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Influence of kaolinite on chiral hydrolysis of methyl dichlorprop enantiomers*

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Abstract: The effect of kaolinite on the enzymatic chiral hydrolysis of methyl dichlorprop enantiomers ((R,S)-methyl-2-(2,4-dichlorophenoxy) propanoic acid, 2,4-DPM) was investigated using chiral gas chromatography. Compared with the control without kaolinite, the enantiomeric ratio (ER) increased from 1.35 to 8.33 and the residual ratio of 2,4-DPM decreased from 60.89% to 41.55% in the presence of kaolinite. Kaolinite likely had emotion influence on lipase activity and its enantioselectivity. Moreover, the amount of kaolinite added was also found to be a sensitive factor affecting the enantioselective hydrolysis of 2,4-DPM. Fourier transform infrared (FTIR) spectroscopy studies of the interaction of lipase with kaolinite provided insight into the molecular structure of the complex and offered explanation of the effects of kaolinite on enzymatic hydrolysis of 2,4-DPM. Spectra showed that the effect of kaolinite on the hydrolysis of 2,4-DPM was affected by adsorption of lipase on kaolinite and changes of adsorbed lipase conformation, which led to the modified enantioselectivity.

Key words: Enantioselectivity, Kaolinite, Chiral gas chromatography, Methyl dichlorprop, Lipase
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INTRODUCTION

Lipase (EC 3.1.1.3) is an important hydrolysis enzyme (Jaeger *et al*., 1994) excreted to the environment by plant roots and microorganisms. Many pollutants such as organophosphorus pesticides and synthetic polyesters and polyamides can be degraded by lipase (Monroy-Noyola *et al*., 1999; Marten *et al*., 2003). In soil, enzymatic reactions occur in a heterogeneous rather than homogeneous environment (Huang *et al*., 1998). Catalytic function of lipase, an extracelluar enzyme, is associated with soil clays. Consequently, properties and kinetic behavior such as enantioselectivity and activity of enzyme-clay complexes will differ from those of the free enzyme. The modulation of the activity of lipase via immobilization on different phyllosilicates was reported (de Fuentes *et al*., 2001). To the author's best knowledge, the influence of clays on lipase enantioselectivity had not been investigated before.

In the present study, dichlorprop methyl ester ((R,S)-2,4-DPM), a typical chiral contamination, was enzymatically hydrolyzed in aqueous systems by lipase. Kaolinite was applied to the enzymatic reaction system, and its influence on the lipase enantioselectivity and performance was studied with chiral gas chromatography (GC) and Fourier transform infrared (FTIR).

MATERIALS AND METHODS

Chemicals

Kaolinite was purchased from Shanghai Fengxian Fengcheng Agent Factory, China. Lipase was obtained from Shenzhen Leveking Biology En-

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gineering Co., China. Racemate and optically pure enantiomer of methyl dichlorprop (2,4-DPM) was synthesized and confirmed by NMR and mass spectroscopy. Stock solution of (R,S)-2,4-DPM was prepared in methanol at 1.0 mg/ml and stored at 4 °C before use. Working standard solutions were prepared daily by diluting the stock solutions with phosphate buffer. Other solvents and chemicals used in this study were analytical-reagent grade.

Enzyme-catalyzed hydrolysis reactions

The effect of kaolinite on lipase-catalyzed hydrolysis of 2,4-DPM was carried out in the dark. Ten ml of 0.05 mol/L phosphate sodium buffer (pH 7.00) was placed in 50 ml flask, and then 2.5 mg lipase dry powder and 0.1 ml 2,4-DPM stock solution (1.0 mg/ml) were added into the flask. Kaolinite was added to the system at 20 mg per flask, and the mixture was incubated at 25 °C on a rotary shaker at 100 r/min for 8 h. Aliquots (0.2 ml) of the reaction mixture were withdrawn and extracted with 2 ml of *n*-hexane, followed by drying over anhydrous Na_2SO_4. The samples were analyzed using chiral GC. Each treatment was sampled in triplicates, and the enzyme-free samples were included as controls for analytical background of enzyme activity.

Influence of kaolinite addition rate and 2,4-DPM concentration

Kaolinite at rates of 0, 5, 10, 20, 40, 80, or 160 mg per flask was added to 10 ml phosphate buffer (pH 7.00) in 50 ml flasks, followed by addition of 2.5 mg lipase dry powder and 0.1 ml 2,4-DPM stock solution. The mixture was incubated for 8 h under the same conditions as described above. Triplicate samples were extracted with *n*-hexane and analyzed by GC.

In the presence of 20 mg kaolinite, 2,4-DPM stock solutions in the amount of 20, 30, 40, 60, 100, or 160 μl was fortified into 10 ml phosphate buffer in 50 ml flasks. The initial concentration of 2,4-DPM was 2, 3, 4, 6, 10, 16 μg/L, respectively. The other conditions were the same as given above.

GC analytical procedure

Chiral GC analyses were carried out using an Agilent 6890N GC with micro-cell electron capture detector (μ-ECD) equipped with a 30 m×0.25 mm×0.25 μm BGB-176 chiral column (BGB Analytik, Adliswil, Switzerland). The inlet temperature was 200 °C, and 1 μl sample was introduced in the splitless mode. The column was held at 140 °C and the carrier gas (N_2) flow rate was 1.2 ml/min. The detector temperature was 300 °C, and the detector makeup gas was N_2 (60 ml/min). Chiral synthesized (R)-2,4-DPM and (S)-2,4-DPM (US 4622415) were used as the sole criterion for identifying peaks in GC-ECD chromatograms under the conditions used, gave peaks with retention times of 32.6 min and 35.0 min, respectively. The enantiomeric ratio (ER) was expressed as the peak area of R-isomer divided by that of S-isomer.

FTIR measurements

The complex of lipase and kaolinite was prepared according to the procedure described previously with a little modification (de Fuentes *et al*., 2001). Fifty mg lipase dry powder and 250 mg kaolinite were added to a flask containing 50 ml phosphate buffer (pH 7.00), and stirred at 4 °C for 24 h. Filtered samples were then washed twice with distilled water and freeze-dried. The samples of the solid lipase and kaolinite complex, the mechanical mixture of 1/10 (*w*/*w*) lipase with kaolinite and lipase dry powder were mixed with KBr and examined on a Shimadzu FTIR-8900.

RESULTS AND DISCUSSION

Effect of kaolinite on the lipase performance

Our investigation on the influence of kaolinite on lipase performance in hydrolysis of 2,4-DPM (Table 1) showed that kaolinite could enhance the enzymatic hydrolysis rate of 2,4-DPM and that the residual ratio of 2,4-DPM decreased from 60.89% to

Table 1 Effect of kaolinite on the lipase catalyzed reaction

	Additive amount (mg)	Reaction time (h)	*ER*	Residue ratio (%)
Control	0	8	1.04	100
No additive	0	8	1.35	60.89
Kaolinite	20	8	8.33	41.55

41.55%. Our results agreed with the previous reports (de Fuentes *et al*., 2001) that kaolinite added could enhance the lipase activity.

Clay served as an absorbent yielding a stable substrate with a large interfacial area. This may contribute to the improvement of lipase activity, as shown in Table 1. The enhancement effect also might be attributed to the hydrogen-bonding and hydrophobic interaction between the lipase with clay, which could induce an unfolding and open-structure of the enzyme (Fernandez-Lorente *et al*., 2001; Palomo *et al*., 2002).

Effect of kaolinite on the enantioselective ratio of 2,4-DPM enzymatic hydrolysis

Fig.1 shows that the lipase displayed only low enantioselectively (*ER*=1.35), and this provides us with a sensitive system to detect small changes in enantioselectivity.

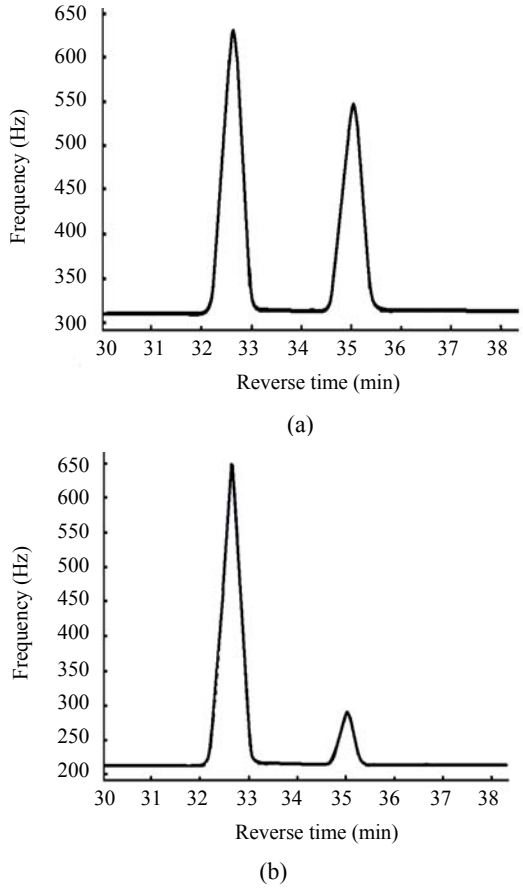

Fig.1 Effect of kaolinite on the enzymatic enantioselectivity (a) no additive, *ER*=1.35; (b) 20.0 mg kaolinite added, *ER*=8.33

The effect of kaolinite on the lipase enantioselectivity is presented in Table 1 showing that in the presence of 20 mg kaolinite, the enantioselectivity of lipase was significantly increased, resulting in the increase of the ER value from 1.35 to 8.33 (Fig.1).

The results suggested a crucial role of kaolinite in the enhancement of enantioselectivity of lipase. In the presence of kaolinite, the S-enantiomer became overwhelmingly the faster reacting enantiomer. From the enantioselective ratio (ER value), it was apparent that the enhancement of enantioselectivity was the consequence of a differential activator phenomenon. Kaolinite acted as enantioselective activator of 2,4-DPM enzymatic hydrolysis.

Effect of addition rate of kaolinite on enantioselectivity of enzymatic reaction

In the process of enzymatic reaction with kaolinite as additive, its addition rate is another important parameter worthy of careful measurement since it may also have influence on enzyme activity and enantioselectivity. The enantioselectivity seems to be the most crucial factor for enzymatic hydrolysis of chiral pesticides in the environment.

The enantioselectivity of the enzymatic reaction depended on the amount of kaolinite added (Fig.2). Within the range of 0~20 mg kaolinite, the ER value of the enzymatic reaction rapidly increased from 1.35 to 8.33. Above 20 mg, the ER value increased more slowly. When the amount of kaolinite added was small, lipase molecules were not completely adsorbed on kaolinite, so the ER value increased rapidly along with the amount of kaolinite. While the amount of kaolinite was over 20 mg, most of the lipase molecules

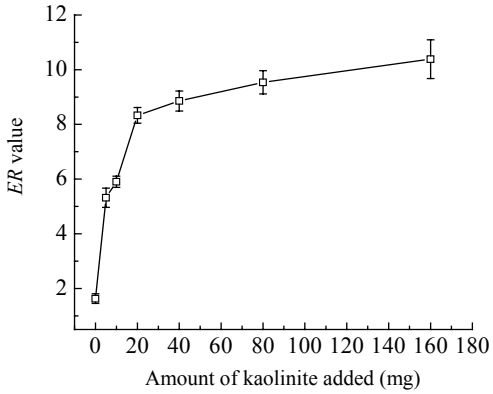

Fig.2 Effect of addition rate of kaolinite on the enantioselectivity

were adsorbed on kaolinite, and the ER value increased more slowly with the amount of kaolinite. These results show that adsorption of lipase on clay is important in lipase enantioselectivity.

Effect of 2,4-DPM concentration on the lipase enantioselectivity

Fig.3 shows that the ER value was almost a constant (*ER*=1.35) in enzymatic system in the range of the given pesticide concentrations. With 20 mg kaolinite were added to the enzymatic system, the adsorption of 2,4-DPM and lipase on kaolinite might occur simultaneously. When the pesticide concentration was very low, the adsorption of lipase on clay was more important. The ER value appreciably increased along with the pesticide concentration. As the pesticide concentration increased gradually, pesticide molecules occupied the adsorptive sites of clay, and the adsorbed lipase became less and the ER value decreased.

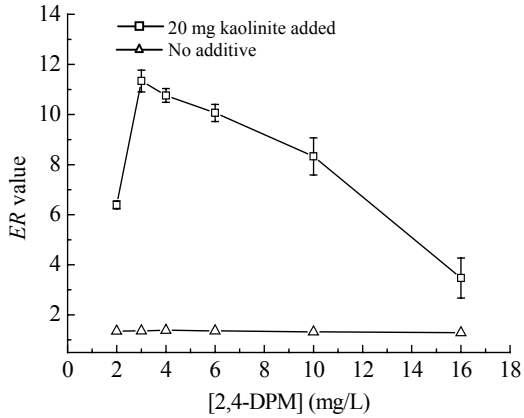

Fig.3 Effect of initial pesticide concentration on the enantioselectivity

FTIR spectra

In order to further understand the effects of kaolinite on hydrolysis, we investigated the IR spectra of the complex of lipase and kaolinite by using Fourier transform infrared (FTIR) spectroscopy. As shown in Fig.4, the characteristic spectrum corresponded to kaolinite covered in 450~4000 cm^{-1}. However, most of the characteristic spectrum corresponding to lipase was masked by the enormous amount of kaolinite. Thus, the carbonyl stretching band of the lipase carbonyl group, centered at 1651.0 cm^{-1}, was analyzed for the spectra. Fig.4 shows that there was no difference between lipase with the 1/10 (*w*/*w*) mixture of lipase and kaolinite at 1651.0 cm^{-1}. This indicates that lipase did not interact with kaolinite in the mechanical mixture.

On the other hand, Fig.4 shows that the maximum of the carbonyl band (1651.0 cm^{-1}) in lipase shifted to higher frequencies (1654.8 cm^{-1}) compared with the lipase-kaolinite complex. This may be attributed to the disruption of the strong hydrogen bonds in the lipase and their replacement by a weak association. It shows that kaolinite induced an unfolding and open-structure of lipase (Fernandez-Lorente *et al*., 2001), which may contribute to the increased lipase activity by kaolinite.

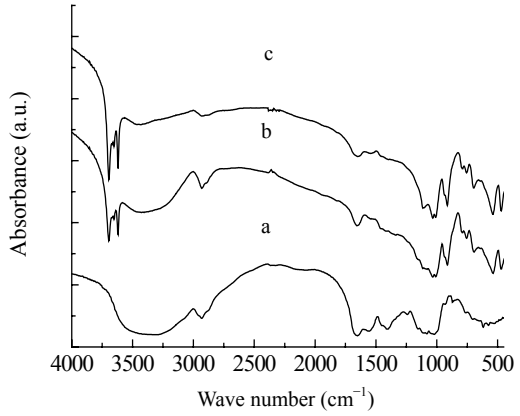

Fig.4 IR spectra of lipase and its related species

a: Lipase; b: Lipase-kaolinite complex; c: 1/10 (*w*/*w*) mixed lipase with kaolinite

CONCLUSION

Investigation of the effect of kaolinite on the lipase-catalyzed chiral hydrolytic degradation of dichlorprop methyl enantiomers in this work showed that kaolinite had potential for greatly enhancing the reaction rate and the enantioselectivity in the lipase-catalyzed reaction. In the presence of 20 mg kaolinite, the enantiomeric ratio (ER) increased from 1.35 to 8.33 and the residual ratio of 2,4-DPM decreased from 60.89% to 41.55%. Moreover, the amount of kaolinite added was found to be a sensitive factor affecting the enantioselective hydrolysis of 2,4-DPM. Within the range of 0~20 mg kaolinite, the ER value of the enzymatic reaction rapidly increased from 1.35 to 8.33, while the increase was slower when

kaolinite was >20 mg. In the test system, clay seemed to play a triple role. First, it served as an absorbent to give a stable, large interfacial area substrate for the enzyme. Second, the nature of clay enabled it to be an activator of the lipase. Finally, an important role of clay was its ability to enhance the enantioselectivity. FTIR spectroscopy analysis of the complex of lipase and kaolinite provided further insight into the molecular structure of the complex and explanation for the effect of kaolinite on enzymatic hydrolysis of chiral 2,4-DPM. Data showed that a complex had formed between lipase and kaolinite and that kaolinite may have induced an unfolding and open-structure of lipase, which led to the increased lipase performance.

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