Reactivity of Medium-Chain Substrates in the Interesterification of Tripalmitin Catalyzed by Papaya Lipase

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ABSTRACT: Reactivity of different medium-chain substrates, i.e., *n*-octanol, caprylic acid, and its alkyl (methyl, ethyl, *n*-propyl, and *n*-butyl) esters, was assessed in the interesterification of tripalmitin catalyzed by papaya (*Carica papaya*) lipase. Alcoholysis with *n*-octanol was the fastest reaction leading to the highest conversion of tripalmitin to *n*-octyl palmitate and concomitant formation of di- as well as monopalmitoylglycerols. This was followed by transesterification of tripalmitin with *n*-butyl and *n*-propyl caprylates, which in turn were faster than transesterification with ethyl and methyl caprylates, yielding in each case the corresponding alkyl palmitates and triacylglycerols containing palmitoyl and capryloyl moieties as the major reaction products. Acidolysis of tripalmitin with caprylic acid yielded palmitic acid and triacylglycerols containing palmitoyl and capryloyl moieties as the major reaction products, however, with the lowest conversion among the three interesterification reactions studied. In each case, interesterification was accompanied by some hydrolysis of tripalmitin.

Paper no. J9893 in *JAOCS 78*, 965–968 (September 2001).

KEY WORDS: Alkyl caprylates, caprylic acid, interesterification, lipase, *n*-octanol, papaya (*Carica papaya*), tripalmitin.

Lipases are enzymes with a great industrial potential, but so far their widespread use in industry has been hampered primarily for two reasons: first, their unavailability in the amounts required for commercial use, and second, the high cost associated with these enzymes (1).

Hence, the lipase selected for study here is papaya (*Carica papaya*) latex lipase (CPL), which is a commercially available plant lipase preparation that is also less expensive than other marketed lipases. Another reason for the selection of a plant lipase is that these enzymes (2–5), including CPL (6), are known to possess pronounced substrate specificities that are comparable to those of microbial lipases (5,7). This makes them well suited for reactions requiring the discriminatory ability of the enzyme. Hence, this study on substrate preference of CPL was undertaken.

CPL has been found to catalyze the hydrolysis of tributyroylglycerol and other medium- and long-chain triacylglycerols (TG) (8) as well as synthetic reactions, such as esterification of fatty acids (FA) (6,9) and interesterification of TG (10–15).

Recent work in our laboratory on CPL-catalyzed transesterification of tripalmitin with ethyl esters of short-, medium- and long-chain FA has indicated that papaya lipase has a preference for medium- and long-chain esters, as well as a regiopreference for the *sn*-1,3 position of the glycerol backbone (16).

We report here CPL-catalyzed interesterification reactions of tripalmitin with different classes of C_8 substrates, i.e., alcoholysis with *n*-octanol, acidolysis with caprylic acid, and transesterification with alkyl (methyl, ethyl, *n*-propyl, and *n*-butyl) esters of caprylic acid. The objective of this study was to determine how the type of C_8 substrate affects the rate and extent of interesterification.

EXPERIMENTAL PROCEDURES

Materials. Crude granular papaya (*C. papaya*) latex, CPL (Sigma-Aldrich-Fluka, Deisenhofen, Germany) was ground in a mortar to a fine powder and sieved to 0.8-mm mesh size. Methyl pentadecanoate, methyl tridecanoate, methyl caprylate, ethyl caprylate, *n*-propyl caprylate, *n*-butyl caprylate, caprylic acid, *n*-octanol, stearic acid, tripalmitin, and 2′,7′-dichlorofluorescein were also procured from Sigma-Aldrich-Fluka. Solvents and silica gel were obtained from E. Merck (Darmstadt, Germany). Trimethylsulfonium hydroxide (TMSH) was purchased from Macherey-Nagel (Düren, Germany).

Interesterification. Tripalmitin (0.5 mmol) was added to 1 mmol of the C_8 substrate (*n*-octanol, caprylic acid, methyl caprylate, ethyl caprylate, *n*-propyl caprylate, or *n*-butyl caprylate) in a magnetically-stirred, Teflon-lined, screw-capped reaction vial immersed in a water bath maintained at 63°C. Once the vials had attained the specified temperature, 27 mg CPL was added to initiate the reaction. Sample aliquots were withdrawn at specified intervals during the 24-h reaction period, taken up in dichloromethane, centrifuged to remove the residual CPL granules, and the supernatants analyzed.

Lipid analysis. A known amount of an internal standard (methyl pentadecanoate in transesterification and stearic acid in acidolysis) was added to aliquots of reaction products. These were then fractionated by thin-layer chromatography (TLC) on 0.3 mm Silica Gel H plates to separate the various

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lipid classes [alkyl esters, TG, diacylglycerols (DG) and monoacylglycerols (MG), as well as free FA] using *i*-hexane/diethyl ether/acetic acid (80:20:1, by vol). Chromatoplates were dried and sprayed with 0.1% (wt/vol) solution of 2′,7′-dichlorofluorescein in ethanol, and the different lipid components marked under ultraviolet light. The latter were scraped off and eluted with water-saturated diethyl ether. The lipid fractions, to which a known amount of methyl tridecanoate as standard was added, were converted to methyl esters *in situ* using TMSH (17) and analyzed by gas chromatography (GC). The peak areas obtained from GC were used for determining the progress of the reactions and for estimation of the relative proportions of different lipid classes.

GC. A Hewlett-Packard (Böblingen, Germany) HP-5890 Series II gas chromatograph equipped with a flame-ionization detector was used. FA methyl esters of lipids were separated on a 0.25 μm CS-FFAP-CB free FA phase column (25 m × 0.25 mm i.d.; J&W, ASS-Chem, Bad Homburg, Germany) using hydrogen as the carrier gas (linear velocity 20 $cm \cdot s^{-1}$) and temperature programming as follows: 5 min at 50°C, followed by heating at 7°C min^{-1} to 220°C, and finally at 220°C for 5 min. The split ratio was 1:10, and the injector as well as the flame-ionization detector temperatures were at 270°C. Peak areas and percentages were calculated using a Hewlett-Packard PC Integration Pack (HP 3365 Series ChemStation Version A.03.21) using response factors.

Calculation of conversions and estimation of different lipid classes. The proportion of alkyl palmitate formed was taken as an indicator of the progress of the alcoholysis and transesterification reactions. In the case of the acidolysis reaction, the proportion of palmitic acid formed was taken as a measure of the progress of the reaction.

The conversion was determined for alcoholysis and transesterification reactions from the peak areas of alkyl palmitate and the internal standard, methyl pentadecanoate, in the alkyl ester fractions, isolated by TLC. The percentage conversion was calculated from the millimoles of alkyl palmitate formed in the alkyl ester fraction with respect to millimoles of substrate, i.e., *n*-octanol or alkyl caprylate.

In the acidolysis reaction, the FA fraction isolated by TLC, consisting of newly formed palmitic acid, unreacted caprylic acid and the stearic acid standard, was converted to methyl esters by treatment with TMSH and analyzed by GC to determine the progress of reaction from the peak areas of methyl palmitate and methyl stearate. The percentage conversion was calculated from the millimoles of palmitic acid in the acid fraction with respect to millimoles of substrate, i.e., caprylic acid added.

The relative proportions of the individual lipid classes in all reaction products were determined from the peak areas of methyl tridecanoate, the internal standard, and those of methyl palmitate and methyl caprylate.

RESULTS AND DISCUSSION

Reactivity of different medium-chain substrates in the interesterification with tripalmitin. The interesterification of tripalmitin with different C_8 substrates, catalyzed by CPL, was monitored over a 24-h period. Progress of the reaction was determined from the appearance of alkyl palmitate in the alkyl ester fraction in the case of alcoholysis with *n*-octanol and transesterification with alkyl caprylates, and that of palmitic acid in the acid fraction in the case of acidolysis. The extent of formation of alkyl palmitate and palmitic acid was found to be almost equivalent to the depletion of the corresponding C_8 substrate (data not shown) and was considered to represent the conversion of the C_8 substrate. It should be pointed out here that in the case of transesterification and acidolysis, the conversions of C_8 substrates thus obtained refer to the total incorporation into TG, DG, MG, and FA fractions. Considerable hydrolysis was found to accompany these interesterification reactions, as can be seen from Table 1. Acidolysis displayed consistently lower values (14% after 24 h), followed by alcoholysis (21%), while transesterification was accompanied by 25–27% hydrolysis (Table 1).

Figure 1 shows the time course of interesterification reactions of tripalmitin with various medium-chain substrates, catalyzed by CPL. The fastest conversion (94% after 24 h) was obtained during alcoholysis with *n*-octanol, while acidolysis with caprylic acid was the slowest reaction (about 28% conversion after 24 h) (Fig. 1). Transesterification of tripalmitin with alkyl esters was intermediate between the above two reactions; propyl and butyl esters gave higher conversions (77–86% after 24 h) than methyl and ethyl caprylate (both 58% after 24 h).

Thus, alcoholysis appears to be the most favored reaction. This is similar to the reported higher rates of interesterification obtained with the *Rhizomucor miehei* lipase when a mixture of TG was reacted with octadecanol as compared to FA or their methyl esters (18). Our results (Fig. 1) are, however, markedly different from the observations of Villeneuve *et al.* (13), who had reported considerably higher incorporation into tricaprylin of lauric acid than ethyl and methyl laurates during interesterification catalyzed by CPL. Ethyl and methyl esters have also been found to be poorer acyl donors than free FA in the esterification of C_{10} substrates with glycerol, catalyzed by the *Rhizopus arrhizus* lipase (19). The above discrepancies, as compared to our present data, appear to be due to the different chain lengths of the acyl donors and acyl acceptors employed (18) and to different lipases used (19).

TABLE 1
Hydrolysis as a Side Reaction During Interesterification of Tripalmitin with Various C_8 Substrates, Catalyzed by Papaya Lipase

	Time		
	4 h	8 h	24 h
Reaction	Degree of hydrolysis (%)		
Alcoholysis: *n*-octanol	19.2	21.4	21.1
Transesterification: methyl caprylate	22.6	23.4	27.6
Transesterification: ethyl caprylate	22.0	21.3	24.5
Transesterification: *n*-propyl caprylate	18.8	24.3	27.7
Transesterification: *n*-butyl caprylate	20.5	26.2	25.0
Acidolysis: caprylic acid	5.4	9.2	14.4

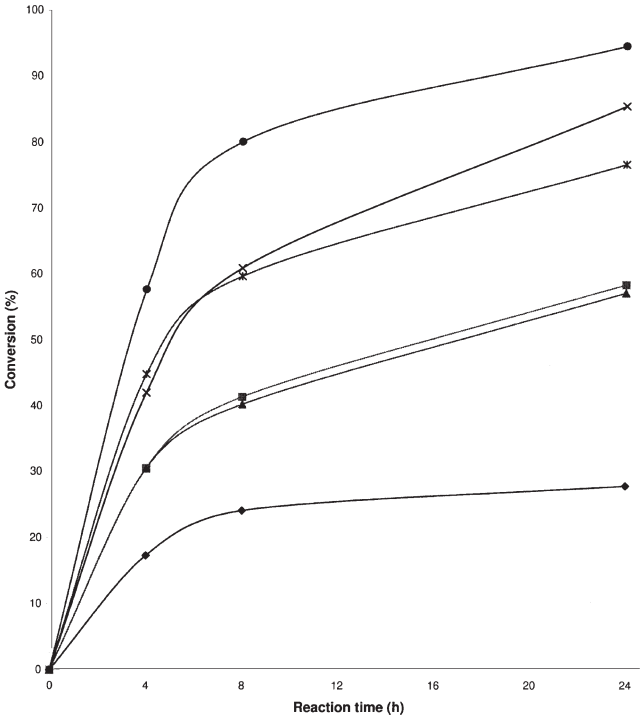

FIG. 1. Time course of interesterification of tripalmitin with various C_8 substrates catalyzed by papaya lipase (CPL) using a tripalmitin/C_8 substrate mole ratio of 1:2. (◆) Caprylic acid; (■) methyl caprylate; (▲) ethyl caprylate; (×) n-propyl caprylate; (*) *n*-butyl caprylate; (●) *n*-octanol.

Composition of the products of interesterification catalyzed by CPL. The products formed by interesterification of tripalmitin with various C_8 substrates were fractionated using TLC and further analyzed by GC. The profile of the various lipid classes in the reaction products as a function of time for different reactions is shown in Figure 2.

The components identified in products formed by alcoholysis were octyl palmitate, unreacted tripalmitin, and the products formed by simultaneous hydrolysis, i.e., dipalmitin, monopalmitin, and palmitic acid, while unreacted *n*-octanol, being volatile, could not be retained during TLC and subsequent isolation procedures (Fig. 2). The products formed by transesterification contained the ester fraction comprising unreacted alkyl caprylate and alkyl palmitate, unreacted tripalmitin, the newly formed TG (dipalmitoyl-monocapryloyl and monopalmitoyl-dicapryloylglycerols), MG, DG, and FA (Fig. 2). In the products of acidolysis, the FA fraction consisted of unreacted caprylic acid and the newly formed palmitic acid, whereas the TG fraction consisted of unreacted tripalmitin as well as the newly formed TG (dipalmitoyl-monocapryloyl- and monopalmitoyl-dicapryloylglycerols); moreover, MG and DG were detected (Fig. 2).

Figure 2 shows, as expected, that the proportion of the alkyl palmitate formed from tripalmitin by both alcoholysis with *n*-octanol and transesterification with alkyl caprylates increased with time. While the decrease in proportion of the

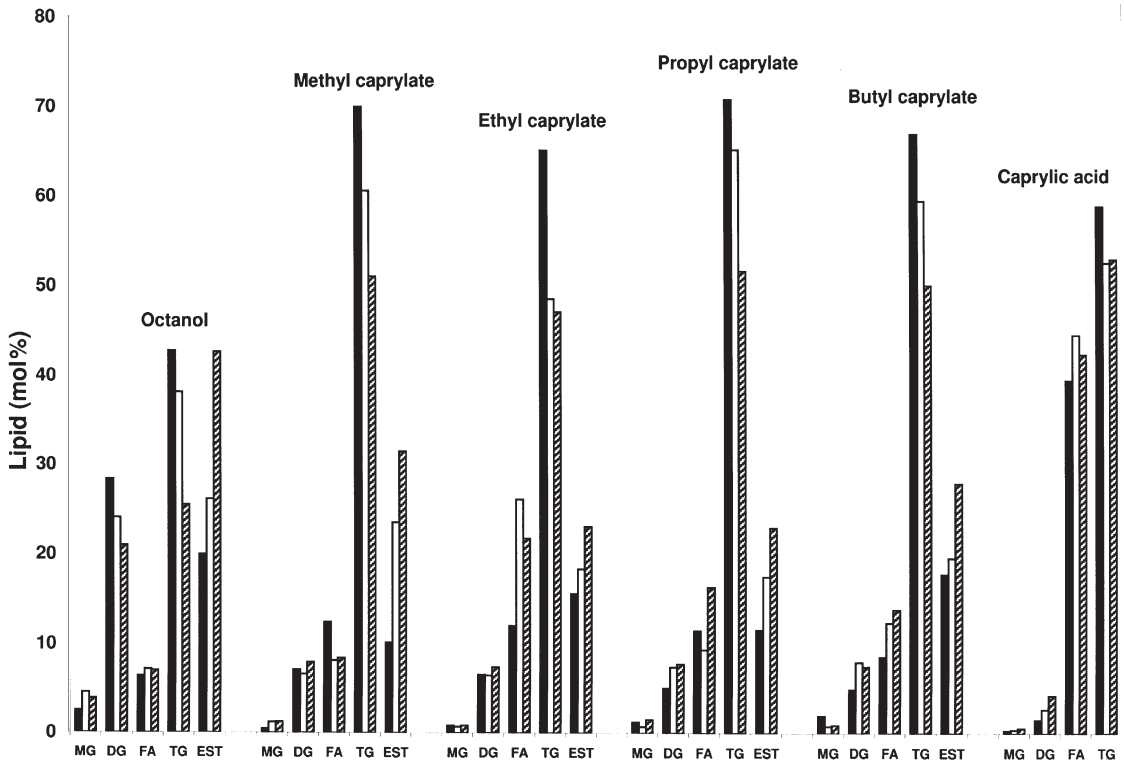

FIG. 2. Percentage molar composition of different lipid classes in reaction products during interesterification of tripalmitin with various C_8 substrates, catalyzed by papaya lipase, CPL, using a tripalmitin/C_8 substrate mole ratio of 1:2. Abbreviations used: TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols; FA, fatty acids; EST, alkyl esters. (solid bar) 4 h; (open bar) 8 h; (cross-hatched bar) 24 h.

TG fraction during alcoholysis corresponded to the increase in octylpalmitate and DG, by-products of hydrolysis such as MG and FA were also obtained (Fig. 2 and Table 1). Concomitantly the proportion of the TG fraction decreased, obviously due to simultaneous hydrolysis leading to the formation of MG, DG, and FA. Interestingly, alcoholysis yielded relatively large proportions (20 to 30%) of DG (Fig. 2). The acidolysis of tripalmitin with caprylic acid yielded much lower proportions of hydrolysis products i.e., 0.6% MG and 4% DG after 24 h as compared to transesterification of tripalmitin with alkyl caprylates (Fig. 2). The high apparent concentration (40–45%) of free FA in this case was due mainly to a large proportion of unreacted caprylic acid as well as to the palmitic acid that was formed as a product of the acidolysis reaction (Fig. 2) rather than to the low amounts of palmitic acid formed as a hydrolysis product. Hence, the use of palmitic acid levels as an indicator of the progress of the acidolysis reaction remains justified.

The data presented here on the reactivity of various medium-chain substrates in the interesterification with tripalmitin using CPL as biocatalyst and the pattern of products formed should be useful in the selection of substrates for the application of this lipase in the preparation of low-calorie products, such as TG and DG containing medium-chain acyl moieties.

ACKNOWLEDGMENT

Neena Gandhi is grateful to the Alexander von Humboldt Foundation for the research fellowship awarded to her.

REFERENCES

1. Gandhi, N.N., Applications of Lipase, *J. Am. Oil Chem. Soc. 74*: 621–634 (1997).
2. Hills, M.J., I. Kiewitt, and K.D. Mukherjee, Lipase from *Brassica napus* L. Discriminates Against *cis*-4 and *cis*-6 Unsaturated Fatty Acids and Secondary and Tertiary Alcohols, *Biochim. Biophys. Acta 1042*:237–240 (1990).
3. Jachmanián, I., and K.D. Mukherjee, Germinating Rapeseed as Biocatalyst: Hydrolysis of Oils Containing Common and Unusual Fatty Acids, *J. Agric. Food Chem. 43*:2997–3000 (1995).
4. Jachmanián, I., and K.D. Mukherjee, Esterification and Interesterification Reactions Catalyzed by Acetone Powder from Germinating Rapeseed, *J. Am. Oil Chem. Soc. 73*:1527–1532 (1996).
5. Jachmanián, I., E. Schulte, and K.D. Mukherjee, Substrate Selectivity in Esterification of Less Common Fatty Acids Catalyzed by Lipases from Different Sources, *Appl. Microbiol. Biotechnol. 44*:563–567 (1996).
6. Mukherjee, K.D., and I. Kiewitt, Specificity of *Carica papaya* Latex as Biocatalyst in the Esterification of Fatty Acids with 1-Butanol, *J. Agric. Food Chem. 44*:1948–1952 (1996).
7. Mukherjee, K.D., I. Kiewitt, and M.J. Hills, Substrate Specificities of Lipases in View of Kinetic Resolution of Unsaturated Fatty Acids, *Appl. Microbiol. Biotechnol. 40*:489–493 (1993).
8. Giordani, R., A. Moulin, and R. Verger, Tributyroylglycerol Hydrolase Activity in *Carica papaya* and Other Latices, *Phytochemistry 30*:1069–1072 (1991).
9. Gandhi, N.N., and K.D. Mukherjee, Specificity of Papaya Lipase in Esterification with Respect to the Chemical Structure of Substrates, *J. Agric. Food Chem. 48*:566–570 (2000).
10. Foglia, T.A., and P. Villeneuve, *Carica papaya* Latex-Catalyzed Synthesis of Structured Triacylglycerols, *J. Am. Oil Chem. Soc. 74*:1447–1450 (1997).
11. Mukherjee, K.D., and I. Kiewitt, Structured Triacylglycerols Resembling Human Milk Fat by Transesterification Catalyzed by Papaya (*Carica papaya*) Latex, *Biotechnol. Lett. 20*:613–616 (1998).
12. Villeneuve, P., M. Pina, A. Skarbek, J. Graille, and T.A. Foglia, Specificity of *Carica papaya* Latex in Lipase-Catalyzed Interesterification Reactions, *Biotechnol. Tech. 11*:91–94 (1997).
13. Villeneuve, P., A. Skarbek, M. Pina, J. Graille, and T.A. Foglia, Catalytic Behaviour of *Carica papaya* Latex in Transesterification Reactions, *Biotechnol. Lett. 11*:637–641 (1997).
14. Mangos, T.J., K.C. Jones, and T.A. Foglia, Lipase-Catalyzed Synthesis of Structured Low-Calorie Triacylglycerols, *J. Am. Oil Chem. Soc. 76*:1127–1132 (1999).
15. Lee, K.-T., and T.A. Foglia, Synthesis, Purification, and Characterization of Structured Lipids Produced from Chicken Fat, *Ibid. 77*:1027–1034 (2000).
16. Gandhi, N.N., and K.D. Mukherjee, Structured Lipids—Tailor-Made—Using Lipase from Papaya Latex, in *Lipases & Lipids*, edited by G. Kokotos and V. Constantinou, Crete University Press, Heraklion, 2000, pp. 365–371.
17. Schulte, E., and K. Weber, Schnelle Herstellung der Fettsäuremethylester aus Fetten mit Trimethylsulfoniumhydroxid oder Natriummethylat, *Fat Sci. Technol. 91*:181–183 (1989).
18. Schuch, R., and K.D. Mukherjee, Interesterification of Lipids Using an Immobilized *sn*-1,3-Specific Triacylglycerol Lipase, *J. Agric. Food Chem. 35*:1005–1008 (1987).
19. Millqvist Fureby, A., P. Adlercreutz, and B. Mattiasson, Glyceride Synthesis in a Solvent-Free System, *J. Am. Oil Chem. Soc. 73*:1489–1495 (1996).

[Received February 12, 2001; accepted June 20, 2001]