Enzymatic Synthesis of L-Menthyl Esters in Organic Solvent-Free System

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ABSTRACT: L-Menthol has been widely used as a food additive and an ingredient of cosmetics, and it is esterified to moderate the strong flavor. We attempted esterification of L-menthol with long-chain unsaturated fatty acid in an organic solvent-free enzymatic system. Commercially available lipases were screened, and *Candida rugosa* lipase was selected as a catalyst. Several factors affecting the esterification were investigated, and the reaction conditions were determined as follows: A reaction mixture of L-menthol/fatty acid (1:3, mol/mol), 30% water, and 700 units of the lipase per gram of reaction mixture was incubated at 30°C with stirring. After 24 h under these conditions, the esterification extents of L-menthol with oleic, linoleic, and α-linolenic acids reached 96, 88, and 95%, respectively. The structure of the esterified product was confirmed by mass, infrared, and nuclear magnetic resonance spectroscopies. Because *Candida* lipase acted strongly on L-menthol and very weakly on D-menthol, DL-menthol was esterified with oleic acid under the same conditions. The reaction showed high enantioselectivity; the enantiomeric ratio (*E*) was 31, and enantiomeric excess (ee) of L-menthyl oleate reached 88% after 32 h.

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KEY WORDS: *Candida rugosa*, esterification, fatty acids, lipase, menthol, optical resolution.

Ester synthesis by chemical process is achieved by the reaction of alcohol with fatty acid derivative in an organic solvent. Compared with the chemical method, a biochemical process has many advantages: (i) The reaction proceeds efficiently, with only mixing required of alcohol and fatty acid using lipase as a catalyst; (ii) low reaction temperature depresses the denaturation of substrates; (iii) lipase catalyzes only the desired reaction because of the substrate specificity; (iv) when the desired ester is to be used as a food, an organic solvent-free enzymatic process is valuable because organic solvents available for use in food processing are restricted. However, lipase acts well on liquid substrates, but very weakly on solid ones (1). In addition, water generated by the reaction disturbs the ester synthesis *via* lipase. It has been reported that the generated water can be removed by the use of a large amount of *n*-hexane (2,3), the addition of molecular sieves (4), and the use of a vacuum pump (5). We recently reported that lipase not acting on esterified product did not disturb the esterification even though the generated water was present in the reaction mixture (6–9).

L-Menthol is a component of peppermint oil and is also produced industrially by optical resolution of DL-menthol that has been synthesized chemically. Because of its flavor and refreshing coolness, it has been widely used as a component of candy, beverages, toothpaste, tobacco products, and so on. It has also been used as an ingredient of cosmetics to enhance absorption into the skin, and therapeutically as an analgesic, an antipruritic, and as a mild local anesthetic. Furthermore, L-menthol can be esterified with short-chain fatty acids to moderate the strong flavor. Esters of L-menthol with long-chain unsaturated fatty acids can be expected to have interesting characteristics: (i) The fragrance is released as the ester bond is hydrolyzed, and absorption into skin is accelerated; (ii) the ester is easily dissolved and emulsified because it is in the liquid state; (iii) the ester with long-chain fatty acid is more stable than that with short-chain fatty acid; (iv) because unsaturated long-chain fatty acids are important components of skin fats, their supplementation may protect the surface of skin. These properties are valuable as an ingredient of cosmetics.

In this paper, we describe esterification of L-menthol with long-chain unsaturated fatty acids in an organic solvent-free system using *Candida rugosa* lipase without removing the generated water. Furthermore, we show that optical resolution of DL-menthol can be accomplished using the reaction system.

MATERIALS AND METHODS

Materials. Oleic acid (purity, 92.9%), linoleic acid (purity, 97.5%), α-linoleic acid (purity, 76.8%), and L-, D-, and DL-menthol were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Lipases were gifts from the following companies: *Candida rugosa* (Lipase-OF; Meito Sangyo Co., Aichi, Japan); *Alcaligenes* sp. (Lipase-QL; Meito Sangyo); *Pseudomonas* sp. lipase (Lipase-PS; Amano Pharmaceutical

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Co., Aichi, Japan); *P. aeruginosa* lipase (LPL; Toyobo Co. Ltd., Osaka, Japan), and *Pseudomonas* sp. KWI-56 lipase (Kurita Water Industries Ltd., Tokyo, Japan). Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical Ind. Co., Osaka, Japan) with 50 mM KOH as described previously (10). The reaction was performed at 30°C for 30 min with stirring at 500 rpm. One unit (U) of lipase activity was defined as the amount of enzyme needed to liberate 1 μmol of fatty acid per min.

Reaction. A reaction mixture of menthol, fatty acid, water, and lipase was stirred at 500 rpm in a 50-mL screw-capped vial. The detailed reaction conditions are described below. The esterification extent was calculated from the amount of fatty acid consumed during the reaction.

Silica gel column chromatography. L-Menthyl linoleate and L-menthol were extracted with 150 mL *n*-hexane after adding 70 mL of 0.5 N KOH (20% ethanol solution) to 15 g of reaction mixture. The extracts (4.3 g) were applied to a silica gel 60 column (20 × 140 mm; Merck, Darmstadt, Germany), and L-menthyl linoleate was eluted with a mixture of *n*-hexane/ethyl acetate (98:2, vol/vol).

Assay. Fatty acid in L-menthyl ester was methylated by ester exchange in methanol containing Na-methylate, and was analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) equipped with a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Folsom, CA). The column temperature was raised from 150 to 300°C at a rate of 10°C/min. The temperatures of the injector and detector (FID, flame-ionization detector) were set at 245 and 320°C, respectively. The carrier gas was helium at a flow rate of 0.05 mL/s. DL-Isomers of menthol were quantified on a CP Cyclodex β236M (0.25 mm × 50 m; Chrompack, Middelburg, The Netherlands). The column temperature was raised from 115 to 145°C at a rate of 1°C/min after holding for 5 min at the initial temperature. The contents of D- and L-menthyl esters were measured from the content of D- and L-menthol, after purified DL-menthyl esters were methylated in methanol using Na-methylate.

Enantiomeric ratio (*E*) was calculated from the extent of esterification (*c*) and enantiomeric excess (ee) of the product using the equation of Chen *et al*. (11):

$$E = \ln[1 - c(1 + \text{ee})]/\ln[1 - c(1 - \text{ee})] \quad [1]$$

$$\text{ee} = ([\text{L}] - [\text{D}])/([\text{L}] + [\text{D}]) \quad [2]$$

where [L] and [D] are the contents (mol%) of L- and D-menthyl esters in the reaction mixture, respectively.

Infrared (IR) spectra were measured with a Shimadzu Fourier transform IR-8100M spectrophotometer (Kyoto, Japan). Nuclear magnetic resonance (NMR) spectra were measured in $CDCl_3$ using tetramethylsilane as the internal standard with a UNITY 300 spectrometer (Varian, Palo Alto, CA). The molecular mass was analyzed by field desorption mass spectrometry (FD-MS) performed on a JMS-DX303HF (JEOL Ltd., Tokyo, Japan).

RESULTS

Screening for a lipase suitable for synthesis of L-menthyl ester. Because L-menthol is a secondary alcohol, a suitable lipase was selected from positionally nonspecific lipases. A mixture of 4 g oleic acid/L-menthol (3:1, mol/mol), 1 g water, and 1000 U lipase was incubated at 30°C for 24 h with stirring at 500 rpm. The degree of esterification of L-menthol with oleic acid achieved was as follows: *Candida rugosa*, 74.6%; *Pseudomonas* sp., 23.5%; *Pseudomonas* sp. KWI-56, 15.2%; *Alcaligenes* sp., 10.8% and *P. aeruginosa*, 0.5%. Because of its efficiency, *Candida* lipase was selected for the following experiments.

Several factors affecting esterification of L-menthol with oleic acid. L-Menthol was esterified with oleic acid in a mixture containing 5 to 70% water (Fig. 1). A larger amount of water generally slows the esterification rate of L-menthol and decreases the esterification extent, because the equilibrium of the reaction shifts to hydrolysis (12). However, the water content in this reaction mixture did not markedly affect the rate and equilibrium of the esterification. The highest esterification extent was obtained in the mixture containing 20 to 30% water.

Figure 2 shows the effect of the amount of oleic acid on esterification of L-menthol. The esterification extent after 6- and 24-h reaction times depended on the amount of oleic acid, and the extent after 24 h reached a constant value in the reaction containing more than three molar equivalents of oleic acid. To investigate the effect of temperature, a mixture of oleic acid/L-menthol (3:1, mol/mol), 30% water, and a 200 U/g-reaction mixture of *Candida* lipase was incubated over a range of 20 to 50°C with stirring. The esterification extent of L-menthol reached *ca.* 75% after 24 h over the temperature range investigated. Finally, L-menthol was esterified with three molar equivalents of oleic acid using various amounts of *Candida* lipase (Fig. 3). The esterification extent after 6 h

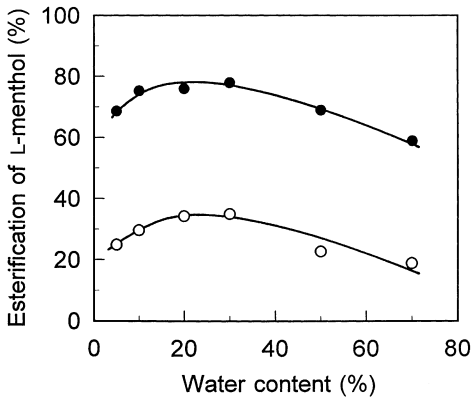

FIG. 1. Effect of water content on esterification of L-menthol with oleic acid. A mixture of oleic acid/L-menthol (3:1, mol/mol), 200 units (U)/g reaction mixture of *Candida rugosa* lipase, and 5–70% water was incubated at 30°C with stirring. ○, Esterification extent after 6 h; ●, esterification extent after 24 h.

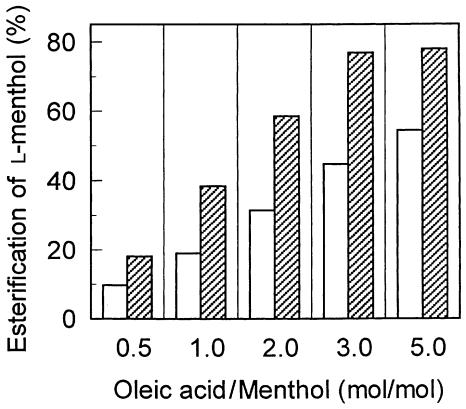

FIG. 2. Effect of oleic acid content on esterification of L-menthol. L-Menthol was esterified at 30°C with various amounts of oleic acid in a mixture containing 30% water and 200 U/g-reaction mixture of *Candida* lipase. The amount of oleic acid was expressed as a molar ratio to that of L-menthol. Open box, esterification extent after 6 h; hatched box, esterification extent after 24 h.

depended on the amount of lipase. The extent after 24 h increased with increasing amounts of lipase, and reached a constant value above 400 U/g reaction mixture.

On the basis of the foregoing results, the reaction conditions were set as follows: Molar ratio of fatty acid to L-menthol, 3:1; water content, 30%; amount of *Candida* lipase, 700 U/g reaction mixture; reaction temperature, 30°C. Under these conditions, L-menthol was esterified with oleic, linoleic, and α-linolenic acids, and the typical time courses are shown in Figure 4. L-Menthol was efficiently esterified with these fatty acids, and the esterification extents after 24 h were 96, 88, and 95%, respectively.

Structure of synthesized product. Because the purity of linoleic acid was the highest (98%) among fatty acids used as substrates, L-menthol was esterified with linoleic acid to analyze the structure of the synthesized product. L-Menthol (1.88 g, 12 mmol) was esterified with linoleic acid (10.12 g, 36 mmol) for 24 h under the above conditions (esterification extent, 89.3%). The esterified product was extracted with *n*-hexane under alkaline conditions and purified by silica gel column chromatography (purified product, 3.6 g). Fatty acid in the product was methylated and then analyzed by gas chromatography. As a result, L-menthol and methyl linoleate were detected at the molar ratio of 50.5:49.5, which showed that the synthesized product consisted of equal amounts of L-menthol and linoleic acid.

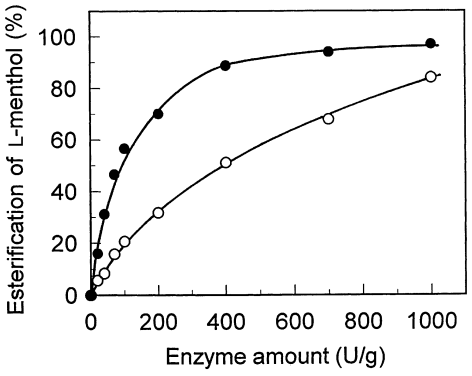

FIG. 3. Effect of enzyme amount on esterification of L-menthol. L-Menthol was esterified at 30°C in a mixture of 3.5 g oleic acid/L-menthol (3:1, mol/mol), 1.5 g water, and various amounts of *Candida* lipase. ○, Esterification extent after 6 h; ●, esterification extent after 24 h.

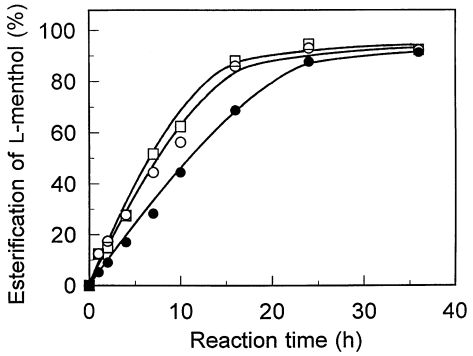

FIG. 4. Typical time course of esterification of L-menthol with oleic, linoleic, and α-linolenic acids. A mixture of 3.5 g fatty acid/L-menthol (3:1, mol/mol), 1.5 g water, and 3500 U *Candida* lipase was incubated at 30°C with stirring. ○, Oleic acid; ●, linoleic acid; □, α-linolenic acid.

The IR absorption maxima of the purified product were observed at the following positions: 2928, 1734 (C=O), 1458, and 1179 cm^{-1}(C–O–). The absorption due to the hydroxy group was observed in L-menthol and linoleic acid, but not in the synthesized product. The absorption of carbonyl group in the synthesized product was shifted to a shorter wavelength by 21 cm^{-1}, indicating the formation of an ester bond.

The purified product was analyzed by ^{1}H and ^{13}C NMR. ^{1}H NMR: δ = 0.76 (3H, *d*, *J* = 6.6 Hz, C$\underline{H}_3$), 0.80–1.14 (13H, *m*), 1.10–1.78 (19H, *m*), 1.88–1.94(1H, *m*, >C$\underline{H}$-), 1.94–2.10 (5H, *m*), 2.24–2.31 (2H, *m*), 2.77 (2H, *J* = 5.7 Hz, -COC$\underline{H}_2$-), 4.63–4.73 (1H, *m*, >C$\underline{H}$O-), 5.27–5.44 (4H, *m*, -C$\underline{H}$ = C$\underline{H}$-); ^{13}C NMR: δ = 14.0, 16.3, 20.7, 22.5, 23.5, 25.1, 25.6, 26.3, 27.2 × 2, 29.10, 29.12, 29.15, 29.3, 29.6, 31.4, 31.5, 34.3, 34.7, 41.0, 47.1, 73.9, 127.9, 128.0, 130.0, 130.2, 173.3. The proton number observed by ^{1}H NMR and the carbon number observed by ^{13}C NMR agreed with those of L-menthyl linoleate, respectively. The signal of carbonyl carbon of linoleic acid shifted from 180.2 to 173.3 ppm, and the signal of C-3 secondary alcohol carbon shifted from 71.3 to 73.9 ppm. These results showed that linoleic acid was esterified to the C-3 hydroxy group of L-menthol. In addition, the molecular weight of the product was *m/z* 418 (M^+) by FD–MS. From these structure analyses, synthesized product was confirmed to be L-menthyl linoleate.

Application to optical resolution of DL-menthol. L-Menthol has a peppermint flavor as well as physiologically useful activities, but D-menthol does not. The isolation of L-menthol from chemically synthesized DL-menthol is a very important industrial process. It was reported that *C. rugosa* li-

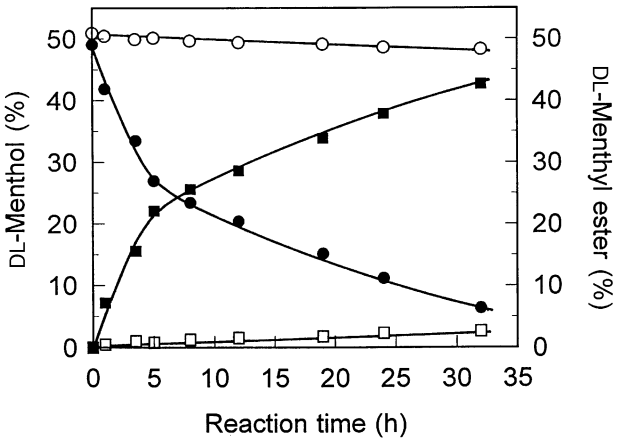

FIG. 5. Time course of esterification of DL-menthol with oleic acid using *Candida rugosa* lipase. A mixture of 4 g oleic acid/DL-menthol (3:1, mol/mol), 1 g water, and 3500 U lipase was incubated at 30°C with stirring. ○, D-Menthol; ●, L-menthol; □, D-menthyl oleate; ■, L-menthyl oleate.

pase acted on L-menthol strongly and on D-menthol weakly in a reaction system containing apolar organic solvent (13). Thus the activity of *Candida* lipase on DL-menthol was investigated in a solvent-free system described here. As shown in Figure 5, *Candida* lipase acted on L-menthol strongly and D-menthol weakly even in the organic solvent-free system. After 32 h of reaction, the esterification extents of D- and L-isomers of menthol were 5.2 and 87.0%, respectively. The reaction showed high enantioselectivity; the enantiomeric ratio (*E*) was 31, and the enantiomeric excess (ee) of L-menthyl oleate reached 88% after 32 h. The result shows that no organic solvent is essential in optical resolution of DL-menthol using *Candida* lipase.

DISCUSSION

We have described the efficient esterification of L-menthol with long-chain unsaturated fatty acids in an organic solvent-free enzymatic system. Lipase acts strongly on liquid substrates and very weakly on solid ones. L-Menthol is a solid at the reaction temperature (30°C), but a mixture of L-menthol and unsaturated long-chain fatty acid was in a liquid state. The efficient esterification *via* lipase may be due to the liquid-state substrates. This result shows that organic solvent is not necessary for the efficient esterification if the substrates are in a liquid state. When an ester to be used as a food is produced, an organic solvent-free enzymatic process is very valuable because organic solvents that can be used in food processing are highly restricted.

Recently, we found that esterification *via* lipase was not disturbed by the presence of a large amount of water. It was furthermore clarified that the equilibrium of the reaction shifted to esterification because the lipase recognized alcohol and fatty acid but not the esterified product. In the present study, L-menthyl oleate was also a poor substrate of *Candida* lipase: When L-menthol oleate was hydrolyzed at 30°C for 24 h in a mixture containing 30% water, the hydrolysis extent was only 8.8%.

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