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Molecular Modeling and its Experimental Verification for the Catalytic Mechanism of *Candida antarctica* Lipase B

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Abstract Quantum mechanical and molecular dynamics simulation analysis has been performed on the model system for CALB (*Candida antarctica* lipase B) with esters to study the reaction mechanism and conformational preference of catalytic hydrolysis and the esterification reaction. Using quantum mechanical analysis, the ping-pong bi-bi mechanism was applied and energies and 3-dimensional binding configurations of the whole reaction pathways were calculated. Further molecular dynamics simulation analysis was performed on the basis of the transition state obtained from quantum mechanical study to observe the effect of structures of the substrates. Calculation results using substrates of different chain length and chiral configurations were compared for conformational preference. The calculated results showed very small influence on chain length, whereas chiral conformation showed big differences. Calculated results from molecular modeling studies have been compared qualitatively with the experimental data using racemic mixtures of (±)-cis-4-acetamido-cyclopent-2-ene-1-ethyl acetate as substrates.

Keywords: Molecular modeling, quantum mechanics, molecular dynamics simulation, *Candida antarctica* lipase B, reaction mechanism, enantiomer

Lipases are most widely used in the commercial production of pharmaceuticals, cosmetics, foods, and fine chemicals [9, 15, 25]. In particular, lipase-catalyzed reactions were considered as an important process of the asymmetric synthesis of enantiomerically pure components [1, 19]. Until now, some experimental results in this area have led to commercial applications, but deeper understandings of the reaction, such as reaction mechanisms and conformational selectivity of substrate, are still far from complete. Computer-based modeling technologies can be used to understand the mechanism and selectivity of enzyme-catalyzed reactions [7, 8, 10, 13, 14, 16]. Depending on the complexity of the model systems and properties required, different types of molecular modeling approaches can be used [17, 18]. However, owing to current computational limits, one cannot model the whole system using most rigorous *ab-initio* HF-SCF calculations. Attempts have been made [4, 22, 24] to calculate enantioselectivity using force field methods, with fair prediction of fast-reacting enantiomers [23]. Monecke *et al.* [20] proposed a method to analyze the catalytic hydrolysis reaction by *Candida rugosa* lipase (CRL) using a simplified model for quantum mechanical and molecular dynamics studies. In this study, simplified quantum mechanical calculations have been performed to identify energies and 3-dimensional configurations of whole reaction pathways for CALB and alkyl ester systems. Furthermore, molecular dynamic simulations were performed to identify selectivity of substrates with different chain lengths and enantiomers. The calculation results were compared with experimental data in a qualitative manner using a racemic mixture of (±)-cis-4-acetamido-cyclopent-2-ene-1-carboxylic ethyl acetate as a substrate.

Materials and Methods

Preparation of the Enzyme Structure

The crystal structure of *Candida antarctica* lipase B (PDB code: 1TCA) [27] was used as the starting point for modeling the CALB-substrate transition state. For complex quantum mechanical calculations, only the catalytic triad (Ser 105, Asp 187, His 224) and anion hole (Thr 40, Gln 106) were selected as skeleton groups and used as a simplified model. The active site of CALB is illustrated in Fig. 1. All positions of non-hydrogen atoms in the active sites were held constant and the positions of hydrogen atoms were optimized during the calculations.

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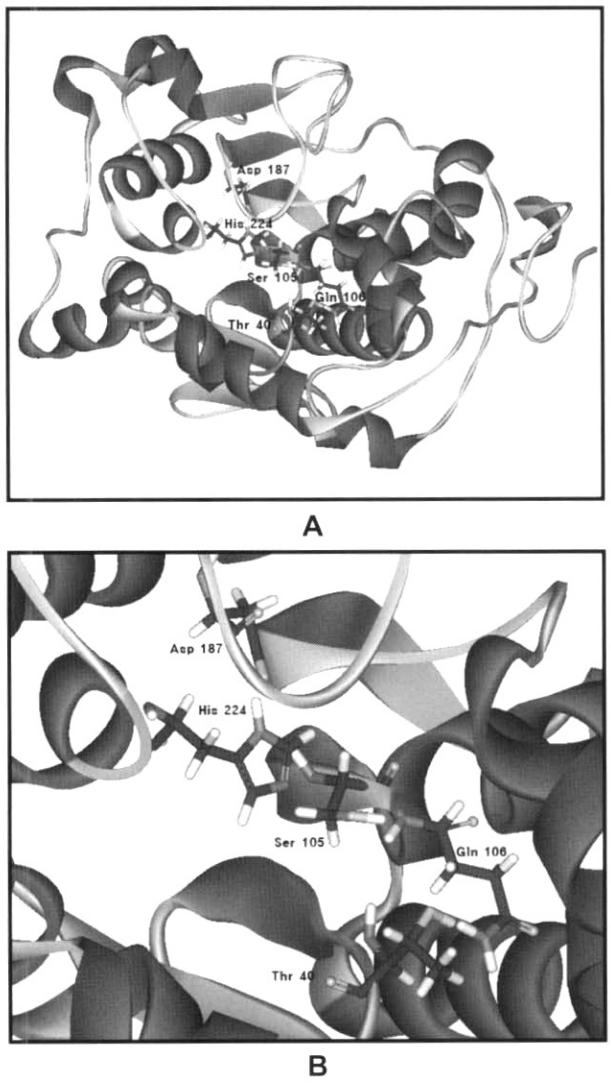

Fig. 1. Active sites of *Candida antarctica* lipase B structure.
A. The whole structure of CALB. **B**. Close-up of the active sites of CALB.

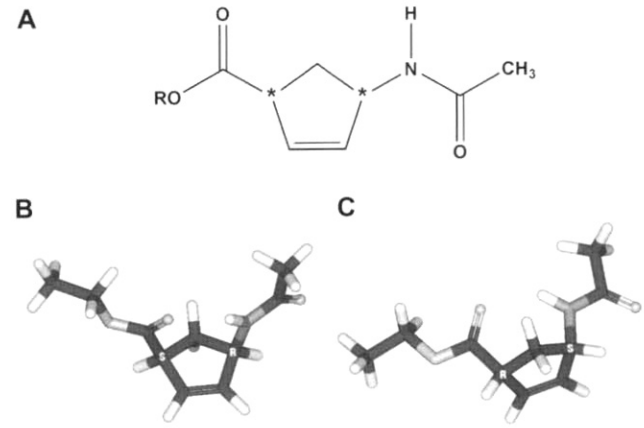

Fig. 2. Structures of substrates; the indication R, S, and * refer to the chirality.
A. Structure of the considered cis-4-acetamidocyclopent-2-ene alkyl esters; R (group of alkyl chain), methyl to n-hexyl group. **B**. Structure of (+)-cis-4-acetamidocyclopent-2-ene ethyl acetate. **C**. Structure of (−)-cis-4-acetamidocyclopent-2-ene ethyl acetate.

Preparation of the Substrates

For the reaction pathway calculations, (±)-cis-4-acetamido-cyclopent-2-ene-1-alkyl esters were taken as substrates for comparing the selectivity of the catalytic reaction. These esters are precursors for the synthesis of aristereomycin. As shown in Fig. 2, components with different side chain lengths (methyl to n-hexyl) and different chiral configurations (+ and − forms) were considered for comparative study. All the substrate molecules were preliminarily optimized for minimum energy using the MM2 method of the HyperChem program [11] before main calculations were performed.

Reaction Pathway Calculation: Quantum Mechanical Calculation Method

The semiempirical PM3 method [26] has been used within the MOPAC 6.1 software package [3] to study the reaction pathway of the simplified model of the CALB enzyme catalyzed reaction. For the verification purpose, the minimum and maximum energy points were compared with rigorous *ab-initio* calculations using GAUSSIAN software [6] with 6-31 basis set.

Reaction Selectivity Calculation: Molecular Dynamic Simulation Method

The CHARMM [2, 21] force fields were used with Accerlys DS Modeling 1.1 software [5] to perform MD simulation of the simplified model system. During the simulations, the positions of non-hydrogen atoms of active sites had been fixed, and positions of substrates and hydrogen atoms were optimized for configurational energy. The simulation process was divided into five steps: (1) The first minimization step (steepest descent method/500 times); (2) The second minimization step (adopted basis Newton Raphson method/ 500 times); (3) Heating (2,000 times, time step=0.001 ps); (4) Equilibrium (1,000 times, time step=0.001 ps); (5) MD-NVT (3,000 times, time step=0.001 ps). During the first period, the temperature was raised stepwise from 50 to 323 K for the method of simulated annealing. A time step of 0.001 ps was used in all simulations. After the heating period, the temperature was kept constant at 323 K during the simulations. A distance-dependent dielectric function was used and a nonbonded cut-off of 1,350 pm was applied. The algorithm used was the Leap-Frog Verlet algorithm [12]. Low energy conformations were collected from the MD trajectory with a time interval of at least 1 ps, in order to avoid structures belonging to the same local minimum. The structures were collected every 100 steps, when the system had equilibrated and exhibited its lowest potential energy.

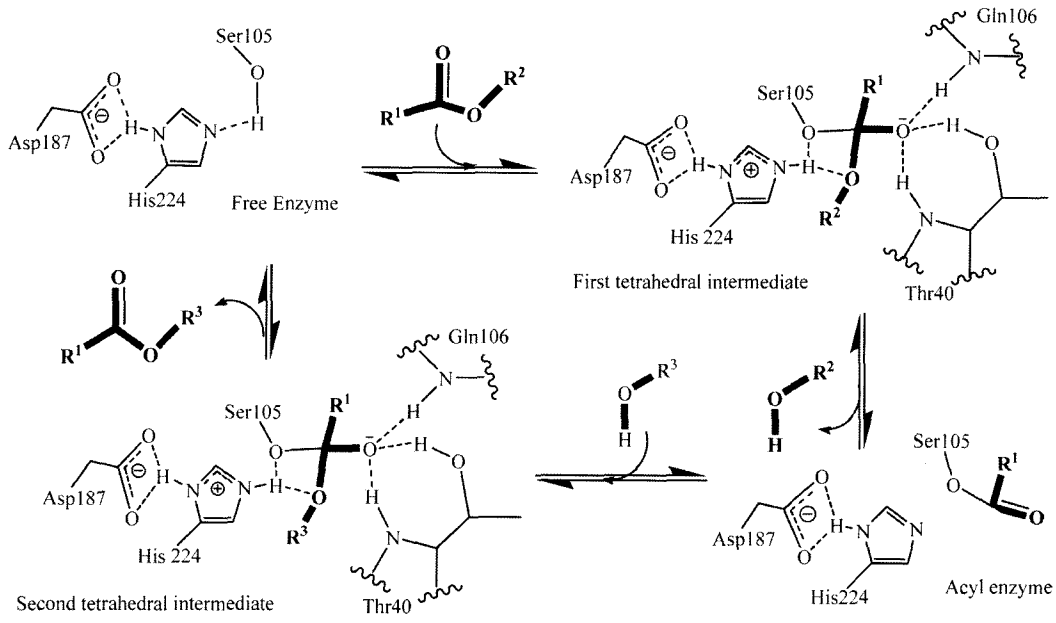

Fig. 3. Reaction mechanism of catalyzed hydrolysis or esterification by CALB.

Materials and Experimental Methods Used to Verify Computational Result

Since little experimental data have been reported for our model system, a simple hydrolysis experiment of racemic mixtures was performed and compared with the molecular modeling calculation result. Our model substrates were composed of (±)-cis-4-acetamido-cyclopent-2-ene-1-carboxylic acid and purchased from Samchully Co. Ltd., Korea. Novozyme 435 was purchase from NOVO Nordisk (Denmark). For determination of enantioselectivity, (±)-cis-4-acetamido-cyclopent-2-ene-1-carboxylic acid was ethylated by adding 2 ml of 14% boron trifluoride in ethanol at 323.15 K for 10 min, and then 40 mg/ml novozyme 435 was added to 5 ml of solvent containing 13.4 mM (±)-cis-4-acetamido-cyclopent-2-ene-1-carboxylic methyl ester. The reaction was carried out in a water-bath for 36 h at 323.15 K with shaking at 200 rpm. Hydrolysis reaction products were analyzed by HPLC (YOUNG-LIN Instrument Co. Ltd., Korea) using a Chiralcel OD column (Daicel Chemical Industries, Japan). Ultraviolet detection (YOUNG-LIN Instrument Co. Ltd., Korea) at 204 nm was for quantification at column temperature of 293.15 K. The mobile phase was a mixture of n-heptane:ethanol (90:10, v/v) at a flow rate of 0.7 ml/min.

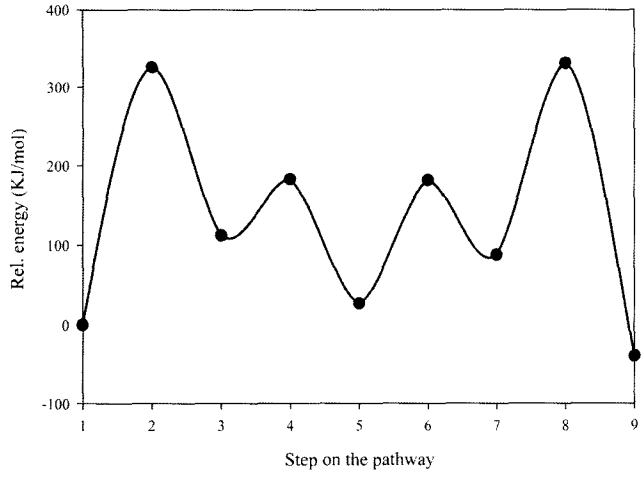

Fig. 4. Complete reaction profile and significant points of the hydrolysis reaction of the CALB-ethyl acetate complex.

Results

Calculation of Complete Reaction Pathways

The reactions catalyzed by CALB follow a ping-pong bi-bi mechanism, as shown in Fig. 3 [19, 20]. The substrate

Table 1. Conformational energies of CALB and ethyl ester complex.

Pathway	Conformational energy (KJ/mol)	Relative energy (KJ/mol)
1	-2,369.48	0
2	-2,043.65	325.83
3	-2,257.08	112.40
4	-2,186.56	182.92
5	-2,342.51	26.98
6	-2,187.70	181.78
7	-2,281.48	88.00
8	-2,038.54	330.94
9	-2,409.30	-39.82

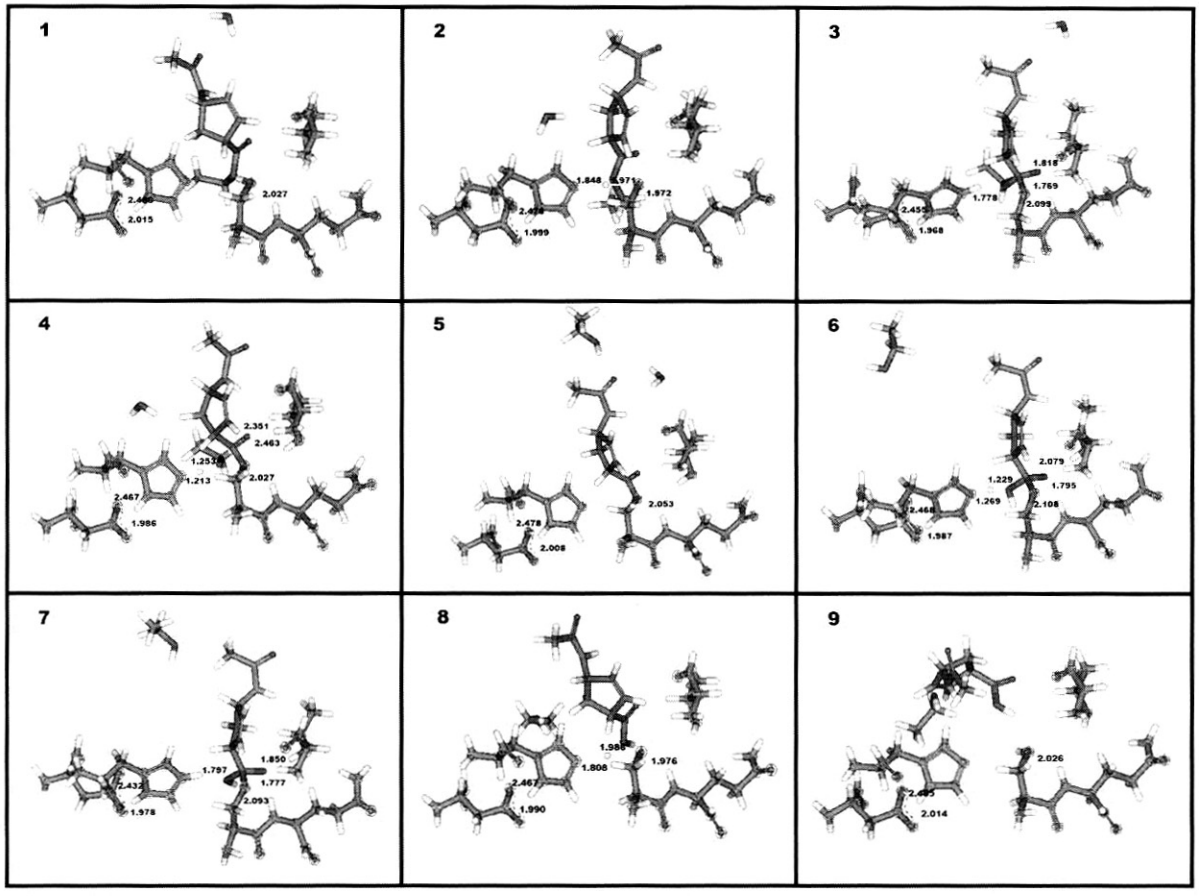

Fig. 5. Structures of minimization and transition states (bond lengths in Å; color code: C, gray; H, white; O, red; N, blue).

enters the active site of the enzyme and the first tetrahedral intermediate is formed (top right). The first product leaves the active site and then the acyl-enzyme is formed (bottom right). The second substrate enters the active site and the second tetrahedral intermediate is formed (bottom left). The product leaves the active site and the enzyme is ready for another catalytic cycle (top left). Based on this theory, the reaction mechanism of the CALB enzyme consists of a total of 9 detailed steps, for which the optimization and calculation of the minimization energy needs to be considered. The energy calculations over a total of 9 steps were carried out using the semiempirical PM3 methods

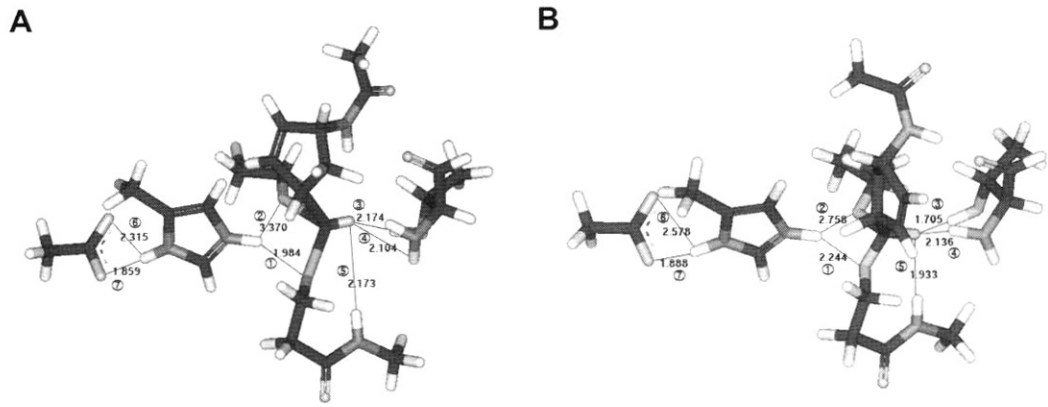

Fig. 6. Hydrogen bond lengths in the CALB-ethyl ester complex.
A. CALB-(+)-ethyl ester. **B**. CALB-(−)-ethyl ester.

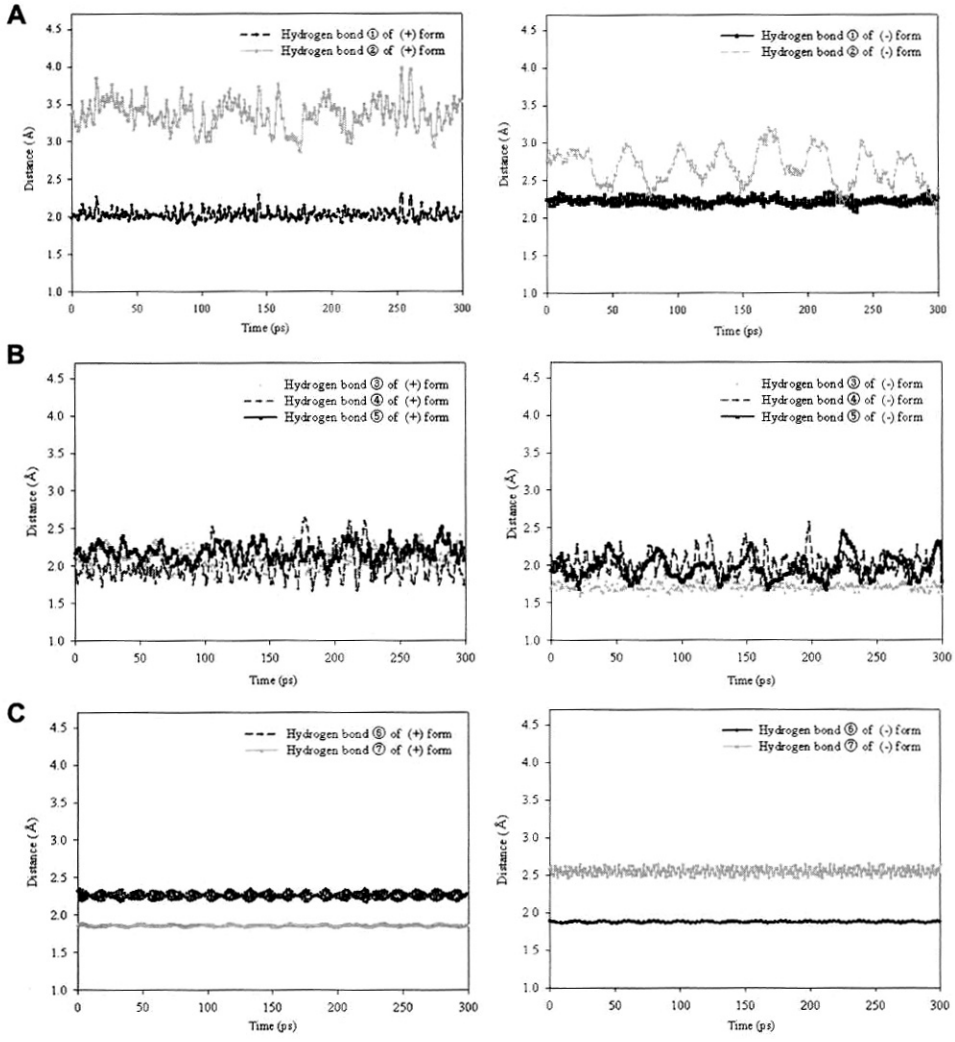

Fig. 7. Trajectories and histograms of the hydrogen bonds of CALB-(±)-ethyl ester.
A. Hydrogen bond lengths of ① and ②. **B**. Hydrogen bond lengths of ③, ④, and ⑤. **C**. Hydrogen bond lengths of ⑥ and ⑦.

implemented in MOPAC (CAChe 6.1) [3]. The map reaction function of the CAChe program was used to determine the transition states of the reaction. The complete reaction profile and significant points of the enzymatic reaction are presented in Fig. 4. The calculated energy values and molecular forms corresponding to each conformation over the 9 minimized structures are shown in Table 1 and Fig. 5.

Effect of Chiral Configuration

The structures of the CALB-(±)-ethyl ester complexes after the molecular dynamics simulation are displayed in Fig. 6. We could confirm the coherence of the hydrogen bond between the substrate and enzyme through the trajectory file of DS modeling. Seven relevant hydrogen bonds in the active site of the CALB-(±)-ethyl ester complexes were also investigated in Fig. 6.

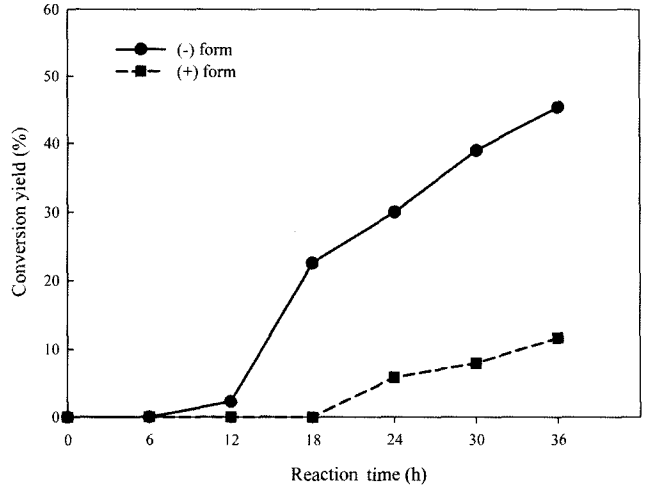

Fig. 8. The experimental results of the hydrolysis reaction by the CALB enzyme.

The trajectories and histograms for the hydrogen bonds for the CALB-(±)-ethyl ester complexes are summarized in Fig. 7. Each distance for the complex was plotted according to the time series. The nature of hydrogen bonds of the CALB-(±)-ethyl ester complexes in Fig. 7A indicate that they exhibit different behavior from those in the complex. The length of the hydrogen bond of the CALB-(−)-ethyl ester complex was shorter than that of CALB-(+)-ethyl ester. Based on this finding, it can be inferred that the CALB-(−)-ethyl ester complex is more stable. The hydrogen bonds (③, ④, ⑤) in Fig. 7B show no significant differences in the two complexes. The trajectories and histograms of the two possible hydrogen bonds and between His 224 and Asp 187 are shown for the CALB-(±)-ethyl ester complexes in Fig. 7C. In these cases, we also found that the predominant difference of the length of the hydrogen bonds did not exist. The analysis of the MD results indicated that the significant hydrogen bonds in the active site are generally more stable in the CALB-(−)-ethyl ester complex.

Experimental Verification of Molecular Dynamics Study

In the case of the molecular modeling simulation, the CALB-(−) form enantiomer complex was more stable than the CALB-(+) form enantiomer complex. For experimental verification of the molecular modeling calculation, an asymmetric hydrolysis reaction was carried out with

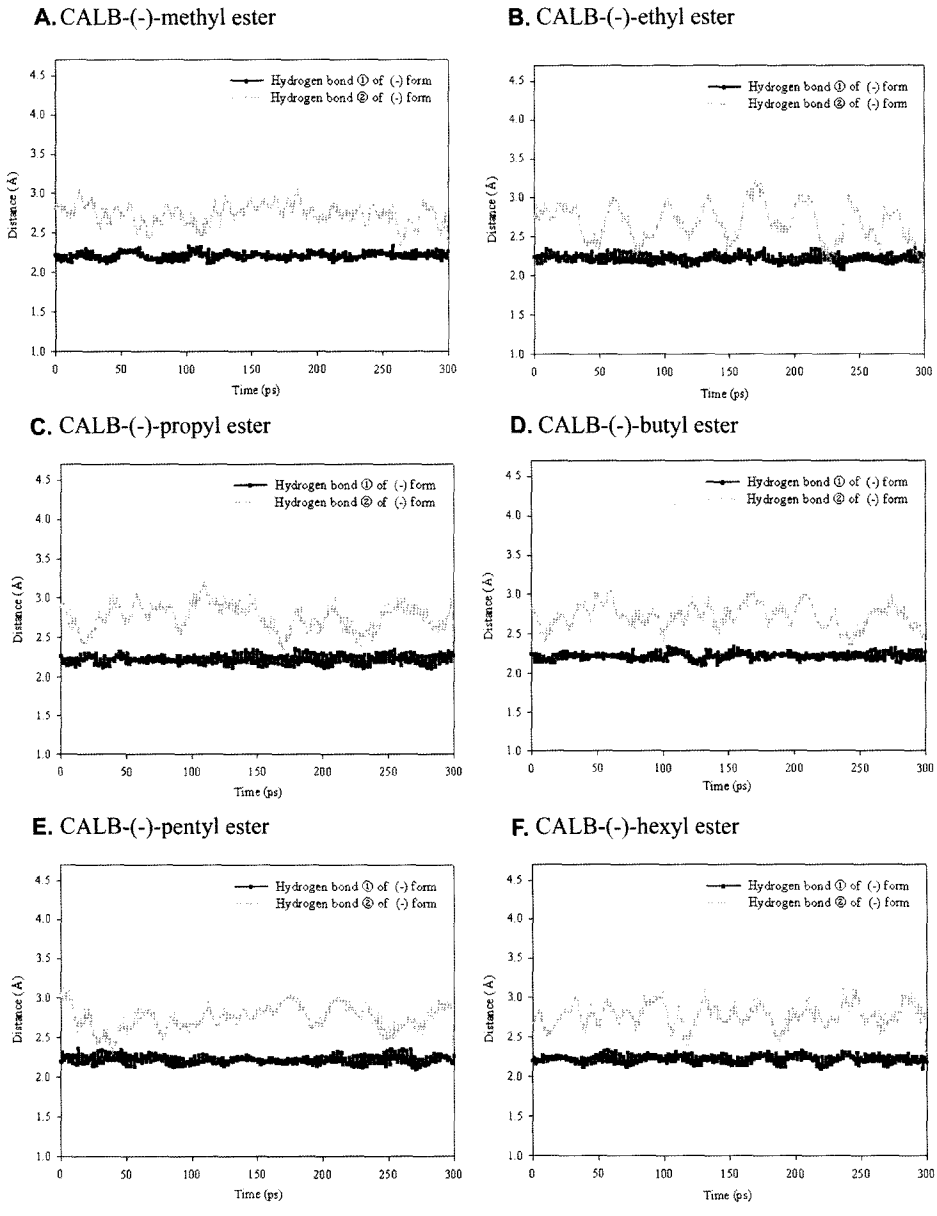

Fig. 9. Effects of alkyl chain length between ① and ② of CALB-(−)-alkyl esters; methyl group to n-hexyl group (**A**–**F**).

racemic mixtures of (±)-cis-4-acetamido-cyclopent-2-ene-1-ethyl acetate by CALB. Fig. 8 shows the conversion yields of the asymmetric hydrolysis reaction. The conversion yields of the ester of the (−) form and (+) form were 45.4% and 11.7%, respectively. These results show higher production of the (−) form than the (+) form and they also prove that hydrogen bonds of the CALB-(−) form enantiomer complex were more stable than that of the CALB-(−) form enantiomer complex.

Effect of Alkyl Chain Length of Substrates

As discussed in a previous section, we have shown that a bi-bi ping-pong mechanism can be assumed for our enzyme-substrate model system. We carried out a molecular dynamics simulation for comparing the selectivity of substrates with different chain lengths and enantiomers additionally. The enzyme-alkyl ester complexes were made by changing the alkyl group size (methyl to n-hexyl) of substrate on the CALB enzyme active site. Fig. 9 shows the variation of hydrogen bond lengths ① and ② of CALB-(−)-cis-4-acetamido-cyclopent-2-ene-1-alkyl ester complexes. We could obtain the similar results about the hydrogen bond length regardless of whether the alkyl chain length was changed or not.

Discussion

Quantum mechanical and molecular dynamics simulation analysis has been performed on the CALB-substrate complex in order to study the mechanical and conformational features of the hydrolysis reaction pathway. 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. After the empirical work had been done, the experimental data could be compared with the simulation results. We could also verify the simulation technique by the results of our experiments.

Computer-based modeling technologies provide a tool that can help to analyze various reaction pathways, and they are useful for calculating the energy of the intermediates and the finding the transition state of the enzyme reaction.

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