Enzymatic Esterification of (–)-Menthol with Lauric Acid in Isooctane by Sorbitan Monostearate-Coated Lipase from *Candida rugosa*

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ABSTRACT: Esterification of (–)-menthol and (±)-menthol with lauric acid in isooctane was successfully catalyzed by a commercial nonionic surfactant (sorbitan monostearate)-coated lipase from *Candida rugosa* (Lipase AY "Amano" 30) at the molar ratio of 1:1 and at 35°C using 1.5 g enzyme/g (–)-menthol and 0.1-g molecular sieves. After 1 h, molar conversion of (–)-menthol reached 81%. Equilibrium was reached after *ca.* 4 h, giving a (–)-menthol molar conversion of 94%. Under the same conditions, native lipase catalyzed the esterification of (–)-menthol and lauric acid to yield a molar conversion of 93% after 72 h. Coating the lipase with sorbitan monostearate increased the esterification rates of both (–)-menthol and (±)-menthol with lauric acid. After 6 h, the molar conversions of (–)-menthol and (±)-menthol were 94, and 62%, respectively.

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KEY WORDS: *Candida rugosa* lipase, esterification, lauric acid, menthol, sorbitan monostearate.

Menthol (*p*-menthan-3-ol) is a secondary terpene alcohol and has eight optically active isomers with different organoleptic properties. (–)-Menthol has a characteristic peppermint flavor and refreshing coolness. Because of its flavor and refreshing coolness, (–)-menthol is widely used in foods, cosmetics, and pharmaceuticals (1). Enzymatic esterification of (–)-menthol is a highly selective method for the resolution of racemic menthol, which is an equal molar mixture (+)- and (–)-menthol. Lipases of different origins have been examined in water–oil emulsions (2,3) and in organic solvents (4–6) to elucidate the effects of temperature, solvents, and fatty acid chain lengths. Kamiya *et al.* (4) investigated the enantioselective esterification of (–)-menthol with lauric acid in isooctane at 35°C using six different lipases. Among them, only the lipase from *Candida cylindracea* (Lipase AY) catalyzed the esterification of (–)-menthol with lauric acid. Moreover, coating Lipase AY with a nonionic surfactant (glutamic acid dioleyl ester ribitol amide) increased the catalytic activity of the lipase. The rate of esterification of (–)-menthol with lauric acid catalyzed by surfactant-coated Lipase AY was more than 100 times that of the native lipase.

Sorbitan monostearate-coated *Rhizopus japonicus* lipase has been shown to catalyze the esterification of long-chain fatty acids and fatty alcohols in a microaqueous *n*-hexane system (7). Therefore we aimed to evaluate the usefulness of a commercial nonionic surfactant "Sorbitan monostearate"-coated lipase from *C. rugosa* (Lipase AY "Amano" 30) for the esterification of (–)-menthol, and to compare its activity toward this substrate with that of unmodified enzyme. Furthermore, we aimed to show that optical resolution of racemic menthol can be carried out by esterification catalyzed by this preparation.

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EXPERIMENTAL PROCEDURES

Materials. Commercial lipase from *C. rugosa* (formerly *C. cylindracea*), 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 39,375 U/g enzyme using olive oil as substrate according to the method of Rosu *et al.* (8). Lauric acid (12:0) of 91% purity was purchased from Hopkin & Williams Ltd. (Essex, England). (–)-Menthol (purity, 99.7%) and (±)-menthol (purity, 99%) were obtained from Haarmann & Reimer GmbH (Holzminden, Germany). Isooctane (purity, 99.5%) was purchased from Carlo Erba reaganti (Milano, Italy). Dehymuls SMS (sorbitan monostearate) was a gift of Henkel Co. (Istanbul, Turkey).

Preparation of surfactant-coated enzyme. Surfactant-coated lipase was prepared using the method of Basheer *et al.* (7). Lipase AY (3 g) was dissolved in 1 L Tris buffer (pH 5.5) and magnetically stirred at 10°C. Sorbitan monostearate in ethanol (0.75 g/20 mL) was added dropwise to the stirred enzyme solution and sonicated in an ultrasonic bath for 10 min. After magnetically stirring for 3 h at 10°C, surfactant-coated enzyme was precipitated by adding acetone at 10°C, collected by centrifugation (21,000 × *g*) and then air-dried. A yellowish powder (the surfactant-coated lipase) was obtained with a yield of *ca.* 13% mass and lipolytic activity of 67,828.7 U/g surfactant-coated lipase.

Esterification reactions. Esterification reactions in isooctane were carried out in a glass reaction flask (25 mL) at the optimal conditions determined for native Lipase AY in our previous study in duplicate (9). Heating of the water bath and stirring of the reaction mixture were performed with a magnetic stirrer equipped with a heating unit (Framo-Geraetetechnik M22/1 5655; Franz Morat KG, Eisenbach, Germany). The stirring rate was adjusted to 500 rpm, and the reaction temperature was 35°C and kept constant with an accuracy of ±1°C

by a temperature controller. The esterification reactions were initiated by adding enzyme (59,000 U/g menthol) either in original or surfactant-coated form to 10 mL isooctane that contained 200 mM menthol and 200 mM lauric acid at the reaction temperature. The 0-h sample (*ca.* 0.4 mL) was taken after 0.5 min. For the removal of the generated water, 0.1 g of 4 Å molecular sieves, which were 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.

Analytical methods. The composition of the esterification product was determined in a capillary gas chromatography apparatus, HP 5890 Series II (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.

RESULTS AND DISCUSSION

Effect of the modified enzyme on the esterification reaction of (–) menthol. Figures 1 and 2 show the time courses of the (–)-menthol conversion catalyzed by lipase in original and sorbitan monostearate-coated forms at 35°C. As previously mentioned by Kamiya *et al.* (4), surfactant-coated lipase was soluble in anhydrous organic solvent and showed high catalytic activity. Indeed it was observed that after 1-h reaction, molar conversion of (–)-menthol reached 81% in the esterification reaction catalyzed with sorbitan monostearate-coated lipase (Fig. 2). The equilibrium conversion was reached after *ca.* 4 h, giving a (–)-menthol molar conversion of 94%. However, the conversion of (–)-menthol was only 77% following 24-h incubation when the lipase in original form was used (Fig. 1). It was recently reported by Kamiya *et al.* (4) that in the esterification reaction of 5 mM (–)-menthol and 10 mM lauric acid in 10 mL isooctane at 35°C using glutamic acid dioleyl ester ribitol amide-coated lipase from *C. cylindracea*, more than 90% of (–)-menthyl laurate was produced within 24 h. In comparing our results with those obtained by Kamiya *et al.* (4), it is very important to point out that the reaction between (–)-menthol and lauric acid could readily reach more than 90% after 4 h instead of 24 h by coating the same lipase with sorbitan monostearate. This can be explained to some degree by the different natures of the surfactants used.

Effect of the modified enzyme on the esterification reaction of (±)-menthol. In order to investigate the effect of coating *C. rugosa* lipase with sorbitan monostearate on the esterification of (–)- and (±)-menthol with lauric acid, these reactions were conducted separately in isooctane under the same reaction conditions mentioned above. Figures 1 and 2 show the time courses of the esterification reactions of both (–)-and (±)-menthol with lauric acid using the lipase from *C. rugosa*, Lipase AY "Amano" 30, and sorbitan monostearate-coated lipase, respectively. The molar conversion of (±)-menthol to its corresponding ester during a 6-h incubation was *ca.* 19% when the lipase in original form was used, compared to more than 62% when the lipase modified with sorbitan monostearate was used (Figs. 1 and 2). Under the same reaction conditions, after 6 h, molar conversion of 94% was obtained with (–)-menthol when the reaction was conducted using sorbitan monostearate-coated lipase while the corresponding molar conversion was only 23% using the lipase in original form (Figs. 1 and 2). Moreover, the lipase in original form catalyzed the esterification of (–)-menthol with an equilibrium molar conversion of 93.1% and that of (±)-menthol with an equilibrium molar conversion of 58.3%, following 72-h incubation (Fig. 1).

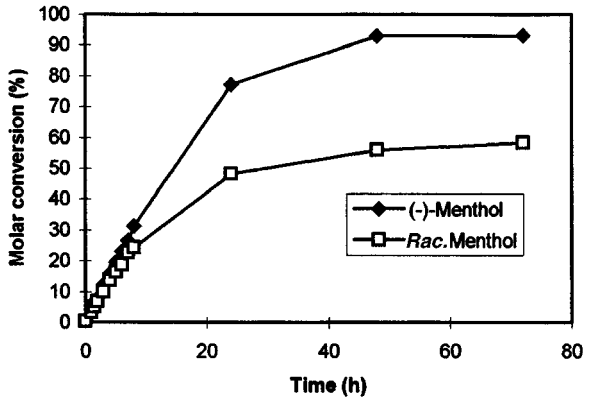

FIG. 1. Time course of the esterification of (–)-menthol and (±)-menthol with lauric acid by the lipase from *Candida rugosa*. Reaction conditions: 200 mM (–)-menthol or 200 mM (±)-menthol and 200 mM lauric acid in 10 mL isooctane, 35°C, 59,000 U/g (–)-menthol.

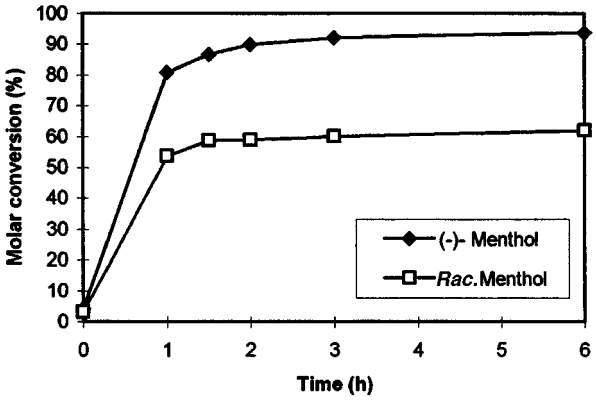

FIG. 2. Time course of the esterification of (–)-menthol and (±)-menthol with lauric acid by sorbitan monostearate coated-lipase from *C. rugosa*. Reaction conditions: 200 mM (–)-menthol or 200 mM (±)-menthol and 200 mM lauric acid in 10 mL isooctane, 35°C, 59,000 U/g (–)-menthol. See Figure 1 for abbreviations.

Thus, the data presented here show that sorbitan monostearate-coated lipase from *C. rugosa* (Lipase AY "Amano" 30) is a highly effective biocatalyst which can be used for the esterification of (–)-menthol and possibly for the resolution of (±)-menthol in a considerably shorter reaction time (4 h) than feasible when using the same enzyme not treated with sorbitan monstearate.

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