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Molecular modeling and experimental verification of lipase-catalyzed enantioselective esterification of racemic naproxen in supercritical carbon dioxide

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Abstract−Experimental and simulation analyses were performed on the lipase-catalyzed esterification reaction of racemic naproxen by CALB (*candida antarctica* lipase B) enzyme in supercritical carbon dioxide. The reaction pathways were investigated by quantum mechanical analysis, and the enantioselectivity of the products was predicted by molecular dynamics simulation analysis. Calculated results from molecular modeling in supercritical carbon dioxide were qualitatively compared with experimental data by using racemic naproxen as a substrate. All molecular modeling results and experimental data were acquired and compared with those in ambient and supercritical condition. Moreover, to verify the stability of enzymatic reaction in each solvent condition, reaction pathways were investigated in several solvent conditions (vacuum, water, hexane and supercritical carbon dioxide), and the stability of enzymatic reaction in supercritical carbon dioxide was compared with other solvent conditions.

Key words: Racemic Naproxen, *Candida antarctica* Lipase B, Enantioselectivity, Supercritical Carbon Dioxide, Quantum Mechanical Analysis, Molecular Dynamics Simulation

INTRODUCTION

Advances in molecular modeling analysis in biological systems are expressed in the more detailed description of reaction mechanism, molecular structures and the prediction of selectivity [1]. Especially, as the use of enzymes in organic solvents has been enhanced, molecular calculation studies using modern computing have been developed with improved algorithms and more reliable approach methods about enzymatic reactions [2]. Some attempts have been made [3,4] to calculate enantioselectivity by experiments, with fair prediction of fast-reacting enantiomer. Recently, Kwon [5] proposed a method to analyze catalytic reaction by *Candida antartica* lipase (CALB) using a simplified model for quantum mechanical (QM) and molecular dynamics (MM) studies.

Supercritical carbon dioxide ($SCCO_2$) is a new reaction medium that offers several advantages as an alternative to organic solvents [6]. Especially, enzymatic reactions in $SCCO_2$ [7] can be achieved with high efficiency, resolution and selectivity compared with conventional organic solvents. In addition, lipase has been recognized as useful biocatalyst for enantioselective hydrolysis or esterification and transesterification because of its wide substrate specificity and ability to recognize chirality [8,9]. In the case of naproxen, lipase has also been used to prepare optically pure naproxen by the enantioselective hydrolysis of its racemic esters [10-13].

Enzyme stability and activity depend on the substrate concentration, amount of enzyme and water, reaction time and solvent, such as organic solvent (hexane) or SCF ($SCCO_2$) [13]. These parameters play an important role in the enhancement of the selectivity. In the last section of this paper, reaction pathways are also investigated in several solvent conditions (vacuum, water, hexane and $SCCO_2$) to verify the solvent effects and the stability of enzymatic reaction in $SCCO_2$.

In the present study, simplified quantum mechanical calculations have been performed to identify energies and configurations of whole reaction pathways for CALB and racemic naproxen in $SCCO_2$. Molecular dynamic simulations were performed to identify selectivity of (S)-form and (R)-form enantiomer. The calculation results were compared with experimental data in qualitative manner by using racemic naproxen as a substrate. This approach is an indispensable complement to both molecular analysis and experimental determination. The goal of this study was to predict the enantioselectivity of the lipase-catalyzed esterification reaction of racemic naproxen by molecular modeling approach. Also, the solvent effects and the stability of enzymatic reaction in $SCCO_2$ were investigated quantitatively for the first time.

MATERIALS AND METHODS

1. Preparation of the Enzyme and the Substrates

The active site of enzyme, CALB, is illustrated in our previous work [5]. Positions of all non-hydrogen atoms in active sites were held constant and positions of hydrogen atoms were optimized during the calculations. Naproxen was used as a substrate to investigate the reaction pathway and the selectivity of the lipase-catalyzed reaction. The structures of (R)-form, (S)-form naproxen used in this molecular modeling study are described in Fig. 1.

2. Reaction Pathway and Enantioselectivity Calculation: QM and MD Simulation Method

The energy values and molecular conformations were calculated in this study by optimization of the molecular structure of each system. To perform this simulation, the semi-empirical PM3 method [15] was used within Accerlys DS Modeling 2.0 software [16] to research the reaction pathway of enantioselective esterification of

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‡This paper is dedicated to Professor Chul Soo Lee on the occasion of his retirement from Korea University.

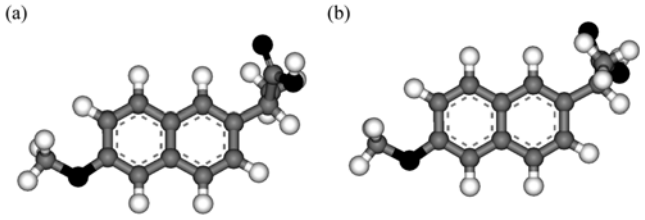

Fig. 1. The structures of the substrates, (a) Substrate of (R)-naproxen, (b) Substrate of (S)-naproxen.

(R)-naproxen and (S)-naproxen. The CHARMM force fields [17] were used with Accerlys DS Modeling 2.0 software to perform MD simulation. All simulation processes to optimize the configurational energy are similar to previous work [5] with the proposed reaction mechanism, ping-pong bi-bi mechanism [18].

3. Experimental Method to Verify Computational Results

3-1. Racemization of Naproxen

To racemize the naproxen, 25 g of naproxen was mixed with sodium hydroxide (16 g) and dissolved in ethylene glycol (150 ml). The mixture was then stirred under reflux at 175 °C for 1 h. After cooling in the ambient condition, the addition of 1 N HCl (200 ml), produced a white sediment. Distilled water was successively added into the resultant solution. After the process of filtering by Whatman GF/C filter, washing with water, drying under a reduced pressure in a dry oven, the racemate was finally obtained in a powder form. A complete racemization was confirmed as evidenced by two peaks of nearly the same area measured from the HPLC system.

3-2. Esterification Reaction

For experimental verification of molecular modeling calculation, an esterification reaction for determination of enantioselectivity was carried out with racemic naproxen by CALB in ambient and supercritical condition. Lipase (Novozyme 435) was purchased from NOVO Nordick (Denmark). Experiments were performed in a high-pressure cell at 325.15 K and 130 bar for 5 hrs in $SCCO_2$, and 48 hrs in the ambient condition with a stirring rate of 150 rpm, 7 g of enzyme amount and 2% water content. The esterification reaction products were analyzed by HPLC (YOUNG-LIN Instrument Co. Ltd., Korea) with a chiralcel OD column (Daicel Chemical Industries, Japan).

4. Molecular Modeling of $SCCO_2$ and Other Organic Solvents Condition

As discussed by some authors [19], the dielectric constant is a key property for presenting the phase behaviors in $SCCO_2$ since it is known that the dielectric constant of CO_2 dramatically changes in supercritical conditions [20]. Supercritical carbon dioxide has an exceptionally low value of the dielectric constant. In this study, the dielectric constant of carbon dioxide was 1.5, and those of organic solvents, water and hexane, were 78.4 and 2.02, respectively. To compare the effect of different solvents, the activation energy and conformational stability of lipase-catalyzed esterification reaction in supercritical carbon dioxide were compared with results of the molecular simulation in each solvent state by using different values of dielectric constants.

RESULTS AND DISCUSSION

1. Complete Reaction Pathway and Chiral Configuration

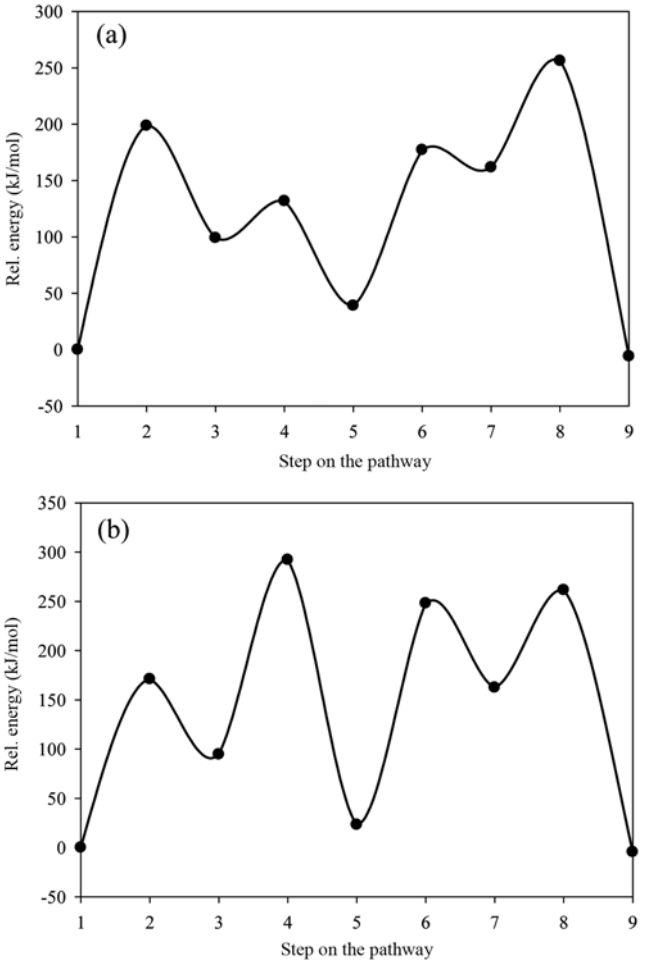

Fig. 2. Complete reaction profile and significant points of the hydrolysis reaction of CALB-naproxen ethyl ester; (a) (R)-naproxen ethyl ester, (b) (S)-naproxen ethyl ester.

Table 1. Energies of CALB and (R, S)-naproxen ethyl ester complex

Pathway	Relative energy (KJ/mol)	
	(R)-naproxen ethyl ester	(S)-naproxen ethyl ester
1	0.00	0.00
2	198.61	171.12
3	99.00	94.70
4	131.82	292.24
5	39.02	23.20
6	177.19	248.10
7	161.65	162.50
8	256.19	261.60
9	−5.90	−4.42

The complete reaction profiles and significant points of this enzymatic reaction of (R)-naproxen ethyl ester and (S)-naproxen ethyl ester are described in Fig. 2. The calculated energy values of (R)-form and (S)-form naproxen ethyl ester are shown in Table 1. Each molecular conformation over the nine minimized structures of (S)-form naproxen ethyl ester is presented in Fig. 3.

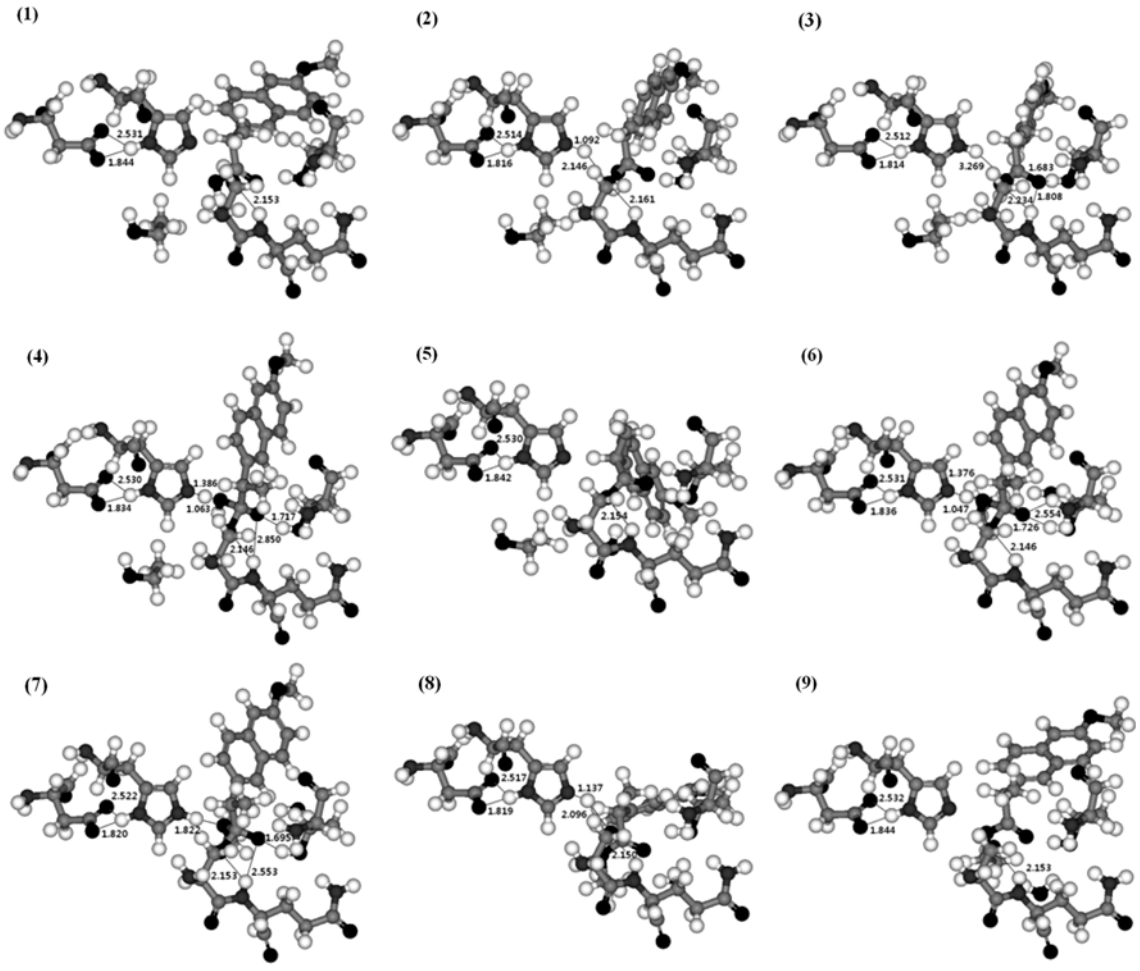

Fig. 3. Structures of minimization and transition states (bond lengths in Å).

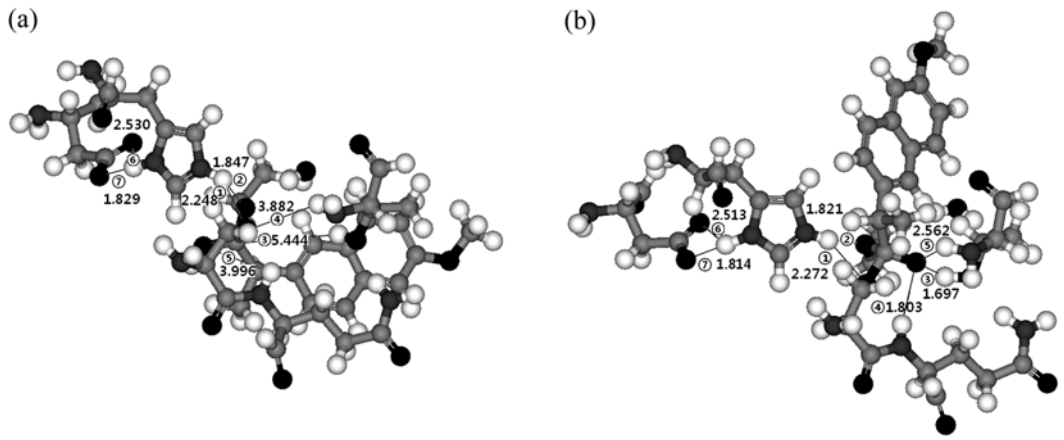

Fig. 4. (a) Structures of the sites of significant hydrogen bonding in the CALB-(R)-naproxen ethyl ester; (b) in the CALB-(S)-naproxen ethyl ester.

To identify the conformational preference of (R, S)-forms, the hydrogen bond lengths were examined on seven parts (from ① to ⑦) of the tetrahedral intermediate (7th reaction step) on the basis of function-based subset in Fig. 4. The significant hydrogen bonding in the active site was studied by analysis of MD results, and the (S)-form ester was more stable than the (R)-form ester in Fig. 5. In this study of molecular dynamics simulation, we could know that the CALB-(S)-form naproxen ethyl ester was more stable than the CALB-(R)-form.

2. Experimental Verification of Molecular Dynamics Study

Conversion yields of (S)-naproxen ethyl ester of the catalyzed reaction in $SCCO_2$ were compared with those at ambient condition.

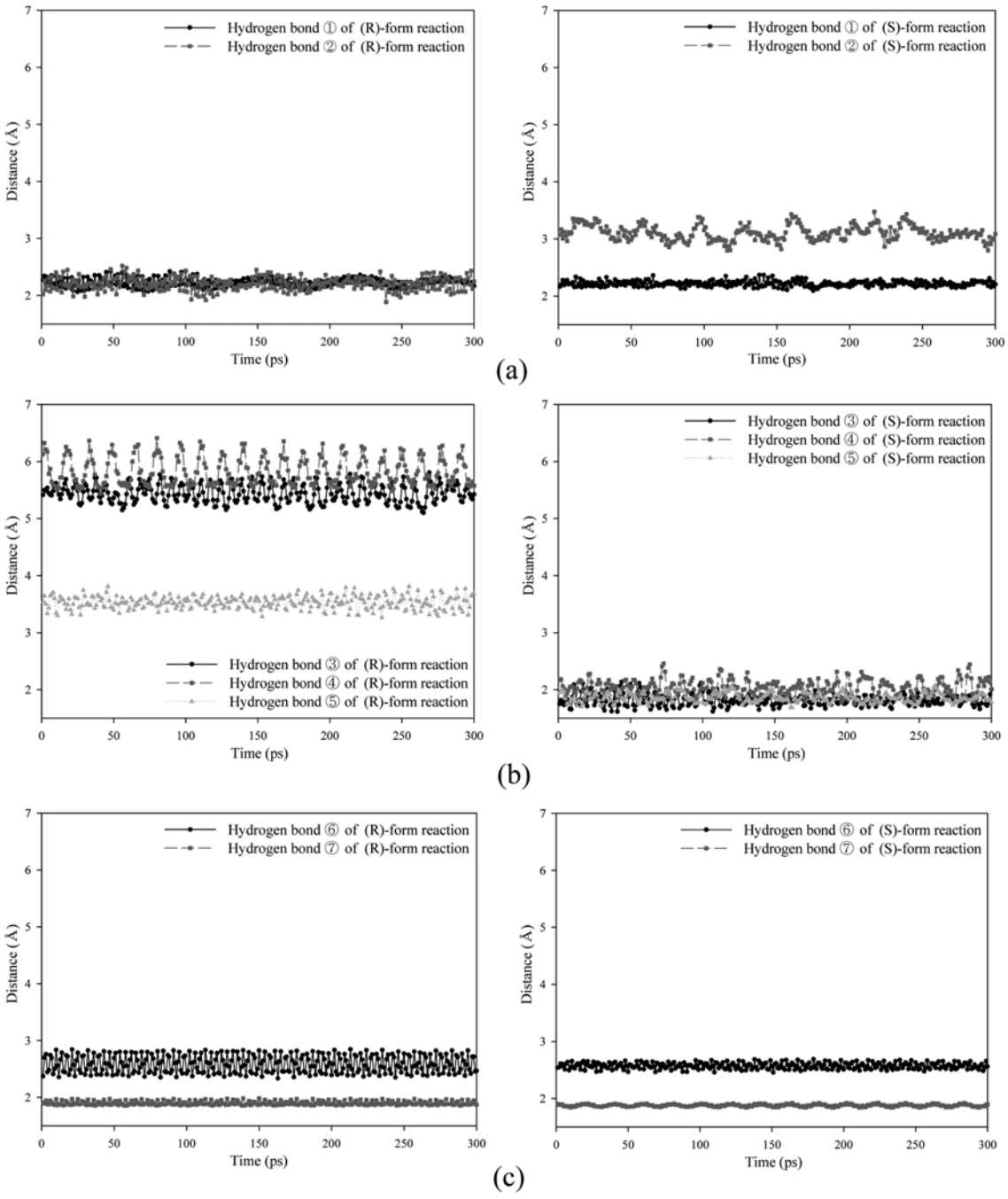

Fig. 5. Trajectories and histograms of the hydrogen bonds of CALB-(R, S)-ethyl ester; (a) hydrogen bond lengths of ① and ② (b) hydrogen bond lengths of ③, ④ and ⑤ (c) hydrogen bond lengths of ⑥ and ⑦.

In Fig. 6(a), (b) the conversion yields of (S) and (R)-form in $SCCO_2$ are represented as 86.6% and 17.3% (a), respectively. At ambient condition, the conversion yield of the esterification reaction was 43.6% and 6.4% (b), respectively. These results show higher production of (S)-form than (R)-form and also prove that hydrogen bonds of CALB-(S)-form enantiomer complex of were more stable than the CALB-(R)-form enantiomer complex. The conversion yield of (S)-form ester in $SCCO_2$ was compared with those in ambient condition as 86.6%, 43.6%, respectively, as shown in Fig. 6(c). According to these results, we could also verify that the productivity in $SCCO_2$ condition is much higher than that in the ambient condition.

3. Stability of Enzymatic Reaction in $SCCO_2$

Most biomolecules are functional in an aqueous environment. The importance of solvent effect on the structure, energetics and dynamics of biomolecules has long been recognized in biochemical reactions. Also, we could focus on the stability of enzymatic reaction under the appropriate solvent condition for obtaining high productivity and selectivity recently. In this work, for investigating the reaction stability in each solvent condition, particularly in $SCCO_2$, the mechanism consisted of an esterification reaction by CALB in $SCCO_2$ to make (R, S)-naproxen ethyl ester. Then, the minimization energy value and the reaction pathway in $SCCO_2$ were compared

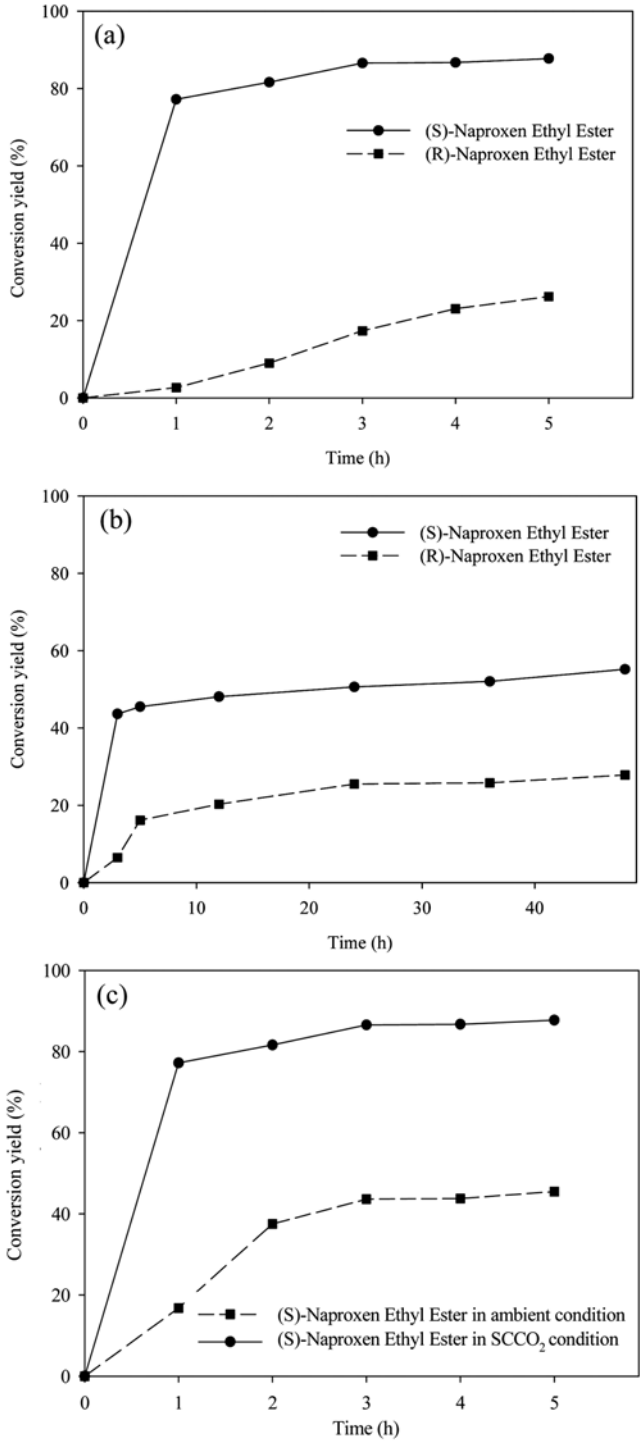

Fig. 6. Comparison of the productivity by the experimental conditions: (a) Esterification of racemic naproxen in $SCCO_2$ at 323.15 K and 130 bar. (b) Esterification of racemic naproxen in ambient condition. (c) Comparison of (S)-naproxen ethyl ester productivity: esterification of racemic naproxen in $SCCO_2$ and atmospheric pressure.

with those in vacuum, water and hexane in Table 2 and Fig. 7, respectively. As shown in this figure, the minimization energy value in $SCCO_2$ is lower than the one in other solvents. In other words, the enzymatic reaction in $SCCO_2$ is still faster and more stable than other solvent's conditions due to the high diffusivity.

Table 2. Conformational energies of CALB in several solvents

Pathway	Rel. energy (KJ/mol)			
	Vacuum	Water	$SCCO_2$	Hexane
1	0	0	0	0
2	171.125	627.248	208.161	257.028
3	94.692	134.450	163.408	163.032
4	292.243	573.116	229.792	192.549
5	23.201	44.993	95.256	88.289
6	248.089	458.863	265.837	342.942
7	162.500	180.775	199.583	192.762
8	261.600	538.008	266.615	337.302
9	−4.422	−6.636	−11.917	−29.578

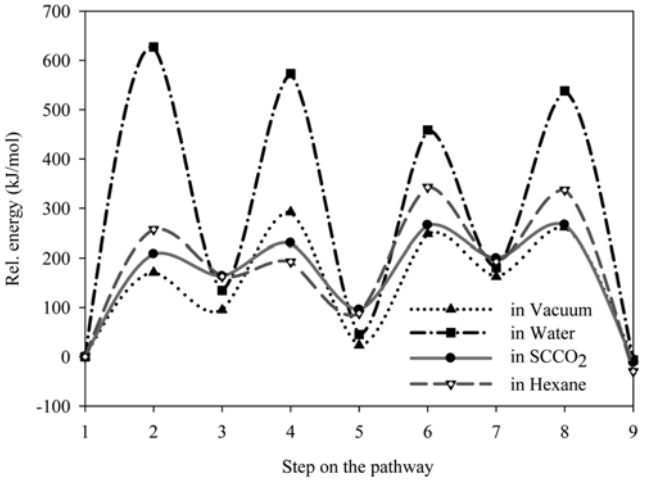

Fig. 7. Complete reaction pathway by the proposed reaction mechanism of (S)-naproxen ethyl ester.

CONCLUSIONS

Quantum mechanical and molecular dynamics simulation analysis has been performed on (R, S)-naproxen ethyl ester by CALB in $SCCO_2$ and other solvent conditions. From the analysis of the trajectories and histograms of the molecular dynamics simulation, the enantioselectivity of lipase could be explained by calculating the binding energy and structural characteristics.

From the results of these molecular modeling works, we could recognize that hydrogen bonds of CALB-(S)-form enantiomer complex were more stable than the CALB-(R)-form complex. These results verified that supercritical conditions produced a higher yield and a more rapid reaction time compared with normal conditions by experimental study. In addition, the minimization energy value in $SCCO_2$ is lower than that of other solvents by molecular modeling technology.

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