bond on the side chain of DHCA.

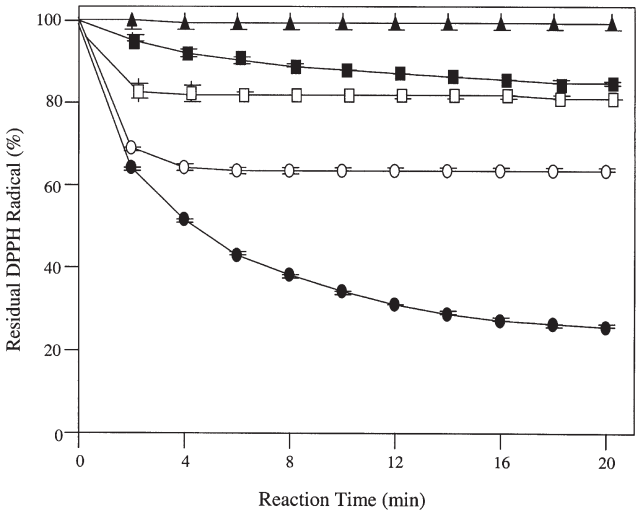


FIG. 5. Time course for diphenylpicrylhydrazyl radical (DPPH$^{\bullet}$) scavenging by dihydrocaffeic acid (●) and α-tocopherol (●) as well as dihydrocaffeoylated linoleyl (□) and linolenyl (■) lipids, using DPPH$^{\bullet}$ (▲) as a control trial.

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