Enzymatic Esterification of (–)-Menthol with Fatty Acids in Solvent by a Commercial Lipase from *Candida rugosa*

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ABSTRACT: Esterification of (–)-menthol with fatty acids in isooctane was successfully catalyzed using a commercial lipase, Lipase AY "Amano" 30 from *Candida rugosa* in original powder form. The esterification reactions were performed to elucidate the effects of temperature, enzyme load, molar ratio of (–)-menthol/fatty acid, and fatty acid type, keeping the (–)-menthol concentration at 200 mM. At the optimal conditions for (–)-menthol esterification, determined at a (–)-menthol/lauric acid molar ratio of 1:1 and 35°C [1.5 g enzyme/g (–)-menthol, 0.1 g molecular sieves], the molar conversion of (–)-menthol after 48 h reached 93%. After 24 h, the lowest and the highest molar conversions of fatty acids at 2:1 molar ratio were obtained with myristic acid (71%) and margaric acid (98%), respectively. After 48 h, the molar conversions of lauric acid at molar ratios 2:1, 1:1, and 1:2 were 98, 93, and 49%, respectively.

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KEY WORDS: *Candida cylindracea* lipase, *Candida rugosa* lipase, enzymatic esterification, fatty acid, (–)-menthol.

Menthol (*p*-menthan-3-ol) is a secondary terpene alcohol; it has eight optically active isomers that have different organoleptic properties. (–)-Menthol has a characteristic peppermint flavor and refreshing coolness. The other isomers of menthol do not have this cooling effect (1). Because of its flavor and refreshing coolness, (–)-menthol is widely used in foods, cosmetics, and pharmaceutics. (–)-Menthol is produced from natural sources such as peppermint oils of *Mentha piperita* and *M. arvenisis*. Their free-menthol contents range between 70 and 80%. It is also obtained industrially by the optical resolution of the racemic mixture produced by organic synthesis, which is an equal molar mixture of (+)- and (–)-menthol (1). The resolution of racemic mixtures can be carried out by esterification, column chromatography, or crystallization.

Enzymatic esterification is a highly selective method compared with other resolution methods. Enzymatic esterification of (–)-menthol and enzymatic resolution of racemic menthol have been investigated with lipases of different origins in a water–oil emulsion (2,3) or in solvent (4–6) to elucidate the effects of temperature, solvents, and fatty acid chain lengths. Lipases (EC 3.1.1.3) have been widely used for the resolution of racemic alcohols through enantiospecific esterification and transesterification. Among the several lipases investigated, lipases originating from *Candida cylindracea* and *Penicillium simplicissimum* were found to be highly selective for the esterification of (–)-menthol. Kamiya *et al*. (4,5) investigated the enantioselective esterification of menthol with saturated and unsaturated long-chain fatty acids in solvent. Surfactant (nonionic, glutamic acid dioleyl ester ribitol amide)-coated lipase from *C. cylindracea* was found to be highly selective for the esterification of (–)-menthol. The rate of esterification of (–)-menthol with lauric acid catalyzed by surfactant-coated lipase was more than 100 times that of the powder lipase. Therefore, (–)-menthol esterification using the surfactant-coated lipase from *C. cylindracea* (also termed *C. rugosa*, commercial name: Lipase AY produced by Amano Pharmaceutical Co.) was investigated to elucidate the effects of temperature, kind of solvent, and *n*-saturated fatty acids with different chain lengths. The optimal temperature was around 35°C. The catalytic activity and enantioselectivity of the lipase were found to depend strongly on the type of organic solvents. Isooctane was found to be the best solvent, giving high initial rates of reaction and enantioselectivity (4).

The initial rate of (–)-menthol esterification increased with increasing fatty acid chain length. Kamiya and Goto (5) concluded that the enzymatic esterification of menthol with lauric acid using surfactant-coated lipase from *C. rugosa* (Lipase AY) conformed to the ping-pong bi-bi mechanism in which the reaction was inhibited by excess menthol. A lipase from *C. rugosa* (Lipase AY-30) catalyzed the enatioselective resolution of racemic menthol using acid anhydrides as acylating agents (6). Thus, stereoselective resolution of racemic menthol with vinyl propionate as the acylating agent was catalyzed in organic solvents by an immobilized lipase from *Pseudomonas cepacia* (PS-30). In reactions conducted in hexane at a 1:1 mole ratio of racemic menthol to vinyl propionate, the mole percentage yield of (–)-menthyl propionate increased from 50 to 86% when the reaction temperature was increased from 20 to 60°C. The highest increase in yield was obtained between 40 and 50°C. However, increasing the temperature decreased the stereospecificity toward (–)-menthol. It is well known that the polarity of organic solvents affects the activity of biocatalyst. The polarity of organic solvents can be measured by the value of log *P* (the logarithm of the partition coefficient of a given solvent between water and 1-octanol). Among the investigated solvents, cylohexane (log *P* = 3.2), toluene (log *P* = 2.5), and benzene (log *P* = 2.0) gave low yields of (–)-menthol propionate but with a good enantiomeric ratio and enantiomeric excess while solvents of log *P* values above 3.5 such as isooctane (log *P* = 4.5), *n*- heptane (log *P* = 4.0), and *n*-hexane (log *P* = 3.5) gave high yields and stereoselectivity (7).

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(–)-Menthol was esterified with long-chain unsaturated fatty acids (oleic, linoleic, and α-linolenic acids) in an organic solvent-free system using commercial lipase from *C. rugosa* (8). The optimal conditions were determined as follows: (–)-menthol/fatty acid molar ratio of 1:3, 30% water, 700 units enzyme/g reaction mixture, 30°C. After 24 h, the esterification yields of (–)-menthol with oleic, linoleic, and α-linolenic acids were 96, 88, and 95%, respectively. The reaction showed high enantioselectivity. After 32 h, the enantiomeric ratio and enantiomeric excess of (–)-menthol were 31 and 88%, respectively.

In this study we used lipase powder from *C. rugosa* (Lipase AY "Amano" 30), without coating with a surfactant, as a catalyst to investigate the enzymatic esterification of (–)-menthol with saturated fatty acids of even and odd numbers of carbon atoms (lauric, myristic, palmitic, margaric, and stearic) and unsaturated (oleic) fatty acid in isooctane. Furthermore, we evaluated the effects of the temperature, type of fatty acid, (–)-menthol/fatty acid molar ratio, and enzyme content of the reaction mixture on the esterification reaction.

EXPERIMENTAL PROCEDURES

Materials. Commercial lipase from *C. rugosa* (Lipase AY "Amano" 30) was a gift of Amano Pharmaceutical Co. Ltd. (Nagoya, Japan) and was used as received. Its lipolytic activity was determined as 39375 U/g_{enzyme} using olive oil as substrate according to the method of Rosu and *et al*. (9). Lauric acid (12:0, 91% pure), and 90.5% pure myristic acid (14:0) were purchased from Hopkin & Williams Ltd. (Essex, England). Palmitic acid (16:0, 97.5% pure) and 89.8% pure stearic acid (18:0) were products of Fluka Chemie AG (Buchs, Switzerland). Margaric acid (17:0, 82% pure) and 77.5% pure oleic acid (18:1) were obtained from Sigma (Deisenhofen, Germany) and Merck Chemical Co. (Darmstadt, Germany), respectively. (–)-Menthol (purity, 99.7%) was obtained from Haarmann & Reimer GmbH (Holzminden, Germany). Isooctane (purity, 99.5%) was purchased from Carlo Erba reaganti (Milano, Italy).

Esterification reactions. Esterification reactions in isooctane were carried out in a glass reaction flask (25 mL) that was placed in a water bath in duplicate. The presented data are the averages of duplicate determinations. Heating of the water bath and stirring of the reaction mixture were performed with a magnetic stirrer equipped with heating unit (Framo-Geraetetechnik M22/1 5655; Franz Morat KGaA, Eisenbach, Germany). The stirring rate was adjusted to 500 rpm and the reaction temperature was kept constant with an accuracy of ±1°C by a temperature controller. In all reactions, the initial (–)-menthol molarity was 200 mM. The molar ratios of (–)-menthol/fatty acid were varied at 2:1, 1:1, and 1:2 in 10 mL isooctane. The reaction mixture was heated to 35°C with stirring. The esterification reaction was started by adding the enzyme to the reaction mixture, and the 0 h sample (approximately 0.4 mL) was taken after 0.5 min. For the removal of the generated water, 0.1 g of 4 Å molecular sieves, previously dried at 120°C for 18 h, were added to the reaction medium 1 h after the incubation started. Samples were taken at selected time intervals and heated in a water bath at 90°C for 15 min to inactivate the enzyme and then centrifuged to separate the ester product. The ester products were dried with anhydrous Na_2SO_4 and analyzed by capillary gas chromatography (see next paragraph). By using the number of micromoles of menthyl esters produced after 1 min per gram of enzyme, the initial rates of the reaction were calculated.

Analysis of the esterification product. The ester products, which were mixtures of menthyl esters, unreacted (–)-menthol, fatty acids and isooctane, were analyzed using an HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldron, Germany) equipped with a flame-ionization detector and Ultra 1 capillary column (25 m × 0.32 mm i.d. × 0.52 μm film thickness, 100% dimethyl polysiloxane; Hewlett-Packard). The temperature program was chosen as follows: 170°C (5 min), 170 to 275°C (10°C/min), and 275°C (10 min). The injector and detector temperatures were 250 and 280°C, respectively. The carrier gas, N_2, flow rate was 4.5 mL/min. The split was 25:1. For identification and quantification of unreacted fatty acids, unreacted menthol and menthyl esters, at first mixtures of these components in different concentrations were prepared using standard fatty acids, standard menthol, and standard menthyl esters prepared according to the method of Wu *et al.* (7). Later these mixtures were analyzed directly without derivatizing the fatty acids by capillary gas chromatography under the same working conditions mentioned above in order to get response factors of reactants and products and to establish calibration curves for them. The response factors of fatty acids and menthol esters were 1–0.96, respectively. However, because the retention time of menthol was very close to that of solvent, the response factor of menthol could not be measured. Since the response factors of fatty acids and menthyl esters varied over a rather small range, the extent of esterification was followed by the amount of fatty acid consumed and the amount of menthyl ester produced during the reaction**,** using just peak areas and calibration curves. The percentage molar conversion of fatty acids, which was not corrected for purity of fatty acid, was defined as (mmol of reacted fatty acid/initial mmol of fatty acid) × 100. Then the composition of the reaction mixtures was calculated using the number of millimoles of reacted fatty acids, the initial weights of reactants, and the mole weights of reactants and menthol esters.

RESULTS AND DISCUSSION

Effect of enzyme amount on the fatty acid conversion. To study the effect of enzyme amount on the esterification of (–)-menthol with lauric acid a set of reactions was conducted at a molar ratio of menthol to lauric acid of 2:1 in 10 mL isooctane using 0.053, 0.5, 1.067, and 1.5 g of Lipase AY "Amano" 30/g (–)-menthol at 35°C for 72 h. As one observes in Figure 1, at the enzyme ratio of 0.053 g/g, (–)-menthol esterification practically did not occur. At the enzyme concentration of 0.5 g/g (–)-menthol, the fatty acid molar conversion reached approximately 11% by 10 h and remained steady out to at least 72 h. Kamiya *et al.* (4) recently observed similar behavior using the same lipase. However, the fatty acid conversion increased

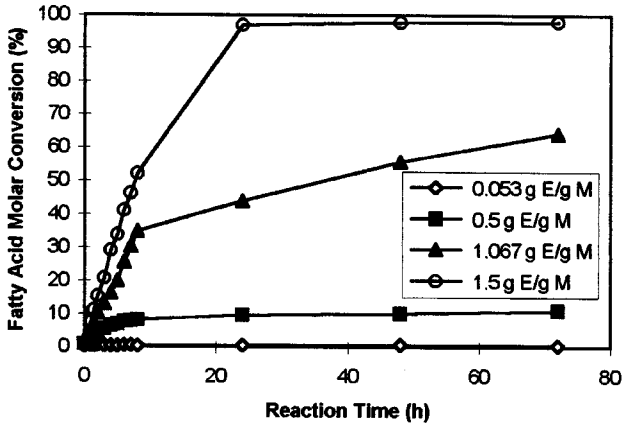

FIG. 1. Effect of enzyme amount on the esterification of (–)-menthol with lauric acid. Esterification reactions were conducted at 35°C with a mixture of 200 mM (–)-menthol and 100 mM lauric acid in isooctane. E, Lipase AY "Amano" 30; M, (–)-menthol.

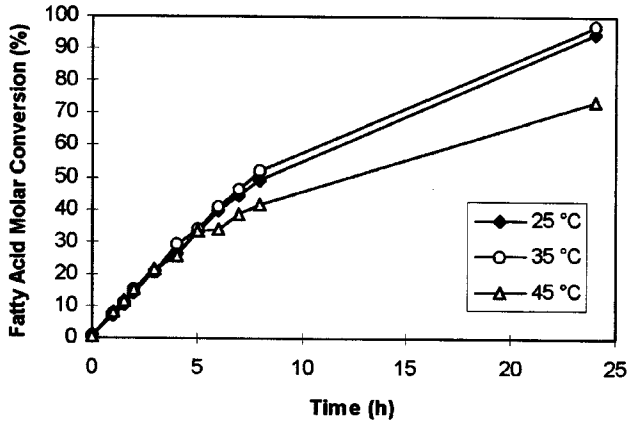

FIG. 2. Effect of temperature on Lipase AY "Amano" 30-catalyzed esterification of (–)-menthol with lauric acid at molar ratio of 2:1 (mmol/mmol) in isooctane. Enzyme concentration was 1.5 g enzyme/g (–)-menthol. Initial menthol concentration: 200 mM.

sharply when the enzyme concentration was increased to the range of 1–1.5 g enzyme/g (–)-menthol. By using 1.5 g enzyme/g (–)-menthol, the lauric acid molar conversion reached approximately 97% in 24 h. Hence, 1.5 g enzyme/g (–)-menthol was chosen as the enzyme amount for further experiments. Table 1 shows changes in the composition of the ester products during the reaction conducted using 1.5 g enzyme/g (–)-menthol as a function of the reaction time. Most of the changes occurred between 0 and 24 h.

Effect of the temperature on the esterification of (–)-menthol with lauric acid. The effect of temperature on the esterification of menthol with lauric acid was studied with reactions conducted at a molar ratio of 2:1 (alcohol/acid) at temperatures ranging from 25 to 45°C. As shown in Figure 2, the conversion of lauric acid was practically the same between 25 and 35°C. Kamiya *et al.* (4) reported that the optimal reaction temperature region for the coated-lipase originating from *C. rugosa* was narrow and that the optimal temperature was around 35°C. To compare our results with those in the literature, 35°C was chosen as the reaction temperature in further experiments.

TABLE 1
Effect of Reaction Time on the Composition of Ester Products[a]

Reaction time (h)	Composition of ester product (wt%)		
	(–)-Menthol	Fatty acids	Menthyl esters
0	60.8	39.0	0.2
1	58.7	36.2	5.1
1.5	57.7	34.9	7.4
2	56.6	33.3	10.1
3	55.0	31.3	13.7
4	52.6	28.0	19.4
5	51.2	26.2	22.6
6	49.1	23.4	27.5
7	47.6	21.3	31.1
8	45.9	19.1	35.0
24	32.5	1.3	66.2
48	32.2	0.8	67.0
72	32.2	0.8	67.0

[a]Reaction was performed in 10 mL isooctane with a 2:1 mole ratio of (–)-menthol to lauric acid using 1.5 g Lipase AY "Amano" 30 per g of menthol, 0.1 g molecular sieves, and a menthol concentration of 200 mM at 35°C.

Effect of fatty acid type on the esterification of (–)-menthol. The effect of the fatty acid type on the esterification of (–)-menthol catalyzed by Lipase AY "Amano" 30 was investigated using saturated fatty acids (lauric, myristic, palmitic, stearic, margaric and the unsaturated fatty acid oleic acid at a 2:1 (–)-menthol/fatty acid molar ratio for 48 h. As can be seen in Figure 3, except for the reaction using myristic acid, the conversions exhausted the supply of fatty acid between 10 and 24 h of incubation. The lowest and the highest conversion rates were obtained with myristic acid and margaric acid, respectively. The long-chain fatty acids with an even number of carbon atoms were converted at higher reaction rates than the even-numbered shorter-chain fatty acids, and the 14:0 fatty acid resulted in the lowest percentage conversion. These results are in agreement with the results of Kamiya *et al.* (4), who concluded that a more hydrophobic substrate is preferred by surfactant-coated lipases. We also observed that margaric acid and oleic acid were converted to their menthyl esters at higher molar conversions than the saturated fatty acids with an even number of carbon atoms. These results suggest that the fatty acid with an odd number of carbon atoms and the unsaturated fatty acid may be preferred substrates of Lipase AY "Amano" 30, resulting in higher substrate selectivity than the even-numbered saturated fatty acids. The initial rates of esterification of lauric, myristic, palmitic, stearic, margaric, and oleic acids were found to be 14, 10, 25, 19, 38, and 30 μmol/min/g of enzyme, respectively. The reason for the lower initial rate of the esterification reaction conducted with stearic acid than that of palmitic acid may be dependent on the lower stearic acid purity (89.8%), since stearic acid contains 8.8% saturated fatty acids with lower carbon numbers, such as 12:0, 14:0, and 16:0 acids. However, margaric and oleic acids were less pure than stearic acid, yet were better substrates, which may be related to their chemical natures.

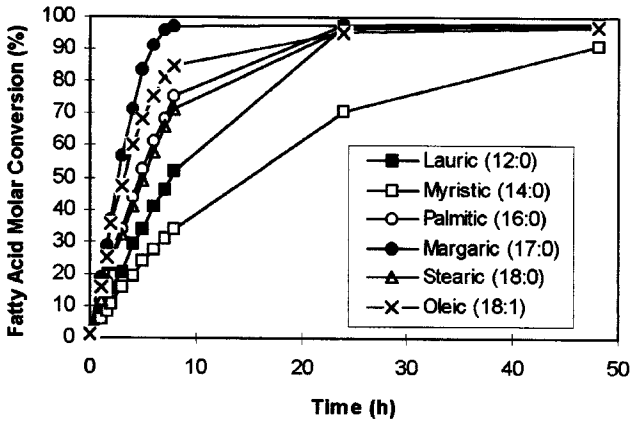

FIG. 3. Effect of fatty acid type on the fatty acid molar conversion. Reactions were conducted at 35°C and 2:1 molar ratio of (–)-menthol/fatty acid in isooctane using Lipase AY "Amano" 30 [1.5 g/g (–)-menthol]. Initial menthol concentration: 200 mM.

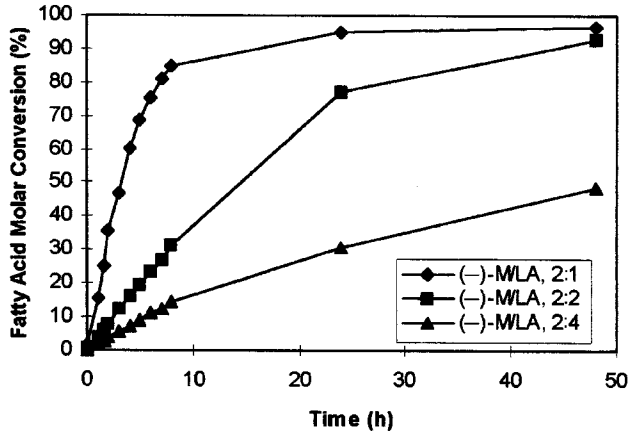

FIG. 4. Effect of molar ratio on the esterification of (–)-menthol with lauric acid. Reactions were conducted at 35°C in isooctane using Lipase AY "Amano" 30 [1.5 g/g (–)-menthol]. LA, lauric acid; see Figure 1 for other abbreviation. Initial menthol concentration fixed at 200 mM in all reactions.

Effect of molar ratio on the esterification of (–)-menthol. The esterification reactions were carried out with 1, 2, and 4 mmol lauric acid, keeping the (–)-menthol content at 2 mmol. Figure 4 shows that the (–)-menthol/lauric acid molar ratio affected the rate of reaction. The highest reaction rate and conversion of fatty acid were obtained with the molar ratio of 2:1 (–)-menthol to lauric acid. After 48 h the conversions of lauric acid at molar ratios of 2:1 and 1:1 reached 98 and 93%, respectively. The compositions of the ester products at 48 h at molar ratios of 2:1 and 1:1 (–)-menthol to lauric acid were 32.2% (–)-menthol, 0.8% fatty acid, 67% menthyl esters, and 3.2% (–)-menthol, 4.1% fatty acid, and 92.7% menthyl esters, respectively. According to the compositions of ester products, the optimal molar ratio of (–)-menthol to lauric acid was established as 1:1 for the resolution of racemic menthol.

The data presented here show that Lipase AY "Amano" 30 from *C. rugosa* without a surfactant coating could be used successfully for the esterification of (–)-menthol with fatty acids in solvent at both high menthol and enzyme concentrations. In general, the molar conversion of fatty acids reached approximately 98% after 48 h. With margaric acid, the molar conversion was found to be about 98% after 8 h. This fact may be related to a possible higher affinity of lipase AY "Amano" 30 for fatty acids with an odd number of carbon atoms. To test this hypothesis, further experiments should be carried out using other odd-numbered fatty acids

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