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Effects of β-cyclodextrins on the enzymatical hydrolysis of chiral dichlorprop methyl ester

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Abstract: The effect of β-cyclodextrins(β-CDs) on the enzymatical hydrolysis of chiral dichlorprop methyl ester (DCPPM) was studied. Four kinds of β-cyclodextrins(β-cyclodextrin, Partly methylated-CD(PM-β-CD), hydroxypropyl-cyclodextrin(HP-β-CD) and carboxymethyl-cyclodextrin(CM-β-CD)) were used. Compared with 100% DCPPM in the absence of β-cyclodextrins, the activity of lipase decreased with the increase of β-cyclodextrin and PM-β-cyclodextrin. However, CM-β-cyclodextrin stimulated the lipase activity. The inhibition effect of β-cyclodextrin and PM-β-cyclodextrin on the hydrolysis of DCPPM is affected by many factors other than degree of the methylation blocking the active site of lipase. UV-Vis and Fourier transform infrared(FTIR) spectroscopy studies of the complexation of aqueous DCPPM with β-CDs provide fresh insight into the molecular structure of the complex and explain the effects of β-CDs on enzymatical hydrolysis of chiral DCPPM. Data showed that inclusion complexes had formed by complexation of the CM-β-CD with DCPPM and the solubility of DCPPM was increased in water, which leaded to the increased lipase activity.

Keywords: β-cyclodextrins; enzyme; hydrolysis; chiral; dichlorprop

Introduction

There is a tremendous interest in using *in situ* bioremediation for the cleanup of contaminated soil and groundwater. However, biodegradation rates in the subsurface are often constrained by a limited oxygen supply and by factors related to bioavailability, such as solubility, dissolution rate, and sorption. Recently, cyclodextrin has been shown to be effective for enhanced biodegradation of organic contaminants(Wang, 1998).

Cyclodextrins are cyclic oligosaccharides with doughnut-shaped cavities and allow the selective formation of inclusion complexes with a variety of guest molecules or ions according to their size and polarity. The inclusion complexes of low-polarity organic pollutants and pesticides may result in considerable improvement of their solubility and bioavailability and then increase removal of persistent pollutants(Tanada, 1999; Brusseau, 1994).

In most cyclodextrins enhanced biodegradation tests, the extent of microbial degradation of organic compounds is usually measured as the elimination of organic carbon. However, in most cases, enantiomers of chiral compounds behave differently to biochemical processes, the length of lag phases as well as the degradation rates may differ for the enantiomers. Therefore, the effects and the environmental fate of the enantiomers of chiral pollutants need to be investigated separately.

Phenoxy acids and their derivatives are widely used as herbicides. Their frequent occurrence in groundwater and soil indicated that they may be critical contaminants that deteriorate drinking water resources. The representative of phenoxyalkanoic acid herbicides is 2, 4-dichlorprop methyl ester, which exits two enantiomeric forms, the(R)- and(S)-forms, but only the(R)- forms are active herbicides(Fig.1). Racemic mixtures of the herbicides have been applied to the fields for many years.

Studies on the hydrolysis of phenoxyalkanoic acid herbicides are important to know their persistencies and fates in natural environments. Also lipase can catalyze hydrolysis of ester in soil, where lipase can be culturable from bacteria in soil(Jaeger, 1994). Thus, the influence of the cyclodextrins on the enzymatical hydrolysis of chiral contaminants need to be explored further.

The objective of this study was to evaluate the influence of β-cyclodextrin and its derivatives on the enzymatical hydrolysis of chiral dichlorprop methyl ester.

1 Materials and methods

1.1 Chemicals

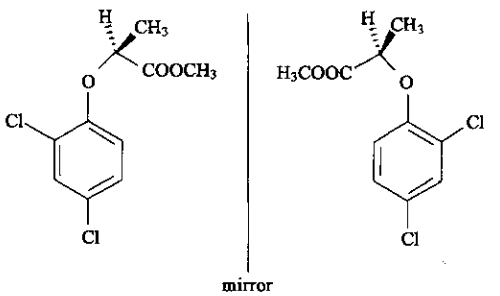

Fig.1 The enantiomers of 2,4-dichlorprop methyl ester

Lipase EC3.1.1.3(enzymatic activity 10000 U/g) were supplied by Shenzhen Lvweikong Biological Engineer Co. Ltd., China. BSA were supplied by Sigma Co. Ltd., USA. Racemic dichlorprop methyl ester (DCPPM) and chiral R form were prepared by ourselves, and were determined by GC-MS and NMR. β-cyclodextrin was purchased from Tianjin Yuanhang Chemicals Co. Ltd. China and was recrystallized three times in distilled water. Partly methylated-CD (PM-β-CD), hydroxypropyl-cyclodextrin (HP-β-CD) and carboxymethyl-cyclodextrin (CM-β-CD) were prepared as described by Yu (Yu, 2001), Feng (Feng, 1997) and Gao (Gao, 1999), respectively. The substitution degree of CM-β-CD was 0.29, which was determined by method of Gao (Gao, 1999).

1.2 Enzymatical hydrolysis of dichlorprop methyl ester

Fifteen ml of sodium phosphate buffer(pH 7.0) was added into 0.1 ml of 500 mg/L DCPPM, quantum of β-CDs and 5 ml of lipase EC 3.1.1.3. The mixture was incubated at 30 ± 1℃ in a rotary shaker at 80 r/min for 5 h. After the completion of the reaction, 30 ml of ethyl acetate was added immediately, and the mixture was shaken in shaker for 5 min. Then, 30 ml(3 × 30 ml) of ethyl acetate was added to the mixture, and the mixture was washed successively with saturated sodium chloride aqueous solution and was dried over anhydrous sodium sulfate, and then the ethyl acetate was distilled off. The residue was redissolved in 10 ml of methanol.

1.3 Enantioselectivity gas chromatography separation

Racemic DCPPM and chiral DCPPM were analyzed by gas chromatography (GC-2010 Shimadzu), equipped with a chiral glass capillary column(β-cyclodextrin BGB-172, 30 m length, outer diameter 0.35 mm, inter diameter 0.25 mm, film thickness 0.25 μm, BGB Analytik AG). The oven temperature of GC was 140℃ for 50 min,

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where that of injection port and the detector 200℃ and 230℃, respectively. The analyses were monitored by electric capture detection. Peaks were identified by comparison the retention times of (R)-DCPPM, (R, S)- DCPPM.

The enantiomeric data were defined as follows (Liu, 2000):

Enantiomeric excess(ee_p), % $= [(B - A)/(B + A)] \times 100$,

Enantioselectivity: $E = \ln[1-c(1-ee_p)]/\ln[1-c(1+ee_p)]$,

where A and B are the concentrations of (S)-DCPPM and (R)-DCPPM (mmol/L), and c represents the conversion ratio.

1.4 UV spectrometry experiments

The complex stability of β-CD and modified β-cyclodextrins with (R, S)- DCPPM was determined by UV spectrometry (UV-2402PC, Shimadzu). Spectral titration of a series of solutions containing DCPPM and β-CDs were performed in buffered aqueous solutions at 25℃.

1.5 FTIR measurements

The solid complex of β-CDs and DCPPM was prepared according to the procedure described previously with a little modification(Li, 2003).

1.135 g of β-CD was dissolved in 100 ml of distilled water, and a solution of 0.249 g of dichlorprop in 2 ml of 95% ethanol was drop wise added. The mixture was shaken at 60℃ in ultrasonic for 5 h and cooled in refrigeratory for 12 h. Then, the mixture was filtered with 0.45 μm of micro-membrane. The sediment was dried at 60℃.

2 g of modified β-CD was dissolved in 100 ml of distilled water, and a solution of 0.249 g of DCPPM in 2 ml of 95% ethanol was drop wise added. The mixture was shook at 60℃ in ultrasonic for 5 h and cooled in refrigeratory for 12 h. Then, the mixture was filtered with 0.45 μm of micro-membrane. The sediment was dried at 60℃.

The samples of the solid complex of the β-CDs and DCPPM, the β-CDs and lipase were mixed with KBr and examined with 8900-FTIR, Shimadzu.

2 Results and discussion

2.1 Effects of β-CD and modified β-CDs

Four kinds of β-CDs(β-CD, CM-β-CD, PM-β-CD, HP-β-CD) were used in this study. The effect of the selected β-CDs on the rate of hydrolysis was systematically investigated (Table 1). CM-β-CD stimulated the lipase activity. Under the same conditions, the conversion ratios of DCPPM in the presence of CM-β-CD achieved 43.9%, compared with 31.8% in the absence of CM-β-CD. However, PM-β-CD inhibited the activity. Under the same conditions, the conversion ratios of DCPPM in the presence of PM-β-CD decreased from 31.8% to 16.5%. As to β-CD, HP-β-CD, the conversion ratios at 5 h were about the same as that of the reaction without β-CDs. This results agreed with the previous reports(Kamiya, 1995) that the methyl groups introduced around the top tours of the host cavity in presence of PM-β-CD lead to restriction of the configuration of the alkyl head portion of DCPPM included. This restriction leaded to the decreased interfacial oil-water area, which is harmful for the improvement of lipase activity, as shown in Table 1. By contrary, the carboxyl groups of CM-β-CD lead to the formation of hydrogen bond between β-CD and DCPPM, which may be helpful for stability of the inclusion complex of β-CD and DCPPM. This result leaded to the increased interfacial oil-water area, and the lipase activity increased. On the other hand, the inclusion complexes of the β-CD and HP-β-CD were not stable, so the increased or decreased activity of the enzyme was not observed in the range of concentration.

Table 1 Effects of β-CD and modified β-CDs on enzymatical hydrolyzation

Additions	Conversions, %	ee_p, %	E
Blank	31.8	11.0	1.30
β-CD	28.9	9.8	1.26
CM-β-CD	43.9	9.8	1.30
PM-β-CD	16.5	5.3	1.12
HP-β-CD	28.5	6.2	1.16

As to enantioselectivity, the β-CD and CM-β-CD have no effects on the enantioselectivity hydrolysis of chiral DCPPM. The enantioselectivity of PM-β-CD and HP-β-CD decreased from 1.30 to 1.12 and 1.16, respectively. This implied that these DCPPM are included in the PM-β-CD and HP-β-CD with some preferred orientation of the DCPPM moiety, which reduces the enantioselectivity hydrolysis of the included DCPPM.

2.2 Effects of concentration of β-CD and modified β-CDs

In the process of enzymatical hydrolysis, the concentration of cyclodextrin may influence the activity of enzyme. Fig. 2 shows the effects of concentration of β-CD and modified β-CDs on the enzymatical hydrolyzation of DCPPM. As it can be seen, at a given time three kinds of β-CDs showed the different patterns of the dependence of the lipase activity on β-CDs concentrations. Compared with 100% DCPPM in the absence of β-CDs, the activity of lipase decreased with the increase of β-CD and PM-β-CD, then increased from 47% to 72% and 42% to 45%, respectively, finally decreased. But it was leveled off when the addition of β-CD reached 80 mg. Apparently, β-CD and PM-β-CD inhibited lipase activity. The inhibition effect of β-CD on the DCPPM hydrolysis is considerably smaller than that of PM-β-CD. When 80 mg of β-CD was added, the lipase activity decreased to 50% and leveled off. However, when 5 mg of PM-β-CD was added, the lipase activity decreased to 41.3% and then level off in 40% with increase of addition of PM-β-CD. It indicated that the inhibition effect of β-CD and PM-β-CD on the hydrolysis of DCPPM is affected by many factors other than the degree of the methylation blocking the active site of lipase. As reported above, the lipase activity increased with addition of CM-β-CD unlike the case of β-CD. When 160 mg of CM-β-CD was added, the relative activity reached 145.7%. It indicated that interfacial oil-water area has an important influence on lipase activity.

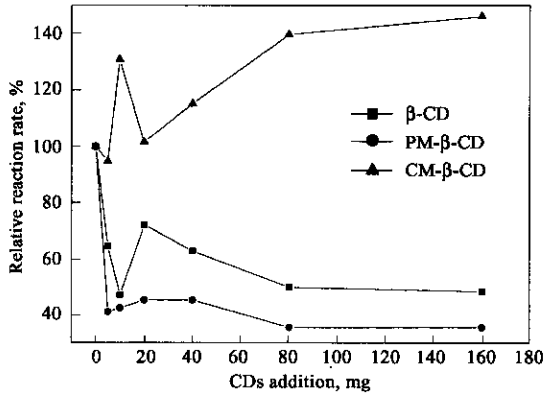

Fig.2 Effects of the addition of β-CDs and their derivatives on activity of enzymatical reaction

2.3 Effects of β-CDs addition on enantioselectivity of enzyme

In the process of enzymatical hydrolysis, the concentration of CD may have influences on enantioselectivity of enzyme. Fig.3 shows effects of β-CDs addition on enantioselectivity of enzymatical reaction. Similar to the enzymatical activity, the enantioselectivity of lipase also depends on the concentration of β-CDs. With increase of addition of β-CDs, the enantioselectivity decreased. When 160 mg of β-CDs was added, the enantioselectivity decreased to 1.1 in the presence of β-CD and CM-β-

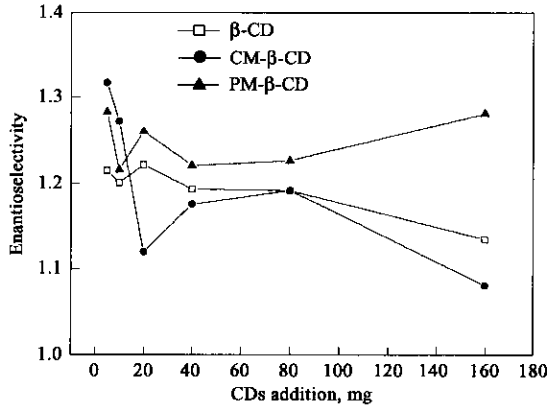

Fig. 3 Effects of β-CDs addition on enantioselectivity of enzymatical reaction

CD. However, the inhibition effect of enantioselectivity of PM-β-CD on the enzymatical hydrolysis is considerably smaller than that in the presence of β-CD and CM-β-CD. Compared with the result above, it also indicated that the enantioselectivity was also affected by many factors other than the degree of methylation of β-CDs. The inclusion depth and inclusion orientation maybe have influence over enantioselectivity.

2.4 UV spectral analysis

In the UV spectrometry experiments, the two absorption peaks of DCPPM are 229 nm, 282 nm, respectively, and the absorption maximum of DCPPM gradually increased under the addition of varying concentration of β-CD. Typical UV-Vis spectral changes under addition of β-CD are shown in Fig.4—Fig.6. It can be seen, the UV-Vis spectra changes can be ranked: CM-β-CD > PM-β-CD > β-CD. No difference in UV-Vis spectral change was observed with increase of the addition of β-CD. This maybe attribute to small diamater of DCPPM, the complex of β-CD with DCPPM was notstable. On the other hand, the maximum absorbance of DCPPM had not been shifted, and its strength increased with the increase of CD concentrations. The result indicated that inclusion complexes had formed by complexation of the CM-β-CD with DCPPM and the solubility of DCPPM increased in water, which leaded to the increased lipase activity reported above.

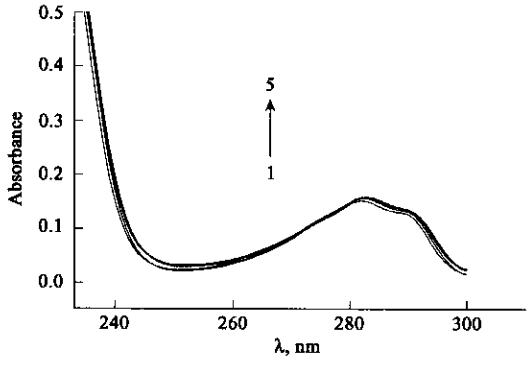

Fig.4 UV spectra of DCPPM in the presence of different concentration of β-CD(mmol/L)
1:0.0; 2: 0.24; 3: 0.48; 4: 0.96; 5: 1.9

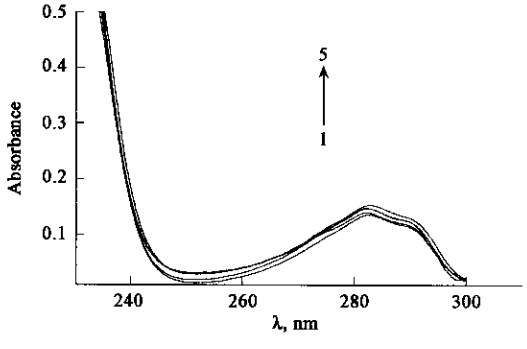

Fig.5 UV spectra of DCPPM in the presence of different concentration of PM-β-CD(mmol/L)
1: 0.0; 2: 0.12; 3: 0.24; 4: 0.48; 5: 0.96

2.5 IR spectroscopy

In order to further reveal the effects of β-CDs on hydrolysis, we have investigated IR spectroscopy of the inclusion complexes of β-CDs and DCPPM by using Fourier transform infrared (FTIR) spectroscopy. Fig. 7—9 show the spectra of the mixtures of DCPPM and β-cyclodextrins.

Fig.7—9 show that the characteristic spectra corresponded to the β-CD cover in 500—4000 cm^{-1}. However, the quality fraction of DCPPM in inclusion complex was less than 25% (w/w), the most of the characteristic spectrum corresponding to DCPPM are masked by the enormous amount of β-CDs (Tong, 2001). Thus, the carbonyl stretching band of the DCPPM carbonyl group, centered at 1751 cm^{-1}, has been analyzed for the complex. Fig. 7 shows that there is not difference between DCPPM and β-CD-DCPPM at 1751 cm^{-1}, this indicated that

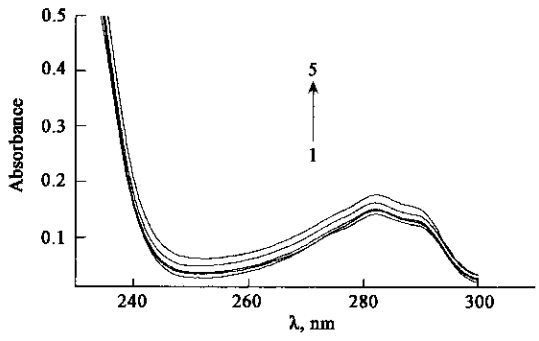

Fig. 6 UV spectra of DCPPM in the presence of different concentration of CM-β-CD(mmol/L)
1: 0.0; 2: 0.12; 3: 0.24; 4: 0.48; 5: 0.96

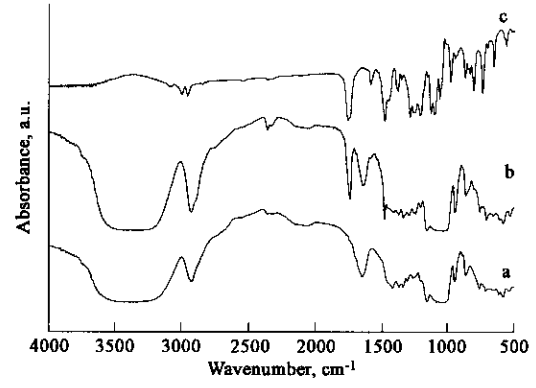

Fig.7 IR spectra of β-CD-DCPPM and its related species
a: β-CD; b: β-CD-DCPPM; c: DCPPM

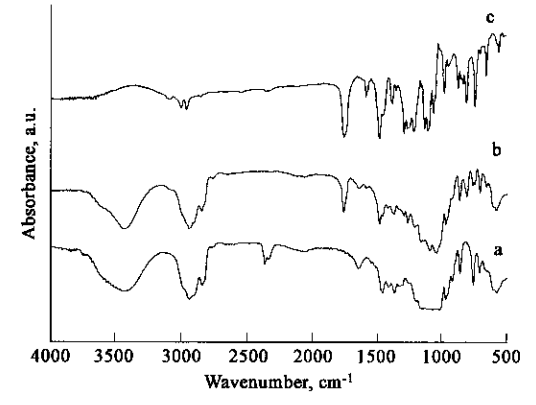

Fig.8 IR spectra of PM-β-CD-DCPPM and its related species
a: PM-β-CD; b: PM-β-CD-DCPPM; c: DCPPM

DCPP did not enter into hydrophobic cavity of β-CD in aqueous solution. However, the spectrum strength of carbonyl group in PM-β-CD-DCPPM decreased, compared with β-CD-DCPPM (Fig. 8). This indicated that DCPPM partly entered into cavity of PM-β-CD.

On the other hand, Fig.9 shows that the maximum of the carbonyl band(ca.1711 cm^{-1}) in CM-β-CD has shifted to higher frequencies for the spectrum corresponding to the complex comparison with that of CM-β-CD-DCPPM. This maybe attributed to the disruption of the strong hydrogen bonds in the crystal and their replacement by a less intense association(Inigo, 2003). Compared with Fig.7 and Fig.8, it can be seen that the spectrum strength of carbonyl group in CM-β-CD-DCPPM obviously decreased, indicating that the carbonyl group of DCPPM might be included in CM-β-CD. This was the reason why CM-β-CD increased lipase activity.

3 Conclusions

The effect of β-CDs on the enzymatical hydrolysis of chiral DCPPM

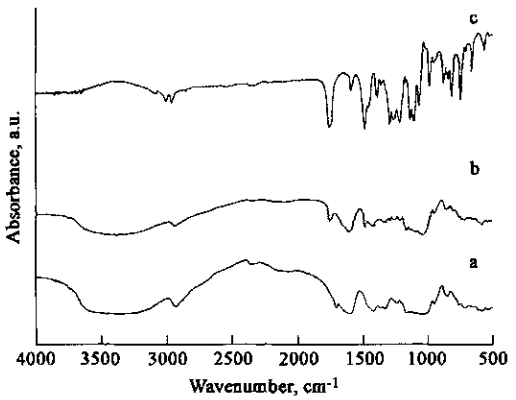

Fig.9 IR spectra of CM-β-CD-DCPPM and its related species
a: CM-β-CD; b: CM-β-CD-DCPPM; c: DCPPM

has been specifically studied. Four kinds of β-CDs were used. Compared with 100% DCPPM in the absence of β-CDs, the activity of lipase decreased with the increase of β-CD and PM-β-CD. However, CM-β-CD stimulated the lipase activity. The inhibition effect of β-CD and PM-β-CD on the hydrolysis of DCPPM is affected by many factors other than degree of the methylation blocking the active site of lipase. UV-Vis and FTIR spectroscopy studies of the complexation of aqueous DCPPM with β-CDs provided the fresh insight into the molecular structure of the complex and explain the effects of β-CDs on enzymatical hydrolysis of chiral DCPPM. Results showed that inclusion complexes had formed by complexation of the CM-β-CD with DCPPM and the solubility of DCPPM increased in water, which leaded to the increased lipase activity

These results maybe helpful for *in situ* bioremediation of chiral organic contaminants using cyclodextrin. The inclusion constant, inclusion depth and orientation of DCPPM need to further study.

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