Stereoselective Acylation of DL-Menthol in Organic Solvents by an Immobilized Lipase from *Pseudomonas cepacia* with Vinyl Propionate

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ABSTRACT: An effective lipase-catalyzed stereoselective transesterification of (±)-menthol in organic solvent with vinyl propionate as acylating agent is described. Immobilization by adsorption and the presence of molecular sieves improved the formation of (±)-menthyl propionate by lipase (PS-30) from *Pseudomonas cepacia*. The reaction time course, mole ratio of substrates, temperature, amount of enzyme, as well as the effect of various organic solvents, were examined for their influence on the enzymatic stereoselective formation of (–)-menthyl propionate. Among the parameters studied, the stereospecificity toward (–)-menthol decreased significantly as temperature increased but the yields of both enantiomers increased. Organic solvents with log *P* (partition coefficient) values above 3.5 gave higher yield and stereoselectivity than solvents with lower log *P* values.

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KEY WORDS: Acylating agent, lipase, (±)-menthol, (±)-menthyl propionate, *Pseudomonas cepacia*, stereoselective transesterification, vinyl propionate.

In recent years, the use of lipases as biocatalysts for enzymatic resolution of racemic substances has increasingly attracted research interest because of their demonstrated high enantioselectivity compared to chemical catalysts (1). In addition, lipases are readily available, relatively inexpensive, and have no cofactor requirement (2).

Among the wide range of lipase-catalyzed reactions that have been studied, certain types of acylating agents, such as acid anhydrides and enol esters, have been recommended because they make the acyltransfer completely irreversible (3–7). Previously, we have successfully achieved the enantioselective resolution of (±)-menthol by a lipase ("AY-30," from *Candida cylindracea*) with acid anhydrides as acylating agents (8). Menthol is a secondary terpene alcohol obtained from *Mentha piperita* and *M. arvenisis*. In the present report, we chose lipase-catalyzed irreversible transesterification with vinyl esters in place of acid anhydrides because of the difficulty we encountered in eliminating the background chemical acylation that resulted from acid anhydrides. Our preliminary screening indicated that lipase PS-30 appeared to be the most promising biocatalyst under the experimental conditions examined, and that high enantioselectivity could be achieved with this lipase when we used vinyl propionate for acylation of (±)-menthol. The (–)-menthol and its esters are more important from an industrial point of view than (±)-menthol. Because of its cooling and refreshing effects, (–)-menthol is an important fragrance and flavor compound that is used largely in cosmetics, toothpastes, chewing gum, cigarettes, sweets, and medicines. It also possesses the characteristic peppermint odor, which is lacking in other isomers. The (±)-menthol cooling effect is not so distinct as that of (–)-menthol, and therefore, it is not highly valued. However, it can be used in medicine and liniments.

In this study, we report the use of an immobilized lipase for the stereoselective transesterification of (±)-menthol with vinyl propionate as acyl donor. Reaction parameters were also studied.

EXPERIMENTAL PROCEDURES

Enzymes and chemicals. Lipase "PS-30" (specific activity 34 IU/mg solid) from *Pseudomonas cepacia* was kindly supplied by Amano Enzyme Co. (Troy, VA). The racemic secondary alcohol dl-menthol, i.e., (±)-menthol, and (–)-menthol were obtained from Eastman Kodak Company (Rochester, NY) and Sigma Chemical Co. (St. Louis, MO), respectively. Vinyl propionate was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All solvents were high-performance liquid chromatography-grade and were from either Fisher Scientific (Norcross, GA) or J.T. Baker Chemical Co. (Phillipsburg, NJ). Molecular sieves, 4Å, were from Davison Chemical (Baltimore, MD) and were activated in a vacuum oven prior to use.

Enzymatic transesterification reactions. Transesterification was carried out in 20 × 125-mm screw-capped culture

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tubes with Teflon liners in a gyratory waterbath shaker (New Brunswick Scientific Co., Inc., Edison, NJ) at 30°C, 200 rpm, for 48 h in 2 mL of an organic solvent. Lipase PS-30 in the amount of approximately 680 units (w/w, soluble enzyme/total reactants) was used as the biocatalyst and added immediately before the incubation started. A control without the presence of a lipase was simultaneously incubated to monitor any chemical background reaction. No product was generated by the control reaction. Molecular sieves (approximately 0.1 g) were added 2 h after the incubation started. The time course study was conducted by taking samples at 1, 2, 3, 4, 5, 6, 12, 24, 36, and 48 h. The effect of various organic solvents, including *n*-hexane, isooctane, *n*-heptane, cyclohexane, benzene, toluene, methylene chloride, and chloroform, on the lipase-catalyzed enantioselective synthesis of menthyl propionate was examined by mixing 1 mmole of (±)-menthol and 1 mmole of vinyl propionate in 2 mL of solvent and incubating at 30°C in the presence of lipase PS-30 for 48 h. The effects of enzyme load, mole ratio of substrates, and reaction temperature on the transesterification were also examined. All reactions were performed in duplicate, and results are reported as means ± standard deviation.

Immobilization of lipase PS onto glass beads by adsorption. Approximately 1 g of powdered lipase PS was mixed well with 2.5 mL of 10 mM phosphate buffer, pH 7, at room temperature to form a slurry. The enzyme slurry was mixed in a 50-mL Pyrex beaker with 5 g of glass beads (0.5-mm diameter, BiospecProducts, Bartlesville, OK) which had been rinsed twice with 5 mL deionized water and dried at 110°C in an oven. The mixture of enzyme and glass beads was then placed in a desiccator filled with Drierite (W.A. Hammond Drierite Company, Xenia, OH) and stirred thoroughly at 4-h intervals for 24 h and then stirred after 18 and 24 additional hours. The activity of immobilized lipase was estimated based on the mass of enzyme adsorbed onto glass beads after water and excess enzyme on the wall of the beaker had been removed.

Quantitation of menthyl esters by gas chromatography (GC). An aliquot of 50 µL of the reaction product in organic medium was pipetted and diluted in 1 mL hexane that contained 0.875 mg ethyl caproate (Aldrich Chemical Company, Inc.) as internal standard. The residual moisture in the solvent and the enzymes was removed by passing the hexane solution through an anhydrous sodium sulfate (Fisher Chemical, Fair Lawn, NJ) column. Quantitation was achieved with a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA), equipped with a flame-ionization detector and DB-5 fused-silica capillary column (30 m × 0.32 mm i.d., film thickness 1 µm; J&W Scientific, Folsom, CA), operated isothermally at 170°C. The injector and detector temperatures were 250 and 260°C, respectively. The carrier gas was helium at a flow of 5 mL/min. The percentage mole yield of menthyl propionate was defined as (mmole of menthyl propionate/initial mmole of menthol present in the system) × 100 and was estimated from peak areas that were integrated by an on-line computer.

Chiral capillary GC separation of (±)-menthols and (±)-menthyl propionates. A Varian 3300 gas chromatograph (Sunnyvale, CA), equipped with a flame-ionization detector and a chiral Beta-DEX^{TM} 120 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Supelco Inc., Bellefonte, PA), was used to separate and identify the optically active menthyl propionate produced by enzymatic reaction. The injector and detector temperatures were 200 and 225°C, respectively. The temperature program used to separate (+)- and (−)-menthyl propionates was held at 110°C for 12 min before being elevated to 150°C at 4°C/min and then held for 5 min. The flow rate of the carrier gas, helium, was 0.8 mL/min. The amount of each enantiomer was estimated from the peak area recorded and integrated by an LDC/Milton Roy CI-10B integrator (Riviera Beach, FL). The retention times for (+)-menthol, (−)-menthol, (−)-menthyl propionate, and (+)-menthyl propionate were 20.4, 20.5, 23.8, and 24.1 min, respectively.

The stereoselectivity and optical purity of the enzymatic reaction were expressed by the enantiomeric ratio E, defined (9) as (−)/(+), as well as the enantiomeric excess ee%, which is equal to the absolute value of percentage excess of one enantiomer over the other: ee% = {[−] − [+]/[−] + [+]} × 100 (10).

Optical purity of substrate (±)-menthol. A 1% (wt/vol) solution of (±)-menthol or (−)-menthol in 95% ethanol was prepared for examination of optical purity. The optical rotation of the substrate (±)-menthol was determined by an Autopol® IV automatic polarimeter (Rudolph Research, Flanders, NJ) and proven to be racemic.

Preparation of standard menthyl propionates. The standard (±)-menthyl and (−)-menthyl propionates were prepared by refluxing (±)-menthol and propionic anhydride in pyridine for 2 h to determine their retention time in capillary GC. The reaction product was washed with water, extracted with petroleum ether, and monitored by capillary GC to ensure complete reaction.

RESULTS AND DISCUSSION

The use of immobilized lipase PS-30 and presence of molecular sieves improved the overall yield of (±)-menthyl propionate from 16 to 35% (Table 1). Immobilization exposes the enzyme more effectively to the substrate (11) and therefore improves the formation of menthyl propionate. The presence of molecular sieves in the system was first recommended by Degueil-Castaing *et al.* (7) to remove water that is liberated from side reactions of acetaldehyde that tautomerizes from vinyl alcohol, a product of the lipase-catalyzed transesterification. The immobilized lipase was used for reaction parameter studies, and molecular sieves were always included in the reaction.

Figure 1 shows the time course of formation of (−)-menthyl propionate. At 30°C, nearly 50% of (−)-menthol was converted to the ester form after 48 h incubation. The reaction rate was comparable to enzymatic resolutions of (±)-menthol obtained previously with lipase from *C. cylindracea* (1,12).

TABLE 1
Percentage Yields of (±)-Menthyl Propionate by Lipase PS-30 at 30°C as Affected by Enzyme Immobilization and Addition of Molecular Sieves[a]

Molecular sieves	Unimmobilized PS	Immobilized PS
No	16.2 ± 2.1	22.6 ± 2.8
Yes	22.6 ± 1.8	34.5 ± 5.3

[a]Reaction was performed in duplicate in hexane at a 1:1 mole ratio of (±)-menthol to vinyl propionate. The amount of enzyme was constant. Values are reported as means ± standard deviation.

The effect of the mole ratio of menthol and vinyl propionate was studied at levels from 1:1 to 1:3 (Fig. 2). There was not much difference across the three levels examined in both yield and stereoselectivity (i.e., E values ranged from 121 to 155). It appears that a 1:1 ratio of the substrates is in fact sufficient for satisfactory resolution of (±)-menthol.

It is widely believed and supported by experimental observations that the stereoselectivity of enzymes is affected by reaction temperature (9). The effect of temperature after 48 h incubation is summarized in Table 2. The mole percentage yield of (–)-menthyl propionate increased from 50 to 86% as reaction temperature increased. However, the differences within the temperature ranges 30–40°C and 50–60°C were not significant. The highest increase in yield occurred between 40–50°C. The stereoselectivity, on the other hand, decreased as reaction temperature increased (Table 2). Apparently, a higher chemical yield could be obtained at elevated temperature at the expense of stereochemical purity of the products. We decided not to go over 60°C to avoid possible loss of terpene esters as reported previously (13).

The amount of enzyme used can be a crucial economical factor when future industrial application is kept in mind. As expected, the percentage yield of (–)-menthyl propionate increased from 20 to 67% when the enzyme load was increased from 100 to 700 units (Fig. 3). The increase was rather slow after reaching 300 units. If the cost of enzyme is a problem, a higher level of enzyme may not be necessary.

It is widely believed that the nature and polarity of organic media in which enzymatic reactions take place affect the activity of biocatalyst (14,15). The polarity of organic solvents can be quantitatively measured by the value of log *P*, the logarithm of the partition coefficient of a given solvent between water and 1-octanol. Organic solvents with log *P* values <2.0 are generally not considered good for biocatalysis (13). In this study, a few commonly used organic solvents were examined for their suitability for lipase-catalyzed stereoselective reso-

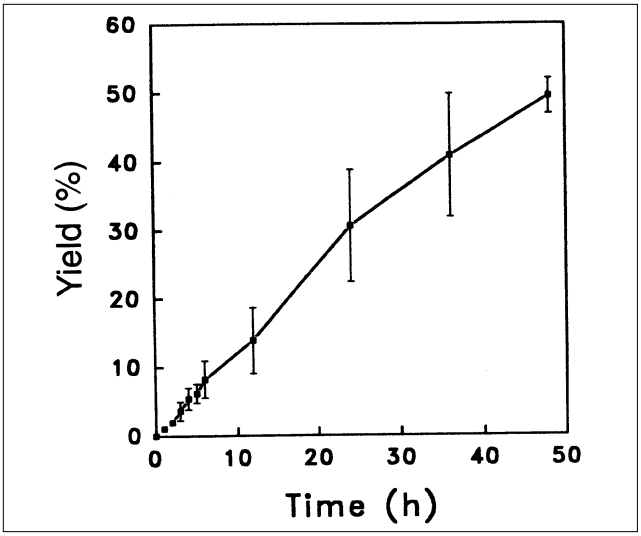

FIG. 1. Time course (0, 6, 12, 18, 24, 36, 48 h) of formation of (–)-menthyl propionate by immobilized lipase PS-30 at 30°C. Reactions were performed in *n*-hexane with a 1:1 mole ratio of (±)-menthol to vinyl propionate. Bars indicate standard deviation.

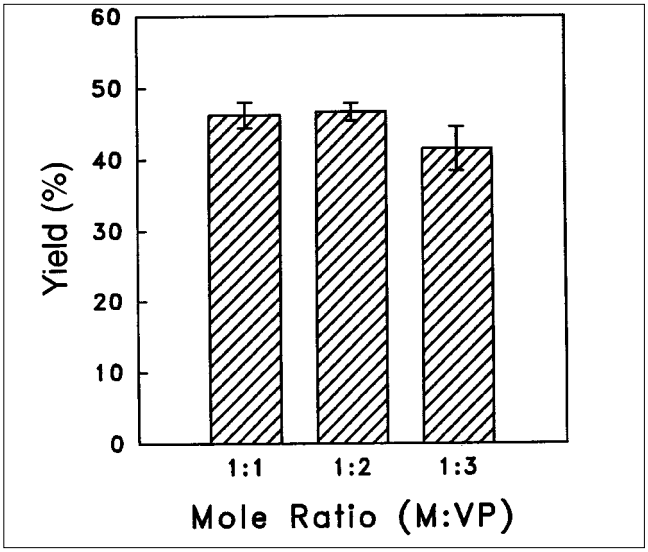

FIG. 2. Effect of (±)-menthol to vinyl propionate ratio (1:1, 1:2, and 1:3) on formation of (–)-menthyl propionate by immobilized lipase PS-30 in *n*-hexane at 30°C. M = menthol and VP = vinyl propionate. See Figure 1 for explanation of bars.

TABLE 2
Values of E, ee%, and Yields of Menthyl Propionate Synthesized by Immobilized Lipase PS as Affected by Temperature[a]

Temperature (°C)	E	ee%	% Yield (–)-menthyl propionate
20	322 ± 10	99.4 ± 0.1	50.2 ± 11.8
30	156 ± 23	98.7 ± 0.2	60.0 ± 10.6
40	97 ± 42	97.7 ± 1.0	58.5 ± 18.4
50	47 ± 18	95.5 ± 1.9	87.2 ± 4.9
60	31 ± 6	93.7 ± 1.1	86.4 ± 3.7

[a]Reactions were performed in hexane at a 1:1 mole ratio of (±)-menthol to vinyl propionate. E, enantiomeric ratio; ee%, enantiomeric excess. The values are reported as means ± standard deviation and were obtained by chiral gas–liquid chromatography with a Beta-DEX™ 120 fused-silica capillary column (Supelco Inc., Bellefonte, PA). See the Experimental Procedures section for separation conditions.

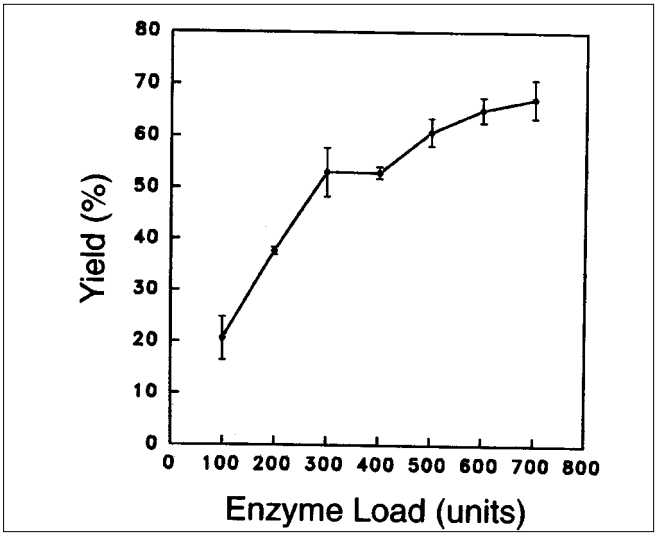

FIG. 3. Formation of (–)menthyl propionate by immobilized lipase PS at 30°C as affected by levels of enzyme. Reactions were performed in *n*-hexane with a 1:1 mole ratio of (±)-menthol to vinyl propionate. See Figure 1 for explanation of bars.

lution of (±)-menthol with vinyl propionate as acyl donor (Table 3). Synthesis was also attempted in methylene chloride, but no reaction occurred, probably due to its high polarity. Chloroform (log P = 2.0) only gave an overall yield of 2.2% for (±)-menthyl propionate, and the optical purity of the products in chloroform could not be estimated because the amount of (+)-menthyl propionate was below GC detection level. After incubating at 30°C for 48 h, the percentage yield of (–)-menthyl propionate formed in isooctane, *n*-heptane, and *n*-hexane reached approximately 55%, and the E and ee% values were as high as 200 and 99%, respectively, which indicates that these solvents probably can be used as media for stereoselective synthesis of (–)-menthyl propionate. Cyclohexane, toluene, and benzene gave low yields but good E and ee% values. Although the log P values did not always correlate with the degree of enzymatic synthesis, it may still provide valuable information in choosing suitable organic solvents for this type of reactions.

TABLE 3
Percentage Yield, E value, and ee% of (–)-Menthyl Propionate Synthesized from (±)-Menthol and Vinyl Propionate at 30°C by Immobilized PS-30 as Affected by Selected Organic Solvents[a]

Solvent	Log P value[b]	% Yield (–)-menthyl propionate	E	ee%
Isooctane	4.5	57.0 ± 14.9	177 ± 31	98.9 ± 0.2
n-Heptane	4.0	53.4 ± 4.6	215 ± 25	99.1 ± 0.1
n-Hexane	3.5	56.4 ± 9.0	191 ± 83	98.8 ± 0.5
Cyclohexane	3.2	39.7 ± 20.9	160 ± 16	98.8 ± 0.1
Toluene	2.5	27.7 ± 6.5	153 ± 27	98.7 ± 0.2
Benzene	2.0	30.5 ± 3.4	119 ± 2	98.4 ± 0.1

[a]Reactions were performed at a 1:1 mole ratio of (±)-menthol to vinyl propionate. Values are reported as means ± standard deviation. See Table 2 for abbreviations and reaction conditions.
[b]Source: Reference 15.

We have demonstrated an effective means for achieving stereoselective resolution of (±)-menthol with vinyl propionate as the acylating agent and immobilized lipase PS-30 as the biocatalyst. For practical applications, a 50% overall conversion of (±)-menthol to (–)-menthyl propionate at 30°C may represent a suitable index where the reaction can be stopped because the lipase-catalyzed reaction is highly stereospecific in nonpolar solvents.

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REFERENCES

1. Kamiya, N., M. Goto, and F. Nakashio, Surfactant-Coated Lipase Suitable for the Enzymatic Resolution of Menthol as a Biocatalyst in Organic Media, *Biotechnol. Prog. 11*:270–275 (1995).
2. Santaniello, E., P. Ferraboschi, and P. Grisenti, Lipase-Catalyzed Transesterification in Organic Solvents: Applications to the Preparation of Enantiomerically Pure Compounds, *Enzyme Microb. Technol. 15*:367–382 (1993).
3. Berger, B., C.G. Rabiller, K. Königsberger, K. Faber, and H. Griengl, Enzymatic Acylation Using Acid Anhydrides: Crucial Removal of Acid, *Tetrahedron Asymmetry 1*:541–546 (1990).
4. Bianchi, D., P. Cesti, and E. Battistel, Anhydrides as Acylating Agents in Lipase-Catalyzed Stereoselective Esterification of Racemic Alcohols, *J. Org. Chem. 53:*5531–5534 (1988).
5. Wang, Y.F., S.T. Chen, K.K.C. Liu, and C.H. Wong, Lipase-Catalyzed Irreversible Transesterification Using Enol Esters: Resolution of Cyanohydrins and Syntheses of Ethyl(*R*)-2-Hydroxy-4-Phenylbutyrate and (*S*)-Propranolol, *Tetrahedron Lett. 30*:1917–1920 (1989).
6. Wang, Y.F., J.J. Lalonde, M. Momongan, D.E. Bergbreiter, and C.H. Wong, Lipase-Catalyzed Irreversible Transesterifications Using Enol Ester as Acylating Reagents: Preparative Enantio- and Regioselective Syntheses of Alcohols, Glycerol Derivatives, Sugars, and Organometallics, *J. Am. Chem. Soc. 110*:7200–7205 (1988).
7. Degueil-Castaing, M., B. De Jeso, S. Drouillard, and B. Maillard, Enzymatic Reactions in Organic Synthesis: 2-Ester Interchange of Vinyl Esters, *Tetrahedron Lett. 28*:953–954 (1987).
8. Wu, W.H., C.C. Akoh, and R.S. Phillips, Lipase-Catalyzed Stereoselective Esterification of DL-Menthol in Organic Solvents Using Acid Anhydrides as Acylating Agents, *Enzyme Microb. Technol. 18*:536–539 (1996).
9. Phillips, R.S., Temperature Effects on Stereochemistry of Enzymatic Reactions, *Ibid. 14*:417–419 (1992).
10. March, J., *Advanced Organic Chemistry*, John Wiley & Sons, Inc., New York, 1985, p. 82.
11. Gray, C.J., J.S. Narang, and S.A. Barker, Immobilization of Lipase from *Candida cylindraceae* and Its Use in the Synthesis of Menthol Esters by Transesterification, *Enzyme Microb. Technol. 12*:800–807 (1990).
12. Lokotsch, W., K. Fritsche, and C. Syldatk, Resolution of D,L-Menthol by Interesterification with Triacetin Using the Free and Immobilized Lipase of *Candida cylindracea*, *Appl. Microbiol. Biotechnol. 31*:467–472 (1989).

13. Claon, P.A., and C.C. Akoh, Enzymatic Synthesis of Geranyl Acetate in *n*-Hexane with *Candida antarctica* Lipase, *J. Am. Oil Chem. Soc. 71*:575–578 (1994).
14. Dordick, J.S., Enzymatic Catalysis in Monophasic Organic Solvents, *Enzyme Microb. Technol. 11*:194–211 (1989).
15. Laane, C., S. Boeren, K. Vos, and C. Veeger, Rules for Optimization of Biocatalysis in Organic Solvents, *Biotechnol. Bioeng. 30*:81–87 (1987).

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