Specificity of Papaya Lipase in Esterification with Respect to the Chemical Structure of Substrates

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Esterification, catalyzed by papaya (*Carica papaya*) lipase (CPL), was studied with various alcohols and carboxylic acids under competitive conditions. Acids studied were straight-chain saturates of different chain lengths, with octanoic acid as the reference. Alcohols chosen were aliphatic straight-chain, branched, secondary, tertiary, terpene, and aromatic alcohols of different chain lengths, using 1-hexanol as the reference. The initial reaction rate increased with increasing chain length of the acid from C4:0 to C18:0, followed by a slight decrease with C20:0. In the case of alcohols, an optimum chain length of 8 carbon atoms was obtained for the straight-chain aliphatic group (C2 to C16). Ethanol, 1-propanol, and secondary and tertiary alcohols showed rather low reactivity. Branching of the alcohols was found not to affect the reactivity in esterification; among the terpenes, β-citronellol [(2*E*)-3,7-dimethyl-6-octenol] and geraniol [(2*E*)-3,7-dimethylocta-2,6-dien-1-ol] were found to be more reactive than nerol [(2*Z*)-3,7-dimethylocta-2,6-dien-1-ol]. The highest reaction rate was found for the aromatic benzyl alcohol (phenylmethanol).

Keywords: *Papaya (Carica papaya) lipase; esterification; ester; alcohol; acid*

INTRODUCTION

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) in nonaqueous media can efficiently catalyze the reversal of hydrolysis (Zaks and Klibanov, 1985). This possibility has enhanced the prospects of application of lipases in synthetic reactions (Macrae, 1983; Mustranta et al., 1993). Notwithstanding this enormous potential, the actual application of lipases in industry has been restricted mainly due to their high cost and inadequate availability (Gandhi, 1997). With this in mind, one major focus of research efforts, lately, has been channeled toward the identification of inexpensive lipases, which can be obtained in bulk quantities required for industrial applications. One lipase with such a potential is that present in the papaya (*Carica papaya*) latex.

The crude latex preparation (papain) derived from papaya has been found to contain lipolytic activity (Giordani et al., 1991). The activity of papaya lipase (CPL) with respect to different glyceride substrates or their derivatives has been the subject of various research investigations. Focus of research on applications of CPL as biocatalyst in transesterification has mainly been on the synthesis of structured triacylglycerols (Foglia and Villeneuve, 1997; Mukherjee and Kiewitt, 1998; Villeneuve et al., 1995, 1997a,b). Esterification using CPL has received relatively less attention (Mukherjee and Kiewitt, 1996). The subject of the latter study was the enrichment of definite fatty acids from mixtures via selective esterification catalyzed by CPL.

The objective of this study was to further probe the feasibility of utilizing CPL for ester synthesis. Esters have numerous applications notably in food industries as flavoring components (Bauer et al., 1990), in cosmetics, as biodiesel, as synthetic intermediates, and as plasticizers and lubricants, etc. (Gandhi, 1997). Lipase-catalyzed synthesis of these esters has received considerable attention (Claon and Akoh, 1993; Gandhi et al., 1995; Gillies et al., 1987; Iwai et al., 1990; Langrand et al., 1990; Mukherjee and Kiewitt, 1988; Welsh and Williams, 1990). The present study reports the reactivity of CPL with different types of carboxylic acids and alcohols during esterification.

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MATERIALS AND METHODS

Materials. CPL was obtained as a crude powder from Sigma-Aldrich-Fluka, Deisenhofen, Germany. The granular latex preparation was ground in a mortar with a pestle to a fine powder and sieved to 0.8-mm mesh size. Ethyl octanoate, 1-propyl octanoate, 1-butyl octanoate, fatty acids, alcohols, and 2',7'-dichlorofluorescein were also procured from Sigma-Aldrich-Fluka. Solvents and silica gel 60 were purchased from E. Merck, Darmstadt, Germany.

Synthesis of Hexyl Esters. Two reactions were carried out with 1-hexanol and different equimolar mixtures of carboxylic acids: reaction I − butanoic, hexanoic, octanoic, decanoic, and dodecanoic acids; reaction II − octanoic, tetradecanoic, hexadecanoic, octadecanoic, and eicosanoic acids. The reaction mixture consisting of 10 mmol of 1-hexanol and 5 mmol of carboxylic acid mixtures (1 mmol of each acid) was heated to 63 °C in a magnetically stirred, Teflon-lined, screw-capped reaction vial, and the reaction was initiated by the addition of 54 mg of CPL. The temperature was maintained at 63 °C. Sample aliquots of 50 μL were withdrawn at specified intervals during the 24-h reaction period, taken up in dichloromethane, and centrifuged to remove the residual CPL granules, and the supernatants were analyzed.

Synthesis of Alkyl Octanoates. Five reactions were carried out using octanoic acid and the following equimolar mixtures of alcohols: reaction III − 1-butanol, 1-hexanol, 1-octanol, and 1-decanol; reaction IV − 2-butanol, isoamyl alcohol (3-methyl-1-butanol), 1-hexanol, and β-citronellol; reaction V − *tert*-butanol (2-methyl-2-propanol), 1-hexanol, benzyl alcohol, and geraniol; reaction VI − 2-propanol, 1-butanol,

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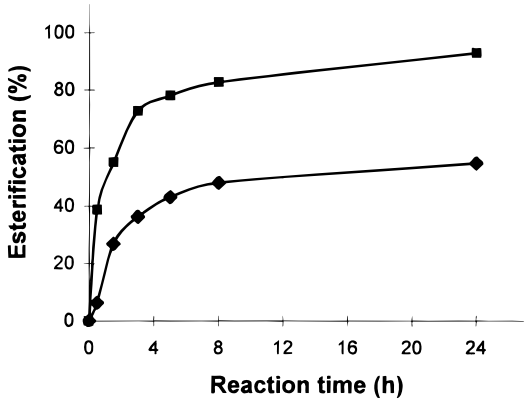

Figure 1. Time course of esterification of 1-hexanol with equimolar mixtures of straight-chain carboxylic acids catalyzed by CPL: reaction I (◆), butanoic + hexanoic + octanoic + decanoic + dodecanoic acids; reaction II (■), octanoic + tetradecanoic + hexadecanoic + octadecanoic + eicosanoic acids.

1-hexanol, and α-terpineol (1-[1-hydroxy-1-methyl]ethyl-4-methylcyclohex-3-ene); reaction VII − ethanol, 1-propanol, 1-hexanol, and nerol; reaction VIII − 1-hexanol, 1-dodecanol, 1-tetradecanol, and 1-hexadecanol; reaction IX − 1-propanol, 1-hexanol, benzyl alcohol, and nerol. Reaction mixtures consisted of 5 mmol of octanoic acid and 10 mmol of alcohol mixtures (2.5 mmol of each alcohol). The rest of the procedure was as for hexyl ester synthesis described above.

Preparation of Standard Esters. Ester standards for the determination of retention times in gas chromatographic (GC) analysis were prepared by reacting an excess of an alcohol with the desired acid at 80 °C for 2 h, using concentrated sulfuric acid (0.4% w/w of the reactants) as a catalyst. Esters were extracted using isohexane, the extract washed till neutral with distilled water, and the organic layer dried with anhydrous sodium sulfate. The esters were subjected to thin-layer chromatography (TLC) on silica gel H using isohexane:diethyl ether:acetic acid (80:20:1, v/v/v) in order to separate the ester fraction from other impurities. Chromatoplates were dried and sprayed with 0.1% (w/v) solution of 2′,7′-dichlorofluorescein in ethanol; the ester fraction was marked under ultraviolet light and scraped off. These were eluted with water-saturated diethyl ether. After removal of diethyl ether by evaporation, the esters were dissolved in isohexane and used as standards for GC analysis.

Gas Chromatography. Aliquots of reaction products to which a known amount of methyl tetradecanoate (reactions I−IV, VIII) or methyl heptadecanoate (reactions V−VII, IX) was added as internal standard were subjected to GC analysis. A Hewlett-Packard (Böblingen, Germany) HP-5890 series II instrument equipped with a flame ionization detector was used. Esters were separated on a 0.25-μm CS-FFAP-CB free fatty acid phase fused silica capillary 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·s^{-1}). The split ratio was 1:10, and the injector as well as flame ionization detector temperatures were 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. The temperature programs used for the different reactions were as follows: reactions I, III−VII, IX − 5 min at 100 °C, followed by linear heating at 4 °C·min^{-1} to 220 °C, finally at 220 °C for 3 min; reactions II, VIII − linear heating at 4 °C·min^{-1} from 160 to 240 °C, finally at 240 °C for 5 min.

RESULTS AND DISCUSSION

Selectivity of Papaya Lipase toward Carboxylic Acids. Esterification reactions were carried out under competitive conditions between 1-hexanol and an equimolar mixture of C4:0 to C12:0 acids (reaction I) and an equimolar mixture of C8:0 and C14:0 to C20:0 acids (reaction II), using CPL as the enzyme. 1-Hexyl ester formation was found to increase with time in both reactions (Figure 1). While the reaction with the mixture of C4 to C12 acids proceeded to 54% after 24 h, a higher esterification yield of 92% was obtained with the mixture of C8 together with the longer-chain C14 to C20 acids.

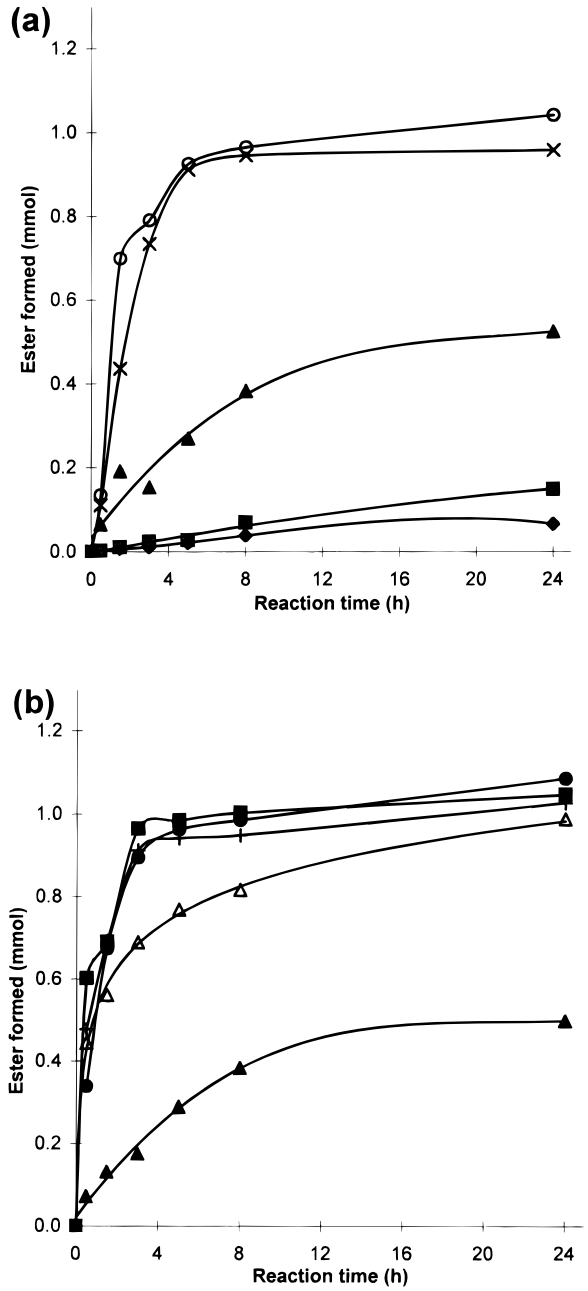

Figure 2. Time course of formation of individual 1-hexyl esters during esterification of 1-hexanol with equimolar mixtures of different carboxylic acids catalyzed by CPL: (a) reaction I, butanoic (◆) + hexanoic (■) + octanoic (▲) (reference standard) + decanoic (×) + dodecanoic (○) acids; (b) reaction II, octanoic (▲) (reference standard) + tetradecanoic (●) + hexadecanoic (+) + octadecanoic (■) + eicosanoic (△) acids.

Figure 2 shows the extent of formation of individual esters during esterification of equimolar mixtures of acids with 1-hexanol, catalyzed by CPL. It is evident from Figure 2a that the plateauing occurred around 2−4

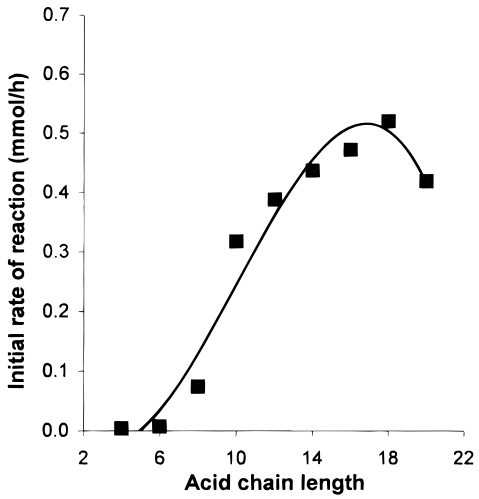

Figure 3. Dependence of initial rate of reaction on acid chain length in the esterification of 1-hexanol with equimolar mixtures of different carboxylic acids catalyzed by CPL.

h in the case of C10:0 and C12:0 acids whereas the longer-chain acids, notably C16:0 and C18:0, showed a linear increase of conversions only within the first 2 h (Figure 2b).

The extent of synthesis of both 1-hexyl butyrate and 1-hexyl caproate was low, and even after 24 h, only about 0.07 and 0.15 mmol of esters, respectively, were formed (Figure 2a). Both the conversions (Figure 2a,b) and the initial reaction rates calculated from the gradient of the tangent to the curves of best fit obtained from the data by statistical analysis using MS Excel software (Figure 3) were found to increase with an increase in chain length of the carboxylic acid, especially from C10 onward. The optimum acid chain length appears to be C18 (Figure 3), and eicosanoic acid gave lower conversion (Figure 2b) and rate (Figure 3) for 1-hexyl ester formation. The extent of 1-hexyl octanoate synthesis under competitive conditions was similar for both reactions I and II (Figure 2a,b).

The evident preference for the longer-chain carboxylic acids in esterification with 1-hexanol (Figures 1−3) was similar to our previous observations on the increase in incorporation with increasing acyl chain length during the interesterification of tripalmitin with ethyl esters of fatty acids as acyl donors (unpublished results). Increase in the extent of esterification with increasing chain length of the acid was also reported for the esterification of C6 to C10 acids with C10 to C18 alcohols using immobilized *Rhizomucor miehei* lipase (Ucciani et al., 1996) and in the esterification of various short-chain acids with C1 to C6 alcohols and terpene alcohols using *Rhizopus arrhizus* and *R. miehei* lipases (Langrand et al., 1990). Also in the geranyl ester synthesis catalyzed by *Rhizopus oryzae* lipase, C2 to C4 acids were converted to the extent of 31%, 86%, and 95%, respectively (Molinari et al., 1995).

Selectivity of Papaya Lipase toward Alcohols. Esterification reactions were carried out between octanoic acid and equimolar mixtures of different combinations of alcohols using CPL as biocatalyst.

Figure 4 shows that a maximum of 75% esterification could be attained after 24 h in the case of reaction III, in which an equimolar mixture of primary C4 to C10 alcohols was taken. In case of the longer-chain alcohols, such as in reaction VIII (C6, C12 to C16), only about 22% esterification was obtained at the end of the

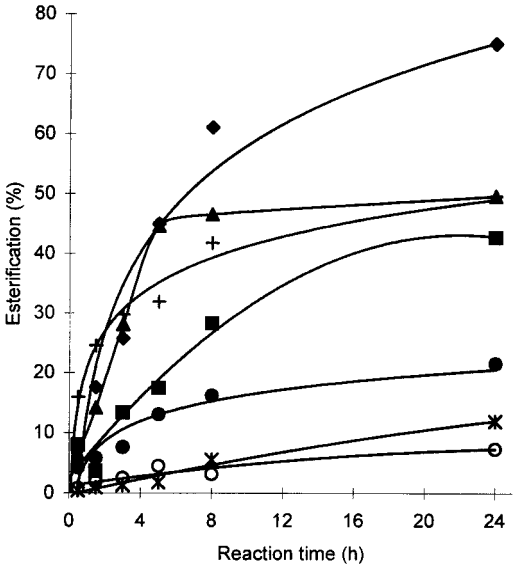

Figure 4. Time course of esterification of octanoic acid with equimolar mixtures of various alcohols catalyzed by CPL: reaction III (◆), 1-butanol + 1-hexanol + 1-octanol + 1-decanol; reaction IV (■), 2-butanol + isoamyl alcohol + 1-hexanol + β-citronellol; reaction V (▲), *tert*-butanol + 1-hexanol + benzyl alcohol + geraniol; reaction VI (○), 2-propanol + 1-butanol + 1-hexanol + α-terpineol; reaction VII (∗), ethanol + 1-propanol + 1-hexanol + nerol; reaction VIII (●), 1-hexanol + 1-dodecanol + 1-tetradecanol + 1-hexadecanol; reaction IX (+), 1-propanol + 1-hexanol + benzyl alcohol + nerol.

reaction, indicating a preference of CPL for alcohols with 4−10 carbon atoms (Figure 4). An optimal chain length of C4 was found in the esterification of C2 to C16 alcohols with oleic (*cis*-9-octadecenoic) acid, catalyzed by another plant lipase, that from rape (*Brassica napus*) (Hills et al., 1990). In the esterification of dodecanoic acid with C2 to C10 alcohols using a lipase from *Humicola lanuginosa* (SP398), an increase in conversion after 24 h with increasing chain length of alcohols from C2 to C8, followed by a marginal decrease in the case of 1-decanol, has been reported (McNeil and Berger, 1995).

Figure 5 shows as examples the time course of alkyl octanoate formation in reactions III, V, and VIII. The following is evident from these figures. With the series of primary aliphatic alcohols (C4 to C16), an increase in the extent of esterification yields with increasing chain length from C4 to C8 was observed; a further increase in the chain length of the alcohol up to C16 led to a gradual decrease (Figure 5). In each case, esterification leveled off after about 6−8 h.

1-Hexyl octanoate synthesis was found to be reduced when 1-hexanol was accompanied by geraniol, benzyl alcohol, and *tert*-butanol (reaction V; Figure 5b). This is very likely due to the high rate of conversion of benzyl alcohol which was found to be the best alcohol substrate for CPL under the experimental conditions used. Thus, the results of Figure 5c might be explained by 'substrate competition'.

A plot of the initial rates of esterification of individual alcohols (calculated from the gradient of the tangent to the curves of best fit obtained from the data by statistical analysis using MS Excel software) relative to that of 1-hexyl octanoate synthesis (the reference reaction) is shown in Figure 6. Salient features are as follows. Among the primary aliphatic alcohols, an optimal reaction rate for a chain length of C8 was found, yielding

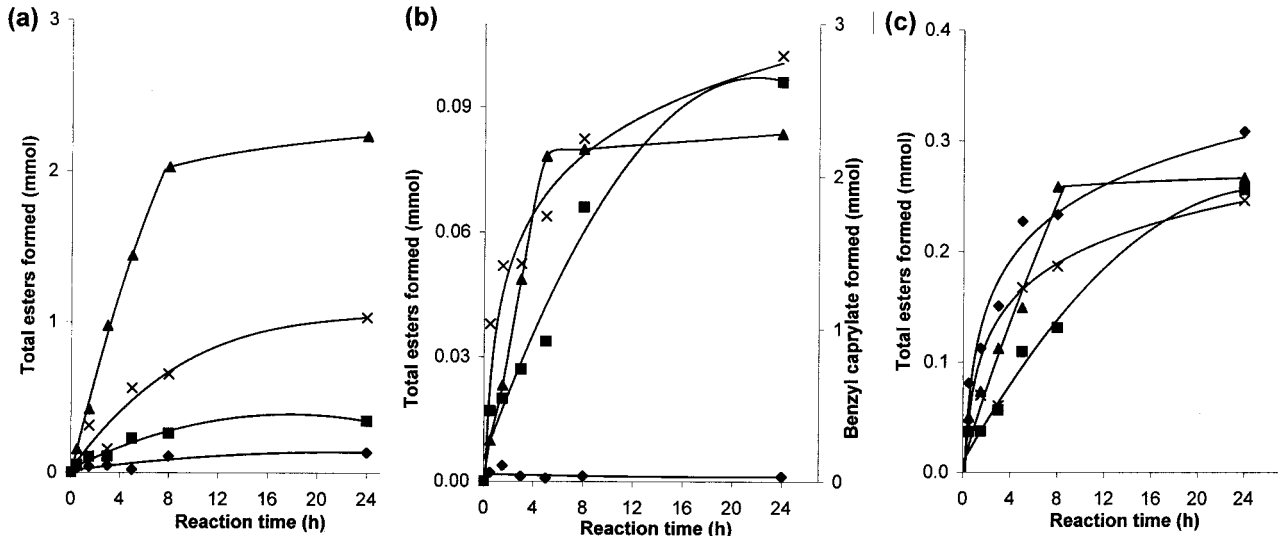

Figure 5. Time course of formation of alkyl octanoates during esterification of octanoic acid with equimolar mixtures of various alcohols catalyzed by CPL: (a) reaction III, 1-butanol (◆) + 1-hexanol (■) (reference standard) + 1-octanol (▲) + 1-decanol (×); (b) reaction V, *tert*-butanol (◆) + 1-hexanol (■) (reference standard) + benzyl alcohol (▲) + geraniol (×); (c) reaction VIII, 1-hexanol (■) (reference standard) + 1-dodecanol (◆) + 1-tetradecanol (▲) + 1-hexadecanol (×).

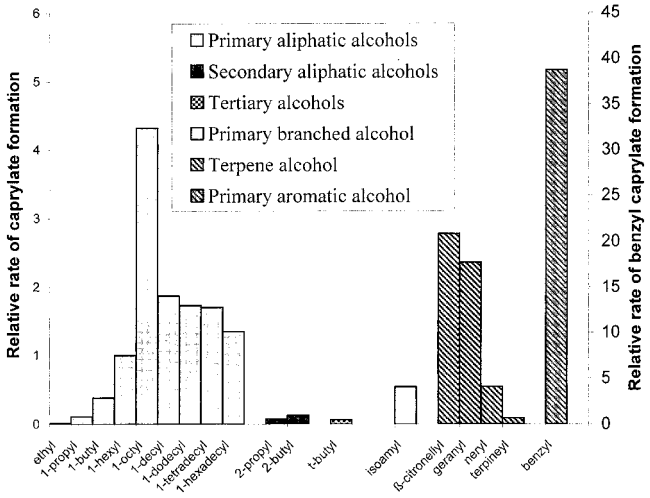

Figure 6. Rates of formation of alkyl octanoates relative to rate of formation of 1-hexyl octanoate during esterification of octanoic acid, catalyzed by CPL, with equimolar mixtures of various alcohols containing 1-hexanol as reference standard.

a bell-shaped profile (Figure 6). This is in contrast to the linear decrease of initial rate of esterification with increase in alcohol chain length obtained for the synthesis of alkyl oleates ((*Z*)-9-octadecenoates) of C3 to C16 alcohols using the immobilized *R. miehei* lipase (Habulin et al., 1996). Similarly in the synthesis of alkyl oleates, gondoates ((*Z*)-11-eicosenoates), and erucates ((*Z*)-13-docosenoates), the above lipase gave an increase in synthetic yields with increasing alcohol chain length from C10 to C18 (Ucciani et al., 1996). However, with C6, C8, or C10 acids, the esterification appeared to be independent of the chain length of the alcohol (Ucciani et al., 1996). On the other hand, in an earlier study (Gandhi et al., 1995), a bimodal pattern for alcohol specificity was found for synthesis of alkyl laurates and oleates using the *R. miehei* lipase with maxima for 1-hexanol and 1-decanol.

The highest relative reaction rate was obtained in the case of benzyl alcohol which was more than 9 times as reactive as 1-octanol (Figure 6). This could imply a high affinity of CPL for aromatic alcohols as compared to aliphatic ones. In contrast, the rape lipase did not accept benzyl alcohol as substrate (Hills et al., 1990).

Among the terpene alcohols, both β-citronellol and geraniol showed higher rates of esterification than 1-decanol although they have the same carbon number. The order of reactivity was found to be β-citronellol $\gg$ geraniol > nerol $\gg$ terpineol (Figure 6). The last one is a tertiary alcohol, and CPL behaves similar to most known lipases, which do not accept tertiary alcohols as substrates due to steric effects. β-Citronellol, geraniol, and nerol, on the other hand, are primary alcohols with basically a C8 template similar to 1-octanol and differing in unsaturation, substitution, and orientation of the substituents. β-Citronellol [(2*E*)-3,7-dimethyl-6-octenol] has one double bond at the sixth carbon and has two methyl group substituents at the third and seventh carbons. Its reduced reactivity relative to that of 1-octanol (Figure 6) may therefore imply improper fitting into the alcohol binding site of CPL. Geraniol [(2*E*)-3,7-dimethylocta-2,6-dien-1-ol] has another double bond even closer to the hydroxy group making it an even weaker substrate for CPL (Figure 6). The only difference in the structures of nerol [(2*Z*)-3,7-dimethylocta-2,6-dien-1-ol] and geraniol is the orientation of the substituent methyl groups, with geraniol having an *E*-orientation and nerol being its *Z*-isomer. Obviously, the *E*-isomer is a more preferred substrate for CPL as compared to its *Z*-counterpart. The greater affinity of CPL for β-citronellol as compared to geraniol makes it different from some widely used lipases such as those from *R. miehei*, *Candida antarctica*, and *Aspergillus niger* which demonstrated the reverse reactivity during the synthesis of C2 to C8 esters (Claon and Akoh, 1993).

Isoamyl alcohol yielded an intermediate value of relative reaction rate between those of 1-butanol and 1-hexanol (Figure 6), indicating that it is recognized by the enzyme as a simple C5 alcohol and that the branching possibly has no effect on either alcohol binding to the lipase or esterification.

Little reaction was obtained with 2-propanol, 2-butanol, *tert*-butanol, and terpineol (Figure 6) indicating the poor affinity of CPL for secondary and tertiary alcohols, respectively, owing probably to steric factors.

This effect was similar to that reported for oleate synthesis with these alcohols using the rape lipase (Hills et al., 1990).

In conclusion, it has been demonstrated through this study that CPL can be effectively used as a biocatalyst for the production of esters. In the case of primary aliphatic carboxylic acids, CPL shows a preference for longer chains resulting in a sigmoid curve for rate versus chain length, whereas a bell-shaped curve is obtained in the case of alcohols with a maximum at C8. The lipase has been shown to possess a high discriminating ability toward different alcohols depending upon their chain length and configuration. While certain features, such as the number of double bonds in the alcohols and their geometrical configuration can have a negative influence on the ability of the lipase to accept them as substrates, others such as aromaticity might possibly exert a positive influence. Branching of the alcohol has negligible effect on its reactivity. Both the secondary and tertiary alcohols are poor substrates for CPL, owing to steric effects.

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