# Specificity of Papaya Lipase in Esterification of Aliphatic Alcohols A Comparison with Microbial Lipases

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**ABSTRACT:** Straight-chain saturated C4 to C18 alcohols and unsaturated C18 alcohols, such as *cis*-9-octadecenyl (oleyl), *cis*-6-octadecenyl (petroselinyl), *cis*-9,*cis*-12-octadecadienyl (linoleyl), all-*cis*-9,12,15-octadecatrienyl (α-linolenyl), and all-*cis*-6,9,12-octadecatrienyl (γ-linolenyl) alcohols, were esterified with caprylic acid using papaya (*Carica papaya*) latex lipase (CPL) and immobilized lipases from *Candida antarctica* (Lipase B, Novozym, NOV) and *Rhizomucor miehei* (Lipozyme, LIP) as biocatalysts. With CPL, highest activity was found for octyl and decyl caprylate syntheses, whereas both NOV and LIP showed a broad chain-length specificity toward the alcohol substrates. CPL strongly discriminated against all C18 alcohols studied, relative to *n*-hexanol, whereas the microbial lipases accepted the C18 alcohols as substrates nearly as well as *n*-hexanol. Both petroselinyl and γ-linolenyl alcohol were very well accepted as substrates by NOV as well as LIP, although the corresponding fatty acids, i.e., petroselinic and γ-linolenic acid, are strongly discriminated against by several microbial and plant lipases, including LIP and CPL.

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**KEY WORDS:** Alcohol specificity, *Candida antarctica* lipase B, esterification, Lipozyme, Novozym, papaya (*Carica papaya*) lipase, *Rhizomucor miehei* lipase.

Microbial lipases such as those from *Rhizomucor miehei* (1–7), *Penicillium roqueforti*, *Candida rugosa*, *Aspergillus niger* (8), and *C. antarctica* lipase B (9,10), to name a few, have been widely used for the preparation of alkyl esters by esterification of short- and long-chain acids with alcohols. Other nonmicrobial sources of lipase used for ester synthesis include plant tissues such as cotyledons and endosperm from germinating seeds and cereals (11–14). Less known is the application of lipase associated with protease preparations from plants, such as the papaya (*Carica papaya*) (15) and pineapple (*Ananassa sativa*) (16).

Latex from papaya is commonly used in the food and beverage industries in the form of a protease preparation, papain. The detection of lipase in the papaya latex (CPL) has triggered interest in elucidating its potential for synthetic reactions such as esterification and transesterification (17–24).

The choice of CPL as a biocatalyst for this study arose primarily out of two considerations. First, CPL is a commercially available plant lipase preparation which is also less expensive than other marketed lipases. Second, plant lipases (11–14), including CPL (18), are known to possess pronounced substrate specificities that are comparable with those of microbial lipases (14,25). Consequently, plant lipases are also attractive for reactions where the discriminatory ability of the biocatalyst is called for.

We have shown recently that in esterification reactions the activity of CPL is strongly affected by the chemical structure of the alcohol substrate, i.e., chain length, number and configuration of olefinic bonds, presence of an aromatic ring or a primary vs. secondary or tertiary hydroxy group (24). We report here the selectivity of CPL as biocatalyst in caprylate ester synthesis from a variety of saturated alcohols of different chain lengths as well as unsaturated C18 alcohols in comparison with two widely used commercially available immobilized microbial lipases, i.e., lipase B from *C. antarctica* (Novozym 435; NOV) (26) and lipase from *R. miehei* (Lipozyme IM 20; LIP) (1).

## EXPERIMENTAL PROCEDURES

*Materials.* Crude CPL was obtained from Sigma-Aldrich-Fluka (Deisenhofen, Germany). The granular latex preparation was ground to a fine powder and sieved to 0.8 mm mesh size. NOV (*C. antarctica* lipase B immobilized on an acrylic resin) with an activity of 10,500 Propyl Laurate Units (PLU) $\cdot g^{-1}$ and LIP (*R. miehei* lipase immobilized on a weak anion-exchange resin) with an activity of 23 Batch Interesterification Units (BIU) $\cdot g^{-1}$ were gifts from Novo Industri A/S (Copenhagen, Denmark). Both NOV and LIP were used as obtained. Solvents and Silica Gel 60 were from E. Merck (Darmstadt, Germany). Caprylic acid, *n*-butyl caprylate, short-, medium-, and long-chain alcohols as well as 2′,7′-dichlorofluorescein were procured from Sigma-Aldrich-Fluka.

*Lipase-catalyzed synthesis of C4–C16 caprylate esters.* The following reactions were carried out between caprylic acid and equimolar mixtures of saturated alcohols using CPL, NOV, and LIP as biocatalysts. *n*-Hexyl alcohol was taken as reference standard in each mixture: reactions CPL-I, NOV-I, LIP-I: *n*-butyl, *n*-hexyl, *n*-octyl, *n*-decyl alcohols; reactions CPL-II, NOV-II, LIP-II: *n*-hexyl, *n*-dodecyl (lauryl), *n*-tetradecyl (myristyl), *n*-hexadecyl (cetyl) alcohols.

Caprylic acid (5 mmol) and 10 mmol of alcohol mixtures (2.5 mmol of each alcohol) were taken in a magnetically stirred, Teflon-lined, screw-capped reaction vial and main-

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tained at 63°C. Reactions were initiated by the addition of 54 mg of either of the three enzyme preparations. Sample aliquots were withdrawn at specified intervals during the 24 h reaction period, taken up in dichloromethane, centrifuged to remove the enzyme, and the supernatants analyzed.

*Lipase-catalyzed synthesis of C18 caprylate esters*. The following reactions were carried out between caprylic acid and equimolar mixtures of C18 alcohols with *n*-hexyl alcohol as the reference standard in each case using CPL, NOV, and LIP as biocatalysts: reactions CPL-III, NOV-III, LIP-III: *n*-hexyl, *n*-octadecyl (stearyl), *cis*-9-octadecenyl (oleyl), all-*cis*-9,12,15-octadecatrienyl (α-linolenyl) alcohols; reactions CPL-IV, NOV-IV, LIP-IV: *n*-hexyl, *cis*-6-octadecenyl (petroselinyl), *cis*-9,*cis*-12-octadecadienyl (linoleyl), all-*cis*-6,9,12-octadecatrienyl (γ-linolenyl) alcohols.

In this case, 0.33 mmol of caprylic acid was reacted with 0.66 mmol of alcohol mixtures (0.167 mmol of each alcohol). The quantity of enzymes taken was also correspondingly reduced to 3.6 mg. The rest of the procedure was as above.

*Chemical preparation of standard esters*. Ester standards for gas chromatographic analysis were prepared by reacting an excess of an alcohol with caprylic acid at 80°C for 2 h using concentrated sulfuric acid (0.4% w/w of the reactants) as a catalyst. Esters were extracted with *i*-hexane, the extracts washed till neutral with distilled water, and the organic layers dried with anhydrous sodium sulfate. The esters were purified by thin-layer chromatography on Silica Gel H using *i*-hexane/diethyl ether/acetic acid (80:20:1, by vol). Chromatoplates were sprayed with a 0.1% (wt/vol) solution of 2′,7′-dichlorofluorescein in ethanol, and the ester fraction marked under ultraviolet light and scraped off. The esters were eluted with water-saturated diethyl ether. After removal of diethyl ether by evaporation, the esters were dissolved in *i*-hexane and dried over anhydrous sodium sulfate.

*Gas chromatography*. Aliquots of reaction products were added to a known amount of an internal standard, i.e., methyl myristate (reactions CPL-I, CPL-II, NOV-I, NOV-II, LIP-I, LIP-II) and methyl pentadecanoate (reactions CPL-III, CPL-IV, NOV-III, NOV-IV, LIP-III, LIP-IV) and subjected to GC analysis.

A Hewlett-Packard (Böblingen, Germany) HP-5890 Series II gas chromatograph, equipped with a flame-ionization detector, was used. Separations were carried out on a 0.25 μm CS-FFAP-CB free fatty acid 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 $cm \cdot s^{-1}$) at a split ratio of 1:10. The injector as well as the flame-ionization detector temperature was 270°C. The following temperature programs were used for the analysis of products from different reactions: CPL-I, NOV-I, LIP-I: 5 min at 100°C, followed by linear heating at 4°C·$min^{-1}$ to 220°C, finally at 220°C for 3 min; CPL-II, NOV-II, LIP-II: linear heating at 4°C·$min^{-1}$ from 160 to 240°C, finally at 240°C for 5 min; and CPL-III, CPL-IV, NOV-III, NOV-IV, LIP-III, LIP-IV: linear heating at 4°C·$min^{-1}$ from 160 to 230°C, at 1°C·$min^{-1}$ from 230 to 240°C, finally at 240°C for 5 min.

Peak areas and percentages were calculated using a Hewlett-Packard PC Integration Pack (HP 3365 Series ChemStation Version A.03.21) using response factors. Amounts of esters formed were calculated from the peak areas obtained, relative to peak areas of known amounts of internal standard.

## RESULTS AND DISCUSSION

Figure 1 shows the time course of formation of caprylate esters during esterification, under competitive conditions, of caprylic acid with equimolar mixtures of two sets of saturated straight-chain alcohols, catalyzed by CPL, NOV, and LIP. The relative reactivities of the individual alcohols with respect to

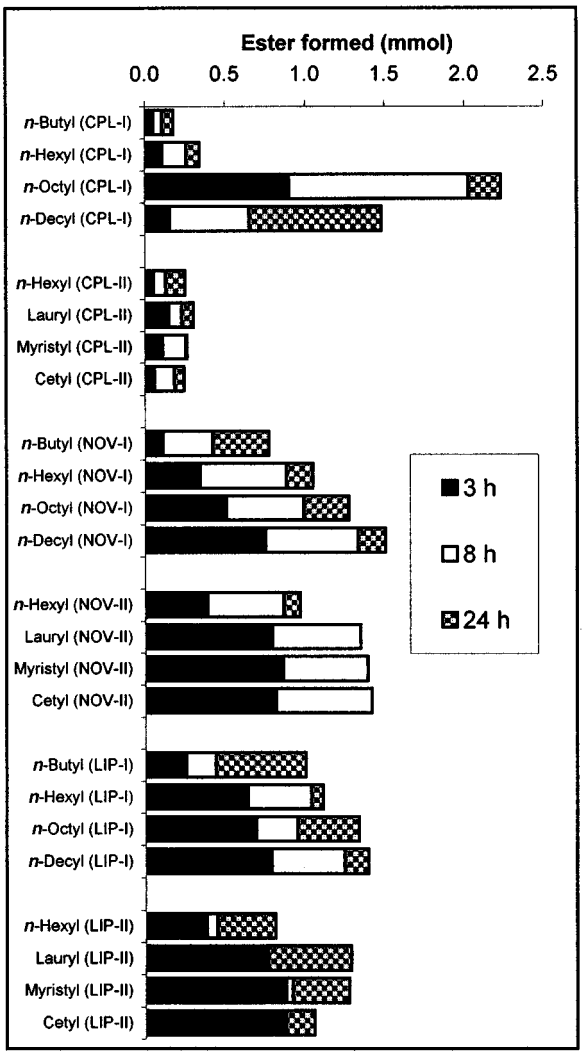


**FIG. 1.** Synthesis of caprylate esters of straight-chain saturated alcohols (C4–C16) by different lipases (CPL, papaya lipase; NOV, Novozym; LIP, Lipozyme) at 63°C using equimolar mixtures of different alcohols and a molar ratio of caprylic acid/alcohol = 1:2. *n*-Hexyl alcohol was used as the reference standard. Reaction I (CPL-I, NOV-I, LIP-I): *n*-butyl, *n*-hexyl, *n*-octyl, *n*-decyl alcohols; Reaction II (CPL-II, NOV-II, LIP-II): *n*-hexyl, *n*-dodecyl (lauryl), *n*-tetradecyl (myristyl), *n*-hexadecyl (cetyl) alcohols.

**TABLE 1**
**Relative Reactivity of Saturated Alcohols and Unsaturated C18 Alcohols in the Esterification with Caprylic Acid, Catalyzed by Papaya Lipase (CPL), *Candida antarctica* Lipase B (NOV), and *Rhizomucor miehei* Lipase (LIP)**

| Alcohol[a] | Relative reactivity[b] | | |
|---|---|---|---|
| | CPL | NOV | LIP |
| *n*-Butyl | 53 | 74 | 90 |
| *n*-Hexyl | 100 | 100 | 100 |
| *n*-Octyl | 644 | 121 | 120 |
| *n*-Decyl | 428 | 143 | 125 |
| Lauryl | 121 | 139 | 158 |
| Myristyl | 104 | 143 | 156 |
| Cetyl | 96 | 146 | 129 |
| Steryl | 5 | 91 | 68 |
| Oleyl | 4 | 165 | 86 |
| Petroselinyl | 5 | 165 | 90 |
| Linoleyl | 4 | 139 | 77 |
| α-Linolenyl | 5 | 101 | 61 |
| γ-Linolenyl | 3 | 142 | 77 |

[a]For designation of alcohols see Figures 1 and 2.
[b]Relative reactivities are calculated as quotient of mmol caprylate ester formed to the corresponding amount of hexyl caprylate formed in 24 h under competitive conditions and expressed as percentage of hexyl caprylate formed.

that of *n*-hexyl alcohol are given in Table 1. In the CPL-catalyzed synthesis of caprylates of C4 to C16 saturated alcohols, the highest ester formation is observed with the C8 alcohol, which is followed by the C10 alcohol, whereas all other alcohols are esterified to a distinctly lower extent (Fig. 1 and Table 1). *n*-Hexyl caprylate yields are similar in both reactions CPL-I and CPL-II (Fig. 1). These results are quite similar to those reported recently (24).

In the esterification of caprylic acid with C4 to C16 saturated alcohols, catalyzed by NOV and LIP, a rather broad specificity with regard to chain length of the alcohols is observed as compared to CPL (Fig. 1 and Table 1). An increase in the extent of esterification with increasing chain length of the alcohol from C4 to C10 is observed with both NOV and LIP. However, further increase in chain length to C16 does not increase the extent of esterification with NOV while a slight reduction in ester yield is obtained in the case of LIP (Fig. 1 and Table 1). In this context, it should be noted that no effect of chain length of alcohols from C12 to C18 on the synthesis of caproates, caprylates, and caprates using LIP was observed earlier (27). These discrepancies are possibly due to different experimental conditions used in the above study as compared to those used in the present study.

Figure 1 also shows an increase in 3-h conversions with increasing chain length of the alcohol from C4 to C10 in the case of both NOV and LIP, implying an increase of initial reaction rate with chain length. This is in contrast to the decrease in initial reaction rate with increasing chain length of the alcohols as observed earlier (28) in the synthesis of oleate esters using LIP. These differences in trend could be attributed to the gross difference in chain lengths of the acid (oleic vs. caprylic) and/or the competitive reaction system used in the present study.

*n*-Hexyl caprylate synthesis in the reactions catalyzed by NOV and LIP is slightly lower when *n*-hexanol is offered as substrate together with the longer chain (C12 to C16) alcohols than with shorter chain (C4, C8 and C10) alcohols (Fig. 1), which is attributed to preferential utilization of the longer-chain alcohols in comparison to *n*-hexanol.

With both NOV and LIP, 90–100% overall caprylic acid conversion in 24 h is observed in all cases; however, in the case of CPL with the longer-chain alcohols (reaction CPL-II) only 22% of the acid is converted in 24 h as compared to 85% with the shorter-chain alcohols (reaction CPL-I) (data not shown). It should be noted here that no attempts have been made to correct for the different activities of the enzymes. The objective of this study was to compare the relative substrate selectivity of the three commercially available enzyme preparations, not their absolute activity.

Figure 2 shows the time course of formation of *n*-alkyl es-

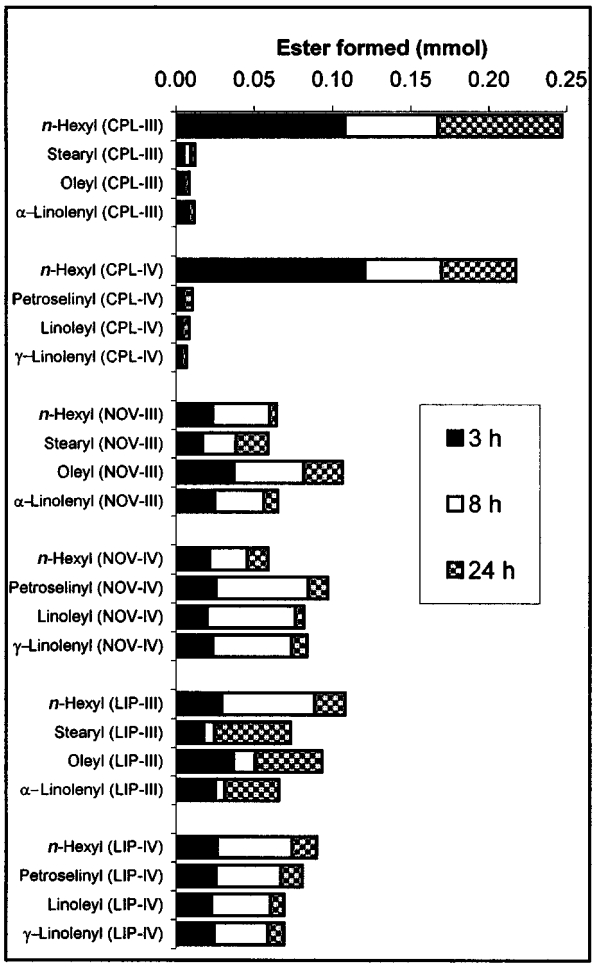


**FIG. 2**. Synthesis of caprylate esters of straight-chain C18 alcohols by different lipases (CPL, NOV, LIP) at 63°C using equimolar mixtures of different alcohols and a molar ratio of caprylic acid/alcohol = 1:2. *n*-Hexyl alcohol was used as the reference standard. Reaction III (CPL-III, NOV-III, LIP-III): *n*-hexyl, *n*-octadecyl (stearyl), *cis*-9-octadecenyl (oleyl), all-*cis*-9,12,15-octadecatrienyl (α-linolenyl) alcohols; Reaction IV (CPL-IV, NOV-IV, LIP-IV): *n*-hexyl, *cis*-6-octadecenyl (petroselinyl), *cis*-9,*cis*-12-octadecadienyl (linoleyl), all-*cis*-6,9,12-octadecatrienyl (γ-linolenyl) alcohols. See Figure 1 for abbreviations.

ters during lipase-catalyzed esterification of caprylic acid with two sets of equimolar mixtures of straight-chain saturated and unsaturated C18 alcohols, each containing equimolar amounts of *n*-hexanol, the reference standard. The relative reactivities of the individual C18 alcohols with respect to that of *n*-hexyl alcohol are given in Table 1. It is evident from Figure 2 and Table 1 that with CPL the C18 alcohols (stearyl, oleyl, petroselinyl, linoleyl, α-linolenyl, and γ-linolenyl) show more than 10-fold lower reactivity as compared to *n*-hexanol. This trend is in line with the decreasing reactivity with increasing alcohol chain length observed in Figure 1 and Table 1 and our recent findings (24). It appears therefore that the alcohol binding site of CPL is suited for shorter-chain alcohols. It should be noted in this context that the rate of esterification of oleic acid, catalyzed by another plant lipase from rape (*Brassica napus*) seedlings, decreases with increasing chain length of the primary aliphatic alcohol from C4 to C16 (11).

In case of the microbial lipases, however, the yields of esters of C18 alcohols are comparable to *n*-hexyl ester yields (Fig. 2 and Table 1). Obviously, all the C18 saturated and unsaturated alcohols are well accepted as substrates by both NOV and LIP. Interestingly, both petroselinyl and γ-linolenyl alcohols having a *cis*-6 olefinic bond are well accepted as substrates for esterification by both microbial lipases (Fig. 2 and Table 1), although several microbial lipases, including LIP, are known to discriminate against fatty acids having *cis*-4, *cis*-6, and *cis*-8 double bonds such as petroselinic and γ-linolenic acids (14,25). Obviously, the fatty acid binding site of LIP strongly discriminates against petroselinic and γ-linolenic acids, whereas the less specific alcohol binding site of this enzyme readily accepts petroselinyl and γ-linolenyl alcohols. CPL, on the other hand, which also discriminates against petroselinic and γ-linolenic acids, but readily accepts oleic, linoleic, and α-linolenic acids (18), apparently has an alcohol binding site with a narrow specificity that discriminates against all C18 saturated and unsaturated alcohols (Fig. 2 and Table 1).

The above substrate specificities of CPL and LIP toward unsaturated long-chain alcohols having the first *cis*-olefinic bond at the $\Delta^9$- or $\Delta^6$-position have not, to our knowledge, been reported for lipases so far.

Our studies also document the good applicability of CPL for the synthesis of caprylate esters of primary medium-chain aliphatic alcohols. The high degree of selectivity for medium-chain alcohols expressed by CPL, as compared to the microbial lipases NOV and LIP, could be exploited in specialized applications such as selective enrichment of one or more constituents from a reaction mixture *via* kinetic resolution.

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