Enantiomeric Synthesis of (*S*)-2-Methylbutanoic Acid Methyl Ester, Apple Flavor, Using Lipases in Organic Solvent

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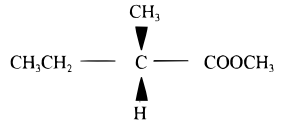
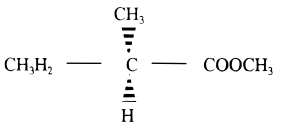
Enantiomeric selective synthesis of (*S*)-2-methylbutanoic acid methyl ester, which is known as a major apple and strawberry flavor, was performed from racemic 2-methylbutanoic acid using lipases in organic solvent. Among 20 lipases, lipase IM 20 (immobilized lipase of *Rhizomucor miehei*), lipase AP (*Aspergillus niger*), and lipase FAP-15 (*Aspergillus javanicus*) exhibited higher enzymatic activities and enantioselectivities and were selected for the synthesis of (*S*)-2-methylbutanoic acid methyl ester. Using these enzymes, the reaction conditions such as temperature and lyophilizing pH were optimized, and kinetic parameters were determined. All of the reactions were performed in isooctane, which was identified as the best reaction media for nonaqueous systems. At 20 °C maximum enantiomeric excess was observed, while synthetic activity increased as the temperature increased. Only lipases lyophilized at pH 5.5, 6.0, 6.5, and 7.0 showed synthetic activity. In this pH range, enantioselectivities were not influenced by the lyophilizing pH. The $K_{M,S}$ and $K_{M,R}$ values for ester synthetic activity of lipase were 1120 and 1240 mM, respectively. Enzyme activity was inhibited by (*S*)-2-methylbutanoic amide, and its K_i was calculated as 84 mM. (*S*)-2-Methylbutanoic amide acted as a competitive inhibitor.

INTRODUCTION

2-Methylbutanoic acid methyl ester (MBE), which is known as the main flavor component of apple or strawberry, is an enantiomer (Maciel et al., 1986) as follows:



(S)-methylbutanoic acid methylester



(R)-methylbutanoic acid methylester

Among these enantiomers, only the (*S*)-form has the characteristic fruit flavor (Maciel et al., 1986; Macleod and Pieris, 1981). Generally, natural flavors such as MBE are produced in limited supply because of the high cost of production, as well as difficulty in separating the active form from the enantiomeric mixture. Therefore, most commercialized flavors available are artificial products. Thus, the development of these high value-added flavors with high enantiomeric purity has been identified as a new research field in food technology.

An enzymatic method to produce the (*S*)-MBE from racemic 2-methylbutanoic acid (*rac*-MBA) using the enantioselectivity of an enzyme is a possible solution to this problem (Fitzpatrick and Klibanov, 1991; Klibanov, 1995; Kwon, 1992; Russel and Klibanov, 1988). Among esterifying enzymes such as esterases and lipases, lipase was found to be suitable for synthesizing the esters from fatty acids and other acids (Klibanov, 1986; Kwon, 1992; Kwon et al., 1996).

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The enzymatic synthetic reaction from the acid to ester is quite different from the hydrolysis reaction, because it is essential to remove the water in the reaction system (Kwon et al., 1996). A nonaqueous organic solvent system is advantageous for the conversion of the water-insoluble acids such as MBA, aliphatic fatty acids, and other aromatic acids to their esters (Klibanov, 1986; Zaks and Klibanov, 1984). In addition to these advantages, the structure of an enzyme in organic solvent is so rigid that the conformation induced before lyophilization cannot easily be changed (Kamiya and Goto, 1998; Klibanov, 1995; Kwon et al., 1996; Vulfson et al., 1997; Zaks and Klibanov, 1988). This ensures that the enantioselectivity of the enzyme is maintained during the reaction (Russel and Klibanov, 1988). Usually the conformation of an enzyme in the aqueous phase is easily induced by the substrate to fit its substrate structure (induced fit) (Koshland and Neet, 1968). Therefore, an organic solvent system is recommended as the best reaction system (Dabulis and Klibanov, 1993; Russel and Klibanov, 1988).

Many lipases have shown their enantioselectivity for the hydrolysis of esters or synthesis of esters from acids and alcohols (Allenmark and Ohlsson, 1992; Russel and Klibanov, 1988; Weissfloch and Kazlauskas, 1995; Wen et al., 1996). Our previous paper (Kwon, 1992) showed that some lipases had a high enantioselectivity for synthesis of esters. The Klibanov group (Fitzpatrick and Klibanov, 1991; Wescott and Klibanov, 1994) reported enzyme specificity was influenced by solvents; however, temperature has also been reported to affect the enantiomeric selectivity of lipase in an organic solvent (Holmberg and Hult, 1991; Phillips, 1992). In dioxane, nitromethane, and acetonitrile, *Rhizomucor miehei* lipase exhibited higher enantioselectivity at low temperature compared to high temperature (Noritomi et al.,

10.1021/jf990871w CCC: $19.00
Published on Web 01/08/2000

1996). Engel (1992) reported that *Candida cylindracea* (CCL) lipase hydrolyzed the (*S*)-2-methylalkanoic alkyl ester in heptane enantioselectively. Water content was also shown to affect the enantioselectivity in organic solvent systems (Bovara et al., 1993; Dudal and Lortie, 1995). Holmberg et al. (1991) showed that enantiomeric resolution depended on the structure of the alcohol in the esterification of 2-methylalkanoic acid by CCL lipase in the aqueous phase.

The objective of this paper was enantiomeric selective synthesis of (*S*)-2-methylbutanoic methyl ester, the major apple flavor component, from racemic mixture 2-methylbutanoic acid by lipases in an organic solvent with elucidation of their synthetic characteristics.

MATERIALS AND METHODS

Materials. *Enzymes.* Lipases used in this experiment were the same lipases as used previously (Kwon et al., 1996). lipase CES, lipase AY, lipase AP-10, lipase GT-20, lipase GC, lipase AP, lipase PS, lipase L, lipase FAP-15, lipase R, lipase D, lipase CE, and lipase G were supplied from Amano Pharmaceutical Co. (Nagoya, Japan). Two lipases from *C. cylindracea* and pancreatic lipase were purchased from Sigma Chemical Co. (St. Louis, MO). Lipase from *R. miehei* and Lipozyme IM-20 (immobilized lipase of *R. miehei*) were obtained from Novo Enzyme Co. (Bagsvaerd, Denmark). The last two lipases, lipase MY and lipase OF-360, were from Meito Sankyo (Osaka, Japan), and lipase CV was from Toyo Jozo (Shizuoka, Japan).

Chemicals. Racemic 2-methylbutanoic acid (*rac*-MBA) and (*S*)-2-methylbutanoic acid ((*S*)-MBA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). (*S*)-2-Methylbutanoic acid methyl ester as a standard was synthesized from (*S*)-MBA by the AOAC method (AOAC, 1995) (Kwon et al., 1999). The chiral column, Chiraldex GTA capillary column for GC, was supplied by Alltech Inc. (Deerfield, IL) (Kwon et al., 1999). Isooctane (2,2,4-trimethylpentane), octane, heptane, cyclohexane, and hexane were purchased from Aldrich Chemical Co. All other chemicals were of analytical grade. Water in solvents was removed by a 3 Å molecular sieve (1/16 in.) (Wako Chemical Co., Osaka, Japan) just before the reaction (Kwon et al., 1996). Silica gel-60 (particle size 0.063−0.2 mm) (Merck, Darmstadt, Germany) was used to remove water produced during the reaction (Kwon et al., 1996).

Enzyme Reactions for MBE Synthesis. Typical enzymatic synthesis of MBE was performed according to the method for synthesis of medium-chain glycerides (Kwon et al., 1996). Each lyophilized lipase (50 mg) was added to 1 mL of solvent in the presence of 700 mM *rac*-MBA and 700 mM methanol. About 50 mg of silica gel was added to the mixture (1 mL of solvent) to remove the water. After 10 s of sonication, the reaction vials were placed in a rotary shaker at 200 rpm at 30 °C. Periodically, aliquots were withdrawn, and the amounts of (*S*)- and (*R*)-2-methylbutanoic acid methyl ester were assayed by GC with the same conditions as used previously (Kwon et al., 1999).

Assay of Enantioselectivity. Enantiomeric excess (ee_s) for (*S*)-MBE was used as a measure of the enantioselectivity of lipase, which was calculated by the following equation (Chen et al., 1982):

$$ee_s\ (\%) = \frac{|[(S)\text{-MBE }(\%)] - [(R)\text{-MBE }(\%)]|}{[(S)\text{-MBE }(\%)] + [(R)\text{-MBE }(\%)]} \times 100$$

For determining the enantiomeric excess, integral area instead of molar concentration of each enantiomer was used because an authentic standard of each enantiomer was not available. Fortunately, the ratio of integral area between (*S*)- and (*R*)-MBE was exactly 1:1 for synthesized racemic MBE from racemic MBA (1:1).

Enzyme Screening and Solvent Screening. For screening the lipases, the ee_s and integral area of each enantiomer for MBE synthesized at 30 °C for 4 h with 20 lipases in organic solvent were determined. Using the same methods the best solvent was screened from isooctane, octane, heptane, cyclohexane, and hexane (Kwon et al., 1996).

Table 1. Enantioselectivities for Various Lipases in Synthesis of (*S*)-2-Methylbutanoic Acid Methyl Ester from *rac*-Methylbutanoic Acid and Methanol in Organic Solvent

		2-methylbutanoic acid methyl ester[a]	
enzyme	ee (%)	(*R*)-MBE	(*S*)-MBE
Rhizomucor miehei	33.3	33 091	66 578
Candida cylindracea	23.0	869	1 478
pancreatic lipase	0.0	17 865	17 617
Geotrichum candidum (GC)	0.0	37 203	36 481
Aspergillus niger (AP)	49.5	23 028	68 371
Pseudomonas aeruginosa (PS)	0.0	38 872	42 494
Chromobacterium viscosum (CV)	0.0		
Candida lipolytica (L)	0.0	34 259	33 725
Aspergillus javanicus (FAP-15)	49.8	20 641	61 429
Penicilium roqueforti (R)	0.0	25 630	25 673
Humicola lanuginosa (CE)	9.0	22 520	26 517
Penicilium cyclopium (G)	9.0	21 608	25 054
Candida rugosa (OF-360)	25.9	34 586	59 914
lipase CES (*Pseudomonas* sp.)	0.0	32 151	31 655
lipase AY (*Candida rugosa*)	7.4	37 019	42 987
lipase AP-10 (*Aspergillus niger*)	20.0	39 645	59 457
Penicilium cyclopium (GT-20)	0.0	41 819	42 452
lipozyme (immobilized *R. miehei*)	33.3	26 795	49 610

[a] Integral area by integrator of GC.

Effects of pH and Temperature on Synthetic Activities. Screened lipases were lyophilized at different pHs. Buffers used in lyophilization of lipases were citrate buffer for pH 3, 4, and 5; succinate buffer for pH 4, 5, and 5.5; phosphate buffer for pH 6, 6.5, 7, and 8; and Tris-HCl buffer for pH 9. After lyophilization, enzymes were washed with acetonitrile to remove the salt on the glass filter (Kwon, 1992). The amount of enzyme loaded was adjusted to 30 mg of protein determined by the Lowry method (Lowry et al., 1951). Temperature effect on enantioselectivity of the lipase was investigated by determining the enantiomeric excess of MBE in various temperatures (15, 20, 25, 30, 40, 50, and 60 °C).

(*S*)-2-Methylbutanoic Amide Synthesis and Identification. The stereoisomer of methylbutanoic amide that acts as an inhibitor on lipase was synthesized as follows (Audrieth and Kleinberg, 1938). (*S*)-2-Methylbutanoic acid (100 mg) was sampled into a round-bottom flask, and 1 drop of dimethylformamide was added. The flask was attached to a reflux condenser, and 3−4 mL of thionyl chloride ($SOCl_2$) was added through the condenser. The mixture was refluxed for about 20 min on a steam bath, and 10 mL of ice-cold concentrated ammonium hydroxide (NH_4OH) was added. The precipitated amide was collected by vacuum filtration and recrystallized from water and aqueous alcohol. The synthetic amide was identified by HPLC (Jasco, Tokyo, Japan) and mass spectrometry (Platform II, Micromass, Manchester, U.K.).

Kinetic Studies. A reaction kinetic study was performed for lipase FAP-15, which exhibited a strong enantioselectivity, with various concentrations of rac-MBA (100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, and 750 mM) at various reaction times (4, 6, 16, and 24 h). Synthetic reactions were performed as described previously. The Michaelis constant ($_{app}K_M$) value and $_{app}V_{max}$ value for each enantiomeric product were calculated from Lineweaver−Burk double reciprocal plots obtained by plotting $1/v$ versus 1/[S] (Lineweaver and Burk, 1934). For the inhibition studies, K_i measurement was carried out with and without 20 mM of (*S*)-2-methylbutanoic amide. All kinetic parameters were the means of at least two determinations and were reproducible.

RESULTS AND DISCUSSION

Screening of Lipase and Solvent for Enantioselectivity. Table 1 shows the results of screening 20

Table 2. Effects of Organic Solvents on the Synthetic Activity and Enantiomeric Selectivity of Lipase (FAP-15) for Synthesis of 2-MBE at 30 °C for 18 h

	2-methylbutanoic acid methyl ester[a]		
solvents	(*S*)-MBE	(*R*)-MBE	ee (%)
isooctane	143 295	47 477	50.2[b]
octane	127 163	42 721	49.7[b]
heptane	113 031	39 789	47.9[b]
cyclohexane	67 820	23 586	48.4[b]
hexane	148 979	49 841	49.9[b]

[a] Integral area by integrator of GC. [b] Not significantly different among solvents at the $P < 0.05$ level.

different lipases for (*S*)-2-methylbutanoic acid methyl ester ((*S*)-MBE) synthesis in isooctane. The enzymes effective for enantioselective (*S*)-MBE synthesis were lipase IM 20 (immobilized lipase of *R. miehei*), lipase AP (*Aspergillus niger*), and lipase FAP-15 (*Aspergillus javanicus*). The ee_s values of the latter two enzymes were about 50%, which means that the production ratio of (*S*)-MBE to (*R*)-MBE is about 3:1. This ee_s was not so high compared to that of the hydrolysis reaction (Engel, 1992). In the hydrolysis reaction, (*S*)-aliphatic acid was produced from *rac*-aliphatic ester by CCL lipase in solvent with 70% enantioselectivity (Engel, 1992). However, lipases OF-360, GC, M-AP10, GT-20, PS, and G showed low enantioselectivity (ee_s), although they had high synthetic activity of *rac*-MBE in isooctane (Table 1). The remaining lipases had poor activities for both synthesis and enantioselectivity. The lipases that were selected as effective for enantioselectivity of (*S*)-MBE or total MBE synthesis were different from the lipases that were good for synthesis of medium-chain glycerides (Kwon et al., 1996) and methyl esterification of trifluoromandelic acid (Kwon, 1992). These results mean that selection of the best lipase for hydrolysis, synthesis, or interesterification depends on the nature of substrate compounds (alcohols as acyl acceptors and acids as acyl donors) and their reaction mechanisms (Holmberg et al., 1991; Kwon et al., 1996). Table 1 also shows that enantioselectivity of lipases was not affected by the lipase specificity (Kwon et al., 1997; Macrae, 1984).

Synthetic activities and enantiomeric selectivities of (*S*)-MBE were determined in various solvents such as isooctane, octane, heptane, cyclohexane, and hexane to select the best solvent for this reaction, using three screened lipases, lipase IM 20, lipase AP, and lipase FAP-15. In a nonaqueous organic solvent system for synthesizing MBE, hexane, and isooctane were the best solvents. This result is consistent with our previous data (Kim et al., 1984; Kwon et al., 1996). The total synthetic activity was the highest in hexane, but the solvent was very volatile. In contrast, isooctane showed activity a little lower than that in hexane but was not evaporated during the reaction. Therefore, isooctane was selected as the optimum solvent in this research to avoid concentration of products during the reaction. For the synthesis of (*S*)-MBE by lipase FAP-15, the enantioselectivity was not affected within these water-immiscible solvents (Table 2), whereas the Klibanov group reported enzyme specificity was affected by the various kinds of solvents (Fitzpatrick and Klibanov, 1991; Wescott and Klibanov, 1994).

Time Course of Lipase Synthetic Reaction. To check how long the reaction rate maintained linearity with increase of the reaction time and substrate concentration, the time course of the synthesis of (*S*)-MBE in isooctane using lipase FAP-15 was investigated for various reaction times (4, 6, 16, 24, and 30 h) with different substrate concentrations (Figure 1). The initial velocity (v_0) for each substrate concentration maintained linearity over 24 h even at low concentration of substrate. Initial velocity was obtained by determining the production amount of (*S*)-MBE up to 12 h for each lipase. Enantioselectivity (ee_s) of lipase FAP-15 with reaction time was not changed (data not shown), while the total synthesis of (*S*)-MBE increased gradually. This was the same for both lipase AP and lipase IM 20.

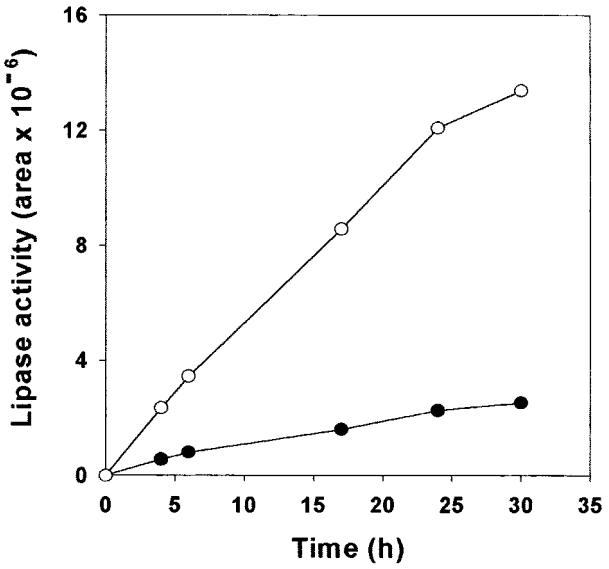

Figure 1. Time course of the synthesis of (*S*)-MBE (○) and (*R*)-MBE (●) by lipase FAP-15 in isooctane from 2-(±)-methylbutanoic acid (*rac*-MBA).

Effects of pH and Temperature on Synthetic Activity and Enantiomeric Selectivity. By checking the linearity of the reaction, synthetic reaction conditions were optimized in terms of lyophilization pH and reaction temperature. The overall synthetic rate of product was increased in proportion to reaction temperature as expected (Kwon et al., 1996). As the temperature increased up to 60 °C, synthetic activity also increased, suggesting that the lipase was stable over 60 °C as a result of the rigidity of the enzyme structure in organic solvent (Klibanov, 1986; Kwon et al., 1996; Ottolina et al., 1992) (Figure 2). The ee_s increased up to 15−20 °C; however, it decreased with the increase of temperature over 20 °C (Figure 2). Thus, the ee_s was the highest around 20 °C at 60%. This was the same as in both lipase AP and lipase IM 20 (Figure 3), which were also highly active and stable over 60 °C.

Enzymatic enantioselectivity usually decreases with temperature (Holmberg and Hult, 1991; Noritomi et al., 1996; Phillips, 1992). Some enzymes become more enantioselective at high temperature in both organic media and aqueous media (Noritomi et al., 1996). Generally, enantio- or stereospecificity of an enzyme is dependent on the structure or conformation of active site of enzyme. Thus, if the conformation of enzyme is easily induced by substrate or changed by circumstance, the enzyme easily loses its specificity. In organic media, the conformation of the enzyme is so rigid that it retains its stereo- or enantiospecificity (Kwon et al., 1996; Russel and Klibanov, 1988). However, at high temper-

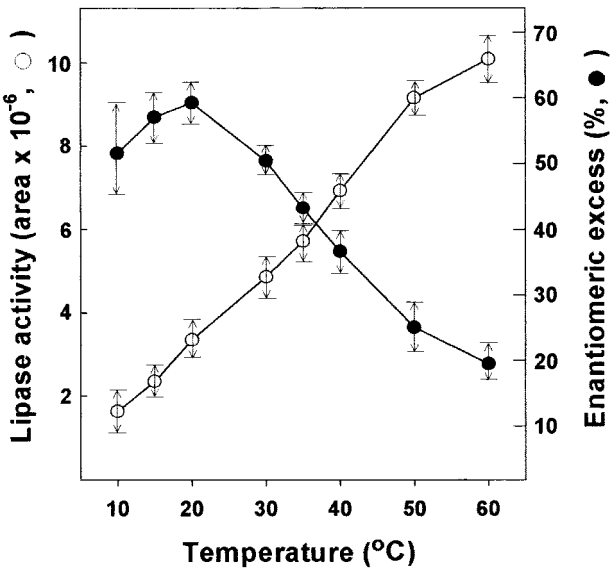

Figure 2. Temperature profile of lipase activity (○) and enantioselectivity (●) for (*S*)-MBE synthesis by lipase FAP-15 in isooctane. Error bars indicate the standard deviation at each point for three determinations.

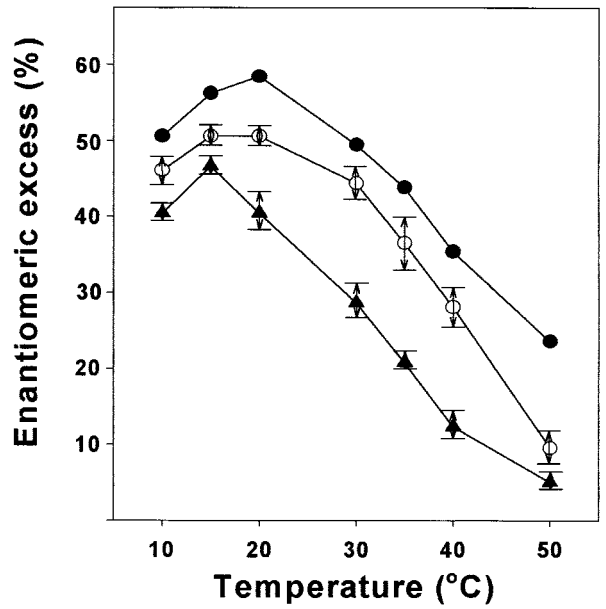

Figure 3. Effect of temperature on enantioselectivity of lipase FAP-15 (●), lipase AP (○), and lipase IM 20 (▲) in synthesizing the (*S*)-MBE in isooctane. Error bars indicate the mean ± SD for three determinations.

ature this rigid structure of the enzyme will be a little bit more flexible compared to its state at low temperature. Therefore, lipase probably loses enantiomeric specificity at high temperature as a result of the increased flexibility. Our data were consistent with the data of Noritomi et al. (1996), who reported that *R. miehei* lipase showed higher enantioselectivity at lower temperature rather than higher temperature (45 °C) in dioxane, nitromethane, and acetonitrile. However, the enantioselectivity of subtilisin Carlsberg remained unchanged at either low or high temperature. Further study is necessary to confirm the conformational change of enzymes in organic solvent as the temperature increases.

The lipases lyophilized at pH 3, 4, 5 8, 9, and 10 showed very poor (*S*)-MBE synthetic activity in organic

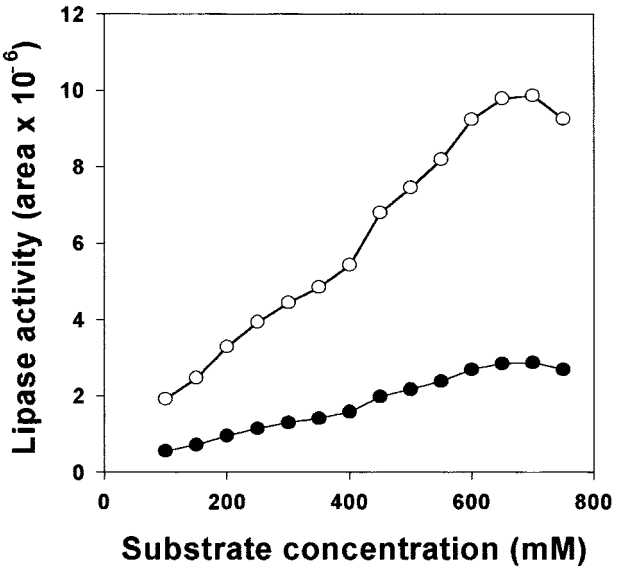

Figure 4. Michaelis−Menten curves for synthesis of (*S*)-MBE (○) and (*R*)-MBE (●) with lipase FAP-15.

solvent. Only lipases prepared at pH 5.5, 6.0, 6.5, and 7.0 showed synthetic activity (data not shown). The optimum pH (6.5) for synthesis in organic media also corresponds the optimum pH of hydrolytic reaction of lipases in aqueous phase (Kwon et al., 1987) and medium-chain triglyceride synthesis in organic solvent (Kwon et al., 1996). Also, they did not show any different enantioselectivity between pHs. These data also support the pH memory theory suggested by Klibanov group (Klibanov, 1995; Kwon et al., 1996; Zaks and Klibanov, 1988).

Reaction Kinetics of Lipase in an Organic Solvent System. In an organic solvent system, enzyme reaction kinetics for enantiomeric synthesis of (*S*)-MBE were investigated by determining the synthetic activity and enantioselectivity of lipases with various concentrations of substrate (*rac*-MBA). First, Michaelis−Menten curves of the lipase reaction in organic solvent with the different substrate concentrations were constructed (Figure 4), which did not show typical saturation curves. Below 80 mM *rac*-MBA, lipase did not show any catalytic activity in organic solvent, which means a minimum substrate concentration is required for the activation of lipase at the interface. At high concentrations of substrate (over 700 mM), synthetic reactivity was decreased with increase of substrate concentration.

Lipase is well known for its interfacial activation, because all of the lipase-catalyzed reactions can take place at the interface between enzyme and substrate in the heterocatalytic reaction system (Brockerhoff, 1973; Derewenda and Sharp, 1993): (1) water-soluble enzyme with water-insoluble substrate in an aqueous or emulsion system (Benzonana and Desnuelle, 1965), and (2) solvent-soluble substrate with solvent-insoluble enzyme in an organic solvent system (Klibanov, 1986; Kwon et al., 1996). For this reason, interfacial activation usually takes place to activate the lipase structure for the reaction (Sarda and Desnuelle, 1958). A minimum substrate concentration for interfacial activation, called the interfacial activation concentration (S_{int}), is required. In this reaction, 80 mM of substrate (racemic form) is the apparent interfacial concentration for synthesis of (*S*)-MBE in organic solvent. This value is

higher than those reported in the aqueous or emulsion phase (Kwon and Rhee, 1983). Usually, in an organic solvent system, the interfacial activation concentration of lipases was greater than in an aqueous or emulsion system with a hydrophobic substrate having a higher value (Martinelle and Hult, 1994). In heterocatalytic reactions involving lipases, especially in organic solvent systems, the diffusion limitation of substrate to the interface in addition to the active site is the main obstacle to reaction. This is because every heterocatalytic reaction including an organic solvent system has a diffusion barrier in transporting the substrate to the interface at low concentration (S_{diff}). Thus lipase reactions in organic solvent systems apparently may have higher interfacial activation concentrations ($S_{int} + S_{diff}$) than those of aqueous or emulsion solvent systems (S_{int}). However, detailed study to discriminate between S_{int} and S_{diff} is necessary. Benzonana and Desnuelle (1965) were unable to discriminate between S_{int} and S_{diff} in an emulsion system. Moreover, interfacial activation in an organic solvent is more obscure than in the aqueous phase or emulsion system, because the S_{int} and S_{diff} should depend on the reaction system. For this interfacial activation and diffusion limitation, the synthetic reaction of lipase in organic solvent might require more than 80 mM concentration of *rac*-MBA (Kwon and Rhee, 1983). Whereas synthetic activity of lipase was increased with the increase of substrate in organic solvent up to 700 mM, enantioselectivity was not changed with increase in substrate concentration (Figure 4). The enantiomeric excess (ee) for (*S*)-enantiomers was about 50−60 when the substrate concentration ranged from 100 to 600 mM. Above 700 mM (racemic form), the synthetic activity of lipase in organic solvent decreased. The same phenomenon was observed with the lipase reaction in an emulsion system (Kwon and Rhee, 1983). This might be due to the physical barrier for substrate transportation into the interfacial area in the case of an excess of substrate or substrate inhibition. However, it is still unclear why the enzyme activity decreased at high substrate concentration.

From these heterocatalytic reactions, reaction kinetic parameters such as K_M and V_{max} were obtained by drawing a double reciprocal plot (Lineweaver−Burk plot) from the Michaelis−Menten curve as an apparent value (Figure 5). The apparent Michaelis−Menten constants for (*S*)-MBE production, such as ${}_{app}K_{M,S}$ and ${}_{app}V_{max,S}$, were evaluated to be 1120 mM and 2.24×10^7 area/h from the intersection points. For the (*R*)-enantiomers, ${}_{app}K_{M,R}$ and ${}_{app}V_{max,R}$ were 1240 mM and 7.09×10^6 area/h, respectively. The K_M values in organic solvent systems were higher than those in aqueous and emulsion systems probably as a result of the rigidity of the enzyme in the organic solvent. In fact, in aqueous and micelle systems, K_M values of lipases for the hydrolysis of esters were about 10−50 mM, and in organic solvent systems, K_M values were around 500 mM (Kikkawa et al., 1989; Martinelle and Hult, 1994).

The data show that there is little difference in ${}_{app}K_M$ but substantial difference in ${}_{app}V_{max}$ between (*R*)- and (*S*)-MBA, indicating that the enzyme could not discriminate (*S*)- from (*R*)-MBA, which can bind at the active site with the same binding affinity. However, the enzyme catalyzed synthesis of MBE with different rates between (*S*)- and (*R*)-MBA. These results indicate that enantioselectivity did not originate from the substrate binding affinity but from the catalytic activity of lipase

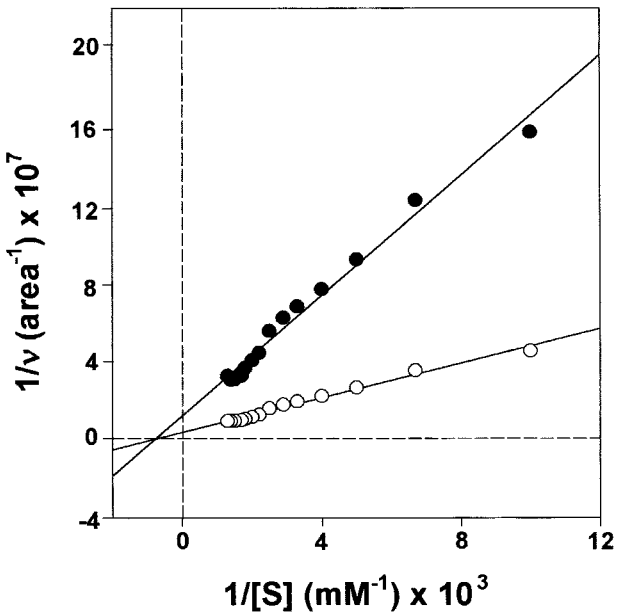

Figure 5. Lineweaver−Burk plot of lipase activity from Figure 4 for (*S*)-MBE (○) and (*R*)-MBE (●) synthesis in organic solvent.

in organic solvent. The $(V_{max,S}/K_{M,S})/(V_{max,R}/K_{M,R})$ for MBE production was 3.48, which corresponded to above 50−60% of enantiomeric excess. In esterification of *n*-dodecanoic acid with secondary alcohols in organic solvent using *Pseudomonas fragi* lipase, $(V_{max,R}/K_{M,R})/(V_{max,S}/K_{M,S})$ varied from 0.96 to 15, depending on the secondary alcohols (Kikkawa et al., 1989).

(*S*)-2-Methylbutanoic Amide Identification and Inhibition Study. It is known that an aliphatic amide, which is an analogue of a lipase substrate (aliphatic acid), acts as a competitive inhibitor of an enzyme (Russell and Klibanov, 1988; Kwon, 1992). In this study, (*S*)-2-methylbutanoic amide, a competitive inhibitor of lipase, was synthesized. This inhibitor was identified by HPLC and mass spectrometry as (*S*)-2-methylbutanoic amide, which is structurally analogous to (*S*)-2-methylbutanoic acid (Figure 6).

Inhibitors are substances that tend to decrease the rate of an enzyme-catalyzed reaction by prohibiting the substrate binding (competitive inhibition) or changing the conformation of the enzyme or active site (uncompetitive inhibition or noncompetitive inhibition). The presence of (*S*)-2-methylbutanoic amide (20 mM) in the reaction mixture reduced lipase activity. A Lineweaver−Burk plot (Figure 7) confirmed that (*S*)-2-methylbutanoic amide acted as a competitive inhibitor of lipase because it changed the ${}_{app}K_{M,S}$ value but not ${}_{app}V_{max,S}$. The apparent dissociation constant (K_i) for the enzyme−inhibitor (EI) complex was 84 mM, which was much smaller than that of the enzyme−substrate complex (${}_{app}K_M$). This means that the binding affinity of the inhibitor ((*S*)-2-methylbutanoic amide) is much stronger than that of the substrate ((*S*)-2-methylbutanoic acid) and that inhibition of (*S*)-2-methylbutanoic amide occurs by prevention of the substrate from binding the enzyme. In general, imprinting of an enzyme by a ligand is recommended as the way to improve the enantiomeric selectivity (Kamiya and Goto, 1998; Kwon, 1992; Vulfson et al., 1997). This enantiospecific competitive inhibitor can be used as a ligand for enzyme imprinting to improve the enantioselectivity of lipase for synthesizing the (*S*)-2-methylbutanoic acid methyl ester. This is the

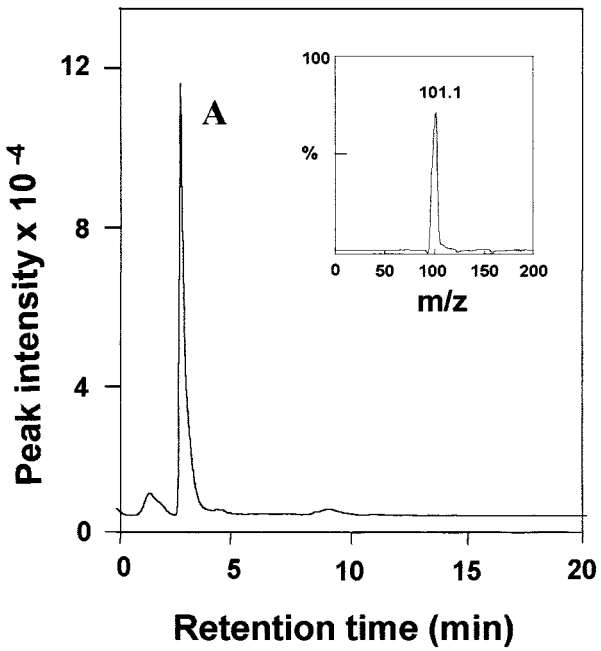

Figure 6. HPLC chromatogram of (*S*)-2-methylbutanoic amide (peak A) after synthesis from (*S*)-2-methylbutanoic acid. HPLC solvent condition was water/acetonitrile (7:3) in an isocratic system. Electrospray mass spectrum of synthesized (*S*)-2-methylbutanoic amide (peak A in HPLC chromatogram) is shown at the top right of the chromatogram. The number on the peak (101.1) indicates the mass for single charge of (*S*)-2-methylbutanoic amide.

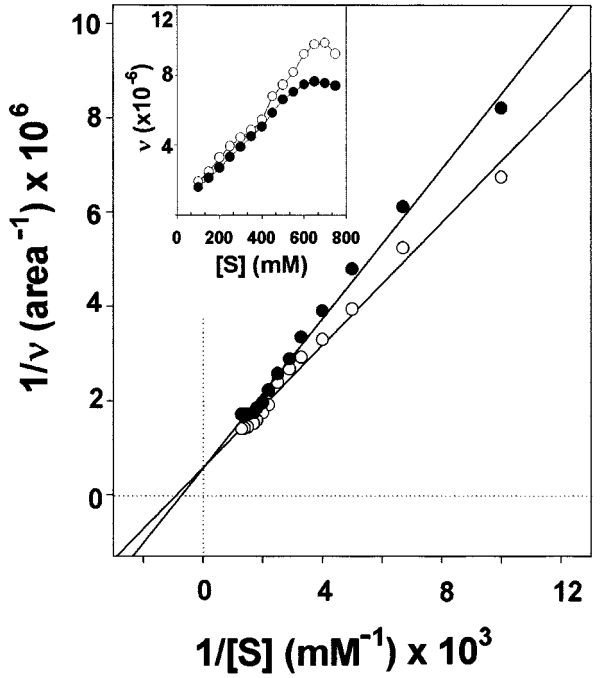

Figure 7. Lineweaver−Burk plot of lipase synthetic activity for (*S*)-MBE without (○) and with (●) (*S*)-2-methylbutanoic amide. The Michaelis−Menten curve for each plot is shown inside the graph.

reason the kinetic study of enzyme inhibition was done prior to the enzyme imprinting study.

In conclusion, the data suggest a way to produce natural apple flavor as a high value-added product using the enzyme. However, further research on the increase of the enantiomeric selectivity and on the selective purification of products from the byproducts will be necessary for commercial production.

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Received for review August 5, 1999. Revised manuscript received November 22, 1999. Accepted November 26, 1999. This work was done with support in part by a grant from the Agricultural Research Promotion Program under the Ministry of Agriculture and Forestry, Korea.

JF990871W