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Factors Affecting the Resolution of *dl*-Menthol by Immobilized Lipase-Catalyzed Esterification in Organic Solvent

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Among 10 lipases tested, *Candida rugosa* lipase exhibited the best ability to catalyze the resolution of *dl*-menthol in organic solvent. The lipase was immobilized on different carriers, and the experiment was carried out with different acyl donors. The high yield and optical purity of the product were achieved in cyclohexane with valeric acid as acyl donor using *C. rugosa* lipase immobilized on DEAE-Sephadex A-25. The conversion of *dl*-menthol depended on the water content of immobilized lipase and on the pH of the aqueous solution from which lipase was immobilized. The operational stability of the DEAE-Sephadex A-25 immobilized lipase in catalysis of the esterification reaction showed that >85% activity remained after 34 days of repeated use. The resolution of racemic menthol in organic medium catalyzed by immobilized *C. rugosa* lipase-catalyzed esterification is very convenient, and it represents a significant improvement in the use of enzyme for the preparative production of optically active menthol. This process is readily applicable to large-scale preparation.

KEYWORDS: Lipase; *dl*-menthol; operational stability; optical resolution

INTRODUCTION

Lipases (EC 3.1.1.3) are highly stereoselective catalysts, which are of great value for the modern chemical and pharmaceutical industries. Many review papers (*1*−*4*) concerning the biochemistry and biotechnological applications of lipases reveal unique opportunities for resolution of racemic mixtures of organic compounds.

Microbial lipases continue to receive attention due to their potential abilities in ester synthesis and ester hydrolysis. Such lipases are widely applied in industry. However, lipases derived from different sources are highly versatile with respect to their substrate and reaction specificities (*5*). Although lipases share the catalytic triad Ser-Asp/Glu-His (similar to serine proteases), their overall performances are usually quite different. Therefore, lipases from various sources must be tested to find the appropriate enzyme for the synthesis of the particular ester (*6*). Furthermore, many factors can affect the lipase-catalyzed synthesis or hydrolysis of esters caused by the enzyme structure, immobilization method, reaction media, and activity of acyl donors. All of these factors should be optimized to obtain the best yield and optical purity of desired products.

l-Menthol is widely used in industry (*1*) because of its refreshing flavor, whereas *d*-menthol has an undesirable taste. *l*-Menthol is used in components of pepperment oil, candy, beverages, toothpaste, tobacco, local anesthetics, and cosmetic products (*1*). Optically pure alcohols can be produced either in aqueous solutions by stereoselective hydrolysis of the corresponding racemic esters (*7*) or in organic solvents by esterification (*8*, *9*) of the corresponding racemic alcohols. The practical application of the method depends on the desired yield and purity of product, rate of reaction, and enzyme operational stability.

Lokotech et al. (*10*) studied the transesterification of *dl*-menthol with triacetin by *Candida rugosa* lipase. They observed that triacetin and other longer chained triglycerides caused changes in higher specific activities but provided poor enantioselectivities of the products. Gary et al. (*8*) assessed the esterification of menthol by *C. rugosa* lipase immobilized on cellulose and on EA-4000 polyethylene. The lipase immobilized on polyethylene was found to be effective in transesterification using tributyrin or triacetin as acyl donor and menthol as an acceptor. Ingaki et al. (*11*) reported the enantioselective esterification of racemic alcohols such as citronellol, isopulegol, and menthol with organic acids using a microbial enzyme derived from *Pseudomonas* sp. NOF-5. They observed that water soluble fatty acids such as acetic acid and butyric acid provide a low yield of esterification, whereas a high yield was achieved with fatty acids containing more than six carbon atoms. Shimadu et al. (*12*) have enzymatically synthesized menthyl ester from menthol and oleic acid by *C. rugosa* lipase. They concluded that *Candida* lipase operates strongly with *l*-menthol and very weakly with *d*-menthol. However, no systematic studies on the effect of various factors on syntheses catalyzed by different lipases, kinetic resolution of *dl*-menthol, and operational stability of the enzyme have been reported. Lipases are rather inexpensive enzymes, and they can be used in organic solvents. However, several factors may essentially affect their application (*13*).

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Table 1. Activity of Lipases from Different Sources on Menthyl Ester Formation

lipase	trade name or brand	hydrolytic activity[a] (units/mg)	conversion[b] × 100 (%)	reaction time (h)	$ee_p \times 100$ (%)	$E^c \times 100$ (%)
Aspergillus niger	Amano AP-6	188 ± 9.5	2 ± 0.12	120	95 ± 5	39.8
Candida rugosa	Sigma Chemical Co.	235 ± 10.9	45 ± 0.31	80	95 ± 5	92.3
Chromobacterium viscosum LP-101-5	Toyo Jozo	231 ± 11.4	0	120		
Mucor sp.	Amano MAP-10	50 ± 2.9	0	120		
Pseudomonas fluorescens	Amano PS	231 ± 12.1	0	120		
Rhizopus sp.	Amano FAP-15	42 ± 1.9	0	120		
Rhizopus sp.	Amano N-conc	26 ± 1.5	0	120		
porcine pancreatic lipase	Sigma Chemical Co.	19 ± 1.3	3 ± 0.16	120	95 ± 5	40.2
cholesterol esterase	Amano	234 ± 11.4	2.5 ± 0.17	120	45 ± 3	2.7
lipoprotein lipase	Amano	227 ± 10.8	0	120		

[a] The lipase hydrolytic activity was determined by *p*-nitrophenyl butyrate as substrate. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute. [b] The lipase (0.05 g/mL) was added to a reaction mixture containing 0.2 M *dl*-menthol and 0.125 M valeric acid at 30 °C and 250 rpm in water-saturated cyclohexane. [c] The *E* value was calculated from the mean values of conversion and enantiomeric excess according to eq 2.

The present paper describes different factors affecting the optical resolution of immobilized lipase-catalyzed esterification in organic solvents, considering the different types of fatty acid donors, pH memory, different carriers for lipase immobilization, critical water content for maximum activity, and operational stability of enzyme.

MATERIALS AND METHODS

Lipases were obtained from available commercial sources. Porcine pancreatic lipase and *C. rugosa* lipase were purchased from Sigma Chemical Co. (St. Louis, MO). All other enzyme lipases were generous gifts from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). DEAE-Sepharose CL-6B, Phenyl-Sepharose CL-6B, Sephadex G-50, and Sephadex G-25 were purchased from Pharmacia Biotech Asia Pacific Ltd. (Hong Kong). Chitin (30 mesh), Amberlite XAD-2, Silica gel 60, and Silica gel 60 G were obtained from Sigma Chemical Co. *dl*-Menthol, *l*-menthol, and *d*-menthol of analytical grade were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemical reagents were purchased from Merck (Darmstadt, Germany). Organic solvents used in this work were of analytical grade and had been previously dehydrated by shaking with 4-Å molecular sieves (Davison Chemical, Baltimore, MD) for 24 h at room temperature.

Lipases were immobilized on different carriers according to the following procedure. Lipase (20 g) was dissolved in 400 mL of 10 mM phosphate, acetate, or Tris buffer and mixed with 80 g of carrier. The preparations were carried out at different pH values ranging from 3 to 10 to optimize the conditions of lipase immobilization. A volume of 1600 mL of cold acetone (−20 °C) was added, and the mixture was stirred for 30 min at 4 °C. The immobilized lipase was filtered, washed with 400 mL of cold acetone (−10 °C), dried in air, and then lyophilized in a vacuum at room temperature. The hydrolytic activity of lipases was measured according to a method described by Rua et al. (*14*).

To study the possible effect of water on the esterification reaction, we have used the carrier's ability to adsorb water. For that purpose, a small amount of water was added to immobilized enzyme and the reaction was monitored to find the optimal conversion of menthol. The initial water content was calculated with respect to the amount of the immobilized enzyme (e.g., enzyme together with carrier) taken for the reaction.

Lipase was added immediately before the start of incubation to a mixture containing 0.2 M *dl*-menthol and 0.125 M organic acid. The reaction was carried out at 30 °C in an orbital shaker at a speed of 250 rpm. The reaction mixture aliquots (1 μL) were withdrawn after each 24 h and analyzed by thin-layer and gas chromatography. The reaction was stopped when conversion extent reached the value of ∼50%. The immobilized lipase was then readily recovered from the reaction mixture by filtration and then reused.

Thin-layer chromatography was carried out for preliminary monitoring reaction on Kieselgel 60 F plates (Merck) using a chloroform/hexane mixture (3:1 v/v) for the chromatogram development. Components were detected using iodine vapor.

Quantitative analysis of the reaction mixture was carried out on a Hitachi gas chromatograph model G-3000. Supelco's Sp 2330 fused silica capillary column (30 m × 0.32 mm i.d.) operating isothermally at 170 °C was used to separate and identify the menthyl esters synthesized by enzymatic reaction. The injector and detector temperatures were 250 and 260 °C, respectively. Nitrogen was used as the carrier gas at the flow rate of 40 cm^3/s. The conversion extent (*c*) of menthol was defined as (millimoles of esters/millimoles of initial menthol) × 100% and was estimated using peak area integrated by computer.

Separation and identification of optically active *d*- and *l*-enantiomers were performed on a Cyclodex-B column (J&W PN 112-2532, 30 m × 0.25 mm i.d.). The injector and detector temperatures were 250 and 300 °C, respectively. The temperature program used for the analysis of *d*- and *l*-menthyl esters was held at 105 °C for 15 min before being elevated to 120 °C at 10 °C/min and then held at 120 °C for 20 min. The amount of each enantiomer was estimated by peak area recorded and integrated by computer.

The enantiomeric excess (ee_p) of the product was determined by gas chromatographic analyses according to the formula

$$ee_p = \frac{[d] - [l]}{[d] + [l]} \quad (1)$$

where [*d*] and [*l*] represent the concentrations of *d*-menthyl and *l*-menthyl ester stereoisomers, respectively.

The enantiomeric ratio (*E*) was calculated from the extent of conversion (*c*) and the enantiomeric excess (ee_p) of the product by using the equation (*15*)

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} \quad (2)$$

RESULTS AND DISCUSSION

The stereoselective formation of menthyl ester from the racemic mixture of menthol and valeric acid by different lipases is shown in Table 1. Among the tested enzymes only *Aspergillus niger*, *C. rugosa*, and porcine pancreatic lipase were found to catalyze the reaction with satisfactory enantiomeric purity. However, of the 10 lipases listed in Table 1, only *C. rugosa* lipase gave results acceptable in terms of reaction rate and optical purity.

The detailed evaluation of the reaction was carried out using extent of conversion (*c*), enantiomeric excess of product (ee_p), and enantiomeric ratio (*E*). The latter is very important for optimizing the enantioselective performance of the enzyme. By definition, *E* is an intrinsic property of the enzyme (*15*), which cannot change unless the intrinsic values of K_m or K_{cat} change (*16*). Chen et al. (*15*) proposed an equation for evaluating *E* values using conversion extent (*c*) and enantiomeric ratio (*E*).

Table 2. Effect of Acyl Donors on Menthyl Ester Formation by Immobilized Lipase

acyl donor	carbon no.	conversion[a] × 100 (%)	relative activity[b] (%)	ee_p × 100 (%)	E^c × 100 (%)
acetic acid	2	7.9 ±	14	95 ± 5	42.3
propionic acid	3	44.6 ±	80	90 ± 5	41.2
butyric acid	4	48.7 ±	87	84 ± 5	27.4
valeric acid	5	35.7 ±	64	95 ± 5	66.4
caproic acid	6	17.1 ±	31	95 ± 5	47.3
capric acid	8	47.9 ±	86	45 ± 2.4	3.9
lauric acid	12	45.6 ±	82	24 ± 1.4	2.0
palmitic acid	16	50.5 ±	91	14 ± 1	1.5
oleic acid	18	55.7 ±	100	8 ± 0.4	1.3

[a] Conversion was determined in 40 h of reaction. The lipase (0.05 g/mL) was added to a reaction mixture containing 0.2 M *dl*-menthol and 0.125 M organic acid at 30 °C and 250 rpm in water-saturated cyclohexane. [b] Conversion in reaction with oleic acid was assumed as 100%. [c] The *E* value was calculated from the mean values of conversion and enantiomeric excess according to eq 2.

However, the calculation of *E* on the basis of eq 2 is very sensitive to the ee_p value. If the ee_p value approaches 100%, the calculation can give an *E* of infinity. The measurement of ee_p >98% requires special methods (*16*) providing an accuracy of at least ±2%. In our case, the standard deviation of ee_p was 5−6% (Table 1). Therefore, only mean of *E* values were calculated. The best yield and enantioselectivity were provided by lipase derived from *C. rugosa* (Table 1). Moreover, among all of the lipases tested, only *C. rugosa* lipase gave $E = 92.3$, which is as effective as $E = \infty$ (*16*). Thus, *C. rugosa* lipase was chosen for the stereoselective esterification of menthol.

The effect of acyl donors on *C. rugosa* lipase activity and specificity was studied to develop the optimal conditions for enzyme reaction. Cyclohexane was chosen as the organic solvent because it is a good solvent for the substrates and for the lipase-catalyzed esterification reaction (*17*). Table 2 shows that acetic, propionic, valeric, and caproic acids were good acyl donors for producing menthyl esters of excellent optical purity. However, the yield of esterification was very low for some acyl donors. For acetic acid, it may be explained by the high polarity of the acid, which strips the essential water from the enzyme and forms the hydrogen bonding. Thus, enzyme molecules become dehydrated and clump together, reducing the interfacial area. These results are in agreement with the data of Ingaki et al. (*11*) showing low esterification of menthol with that acid. In our experiments the best conversion was reached with palmitic and oleic acids, but the optical resolution was poor. Among all of the acyl donors allowing high enantiomeric excess, only valeric acid gave the largest value of *E* (Table 2). Thus, the enzyme−valeric acid system can provide the most appropriate intrinsic properties needed for the esterification of menthol. Therefore, valeric acid was chosen as the best acyl donor for the esterification of menthol among the various fatty acids of different chain lengths.

The stereoselective esterification of *dl*-menthol was carried out by immobilized lipase preparations. The immobilization of *C. rugosa* lipase on 11 carriers was investigated (Table 3). There were essentially no differences found among the different carriers when compared by enantiomeric excess ee_p. However, the highest value of enantiomeric ratio $E = 87.7$ revealed that *C. rugosa* lipase immobilized on DEAE-Sephadex A-25 is the best catalyst compared to free enzyme or to other carriers. This carrier was used for the further experiments.

The conversion of menthol was affected by the pH of the aqueous buffer from which the lipase was vacuum-dried. Such a phenomenon was called pH memory by Kilbanov (*18*). The

Table 3. Effect of Carriers on Menthyl Ester Formation by Immobilized Lipase

carrier	water content in immobilized enzyme[a] (%)	conversion[b] × 100 (%)	ee_p × 100 (%)	E^c × 100 (%)
no carrier	9.7 ± 0.5	36 ± 2.1	90 ± 5	31.4
Amberlite XAD-2	38.1 ± 1.9	42 ± 1.9	92 ± 6	48.0
Amberlite XAD-8	41.7 ± 2.2	39 ± 1.8	90 ± 5	34.0
Celite	5.5 ± 0.2	36 ± 1.8	91 ± 4	35.3
chitin	18.5 ± 0.9	12 ± 0.6	95 ± 5	44.3
chitosan	22.5 ± 1.1	32 ± 1.5	90 ± 6	28.8
DEAE-Sephadex A-25	48.3 ± 2.4	44 ± 2.2	95 ± 5	87.7
DEAE-Sepharose CL-6B	49.1 ± 2.6	9 ± 0.5	94 ± 5	35.4
Phenyl-Sepharose	27.5 ± 1.6	6 ± 0.3	95 ± 5	41.4
Sephadex G-25	19.6 ± 1.0	49 ± 2.6	92 ± 5	70.7
Sephadex G-50	16.0 ± 0.9	38 ± 2.0	90 ± 5	33.1
Silica gel 60	14.2 ± 0.7	30 ± 1.7	91 ± 5	31.1
Silica gel 60 G	10.8 ± 0.6	35 ± 1.9	90 ± 5	30.7

[a] Water content was measured after immobilized enzyme had been dried at 105 °C. [b] The lipase (0.15 g/mL) was added to a reaction mixture containing 0.2 M *dl*-menthol and 0.125 M valeric acid at 30 °C and 250 rpm in water-saturated cyclohexane. [c] The *E* value was calculated from the mean values of conversion and enantiomeric excess according to eq 2.

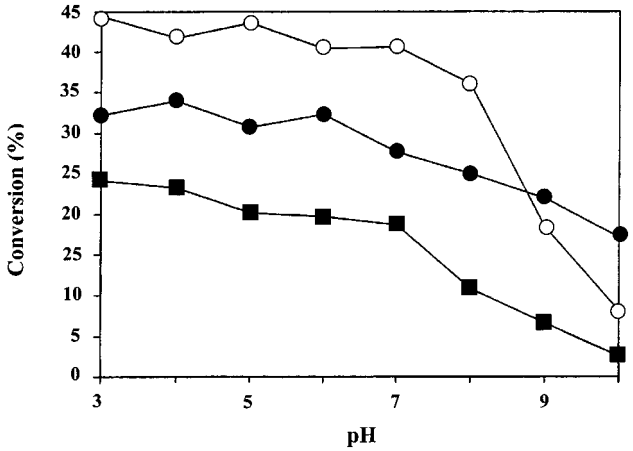

Figure 1. Correlation between the conversion extent of menthol and pH of aqueous solution from which lipase was immobilized: (○) lipase immobilized on Sephadex G-25; (●) lipase immobilized on DEAE-Sephadex A-25; (■) lipase immobilized on CM Sephadex C-25. Experimental conditions: 0.15 g/mL immobilized lipase, 0.2 M *dl*-menthol, and 0.125 M valeric acid were mixed in dry cyclohexane with shaking at 30 °C and 250 rpm during 48 h.

correlation between pH and conversion of menthol is depicted in Figure 1. The optimal pH values for the different carriers of immobilized *C. rugosa* lipase were different. For instance, lipase immobilized on Sephadex G-25 showed an optimal activity over a pH range from 3 to 7.

Another important factor influencing the conversion of menthol by enzyme reaction in the organic solvent is the different abilities of carriers to adsorb water. We have found that a small amount of water can improve the conversion of menthol. The optimal water contents for Sephadex G-25-immobilized lipase and DEAE-Sephadex A-25-immobilized lipase (Figure 2) were 10 and 15−20%, respectively. These data are consistent with the results of Bova et al. (*19*) obtained for PEG−lipase catalysis.

The immobilized enzyme was recycled many times with little loss of activity. The operational stability of DEAE-Sephadex A-25-immobilized lipase is shown in Figure 3. The data reveal high stability of the immobilized enzyme in batch type operation: >85% of the initial activity remained after 34 days of

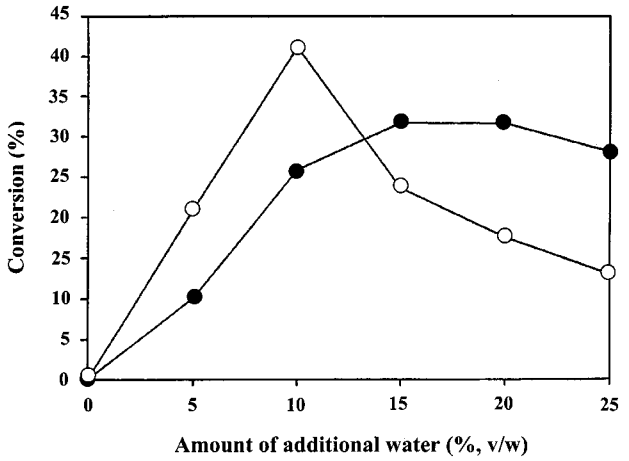

Figure 2. Effect of water content on conversion of menthol by lipase immobilized on Sephadex G-25 (○) and on DEAE-Sephadex A-25 (●). Experimental conditions are similar to those of Figure 1.

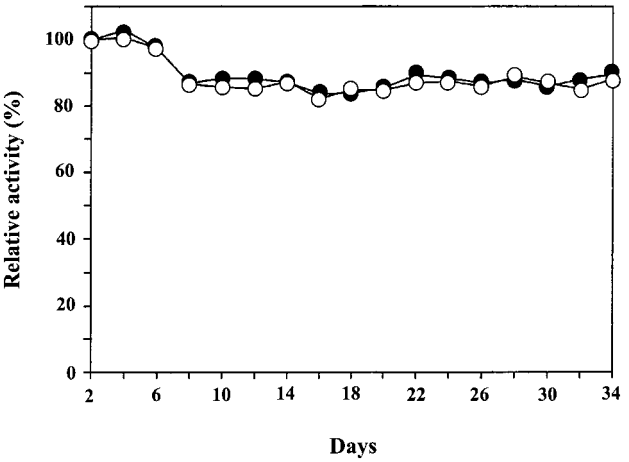

Figure 3. Operation stability of lipase immobilized on DEAE-Sephadex A-25 in esterification reaction of menthol by valeric acid carried out in dry cyclohexane (○) and in cyclohexane saturated with water (●). Experimental conditions are similar to those of Figure 1.

repeated use (1 cycle/day) of the immobilized lipase. This indicates that under reaction conditions the enzyme is quite stable. Such operational stability suggests that the tested system is industrially applicable for the production of optically pure menthol.

From the data reported it is apparent that the resolution of racemic menthol by immobilized *C. rugosa*-catalyzed esterification in organic medium is very convenient, and it represents a significant improvement in the use of enzyme for the preparative production of optically active menthol. This process is readily applicable to large-scale preparation.

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