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Preparation, Stabilization, and Bioefficacy of β-Cyclodextrin Inclusion Compounds of Chloramidophos

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Cyclodextrins are common compounds capable of forming inclusion complexes with a variety of pesticides to improve their solubility, bioavailability, and stability. In this study, chloramidophos (CP) was inclusion-complexed with β-cyclodextrin (β-CD) by a kneading method in an attempt to gain a more stable but equally effecacious formulation compared with CP alone. A 1:1 CP−β-CD complex with an inclusion constant of 203.0 M^{-1} was determined to exist by UV spectrophotometry. The structural identification, thermal stability, and biological assays of the CP−β-CD complex were then carried out with a product with the maximum guest loading efficiency. The data mearsured by differential scanning calorimetry (DSC), Fourier transform infrared (FT-IR), and X-ray diffraction (XRD), where the endothermic peaks of β-CD, the FT-IR bands, and the XRD peaks were generally changed, deduced the formation of complex. Results of the thermal stability assay showed that the degradation rate of CP in 14-day incubation was slowed by a factor of 3.6 when it was complexed with β-CD. Then, activity and toxicity of CP influenced by the encapsulated process of β-CD were evaluated by an in vitro acetylcholinesterase (AChE) inhibition assay and an acute aquatic toxicity assay, respectively. No significant differences were found in both the two biological assays by a *t*-test. This indicated that the encapsulation process greatly improved the thermal stability of the pesticide with no adverse effects on bioefficacy compared to that of CP. There is a promising outlook for CP−β-CD to be produced as the active ingredient of various formulation additives of CP for its continued application.

KEYWORDS: Cloramidophos; cyclodextrin; stabilization; bioefficacy

INTRODUCTION

Chloramidophos (CP, **Figure 1** is a new organophosphorus pesticide (OP) provisionally registered in China in 2005 as an alternative to the highly toxic and persistent OPs which have been banned for use. CP is comparable in efficacy to methamidophos against a broad spectrum of insects and has low potency at acute oral toxicity to rat, making it widely applied in several provinces in China (*1*). However, due to its intrinsic hemiacetal structure, CP is easily decomposed in the processes of storage, transportation, or application, which limits its usage in agriculture. As a result, finding an appropriate formulation of CP to stabilize the active ingredient becomes a valuable challenge to its continuous usage.

One known method for stabilizing the compounds is to form molecular complexes of the clathrate or inclusion type between a "guest" compound and the "host" cyclodextrins (CDs). CDs are toroidally shaped oligosaccharides formed from the enzymatic degradation of starch by bacteria, having a relative hydrophilic outer surface and hydrophobic central cavity. In a hydrated state, the)cavity of the torus is filled with some water molecules. These molecules of water may be replaced with a gain in energy by molecules of a compound that is less polar than water and thus can form noncovalent host–guest inclusion complexes (*2*). This "molecular encapsulation" reaction results in many advantageous modifications of the properties of the complexed substances, such as chemical stability, allowing protection against volatilization, hydrolysis, oxidation, photolysis, and high temperature or retarding degradation in storage (*3, 4*).

In past decades, CDs have aroused considerable attention in agriculture industry for similar virtues (*5*). Pyrethroid is one of the top applied pesticides all over the world. It has been found that both natural and synthetic pyrethroids complexed with CDs improved on the heat and light stability of the active components (*6–8*). Field tests also showed that the inclusion

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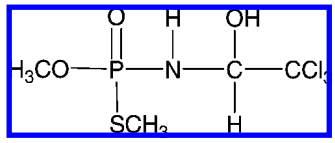

Figure 1. Chemical structure of cloramidophos.

process considerably reduced the oxygen- and photodecomposition without weakening the insecticide activity (*9–11*). CDs were also used for producing new formulations of OPs for eliminating their essential defects in stability. In the GB patent 145380 (*12*), a complex reaction product of dichlorvos and a cyclodextrin was prepared and proven to have greatly brought down the volatility and inflammability of the pesticide. Effects of CDs on the photodecay of parathion and paraoxon were investigated in both aquatic medium (*13*) and methanolic aqueous solution (*14*). Opposite influences of CDs were gained for the two organophosphorus pesticides. For example, in water/methanol (80/20 by volume) solution, the initial photolysis rate of the parathion−β-CD inclusion complex was just about a quarter of that of the pure parathion. However, the photodegradation rate of paraoxon was increased to 1.31-fold by β-CD. This phenomenon can be explained by the difference in the degree of proximity between the catalytic sites of the cyclodextrin host cavities and the reaction centers of the included pesticide guests. Several reports manifested that CDs can inhibit the hydrolysis of OPs, such as parathion, methylparathion, fenitrothion, and DCPE (*15, 16*). Szente's study indicated that the molecular encapsulation of OPs with cyclodextrin in dry solid state effectively preserved the pesticide content even at elevated temperature (*17*). Actually, the application of CDs in pesticide formulations is very modest. However, a new several thousand ton section of the cyclodextrin market will be opened as the price of CDs being perfectly acceptable for the pesticide industry (*18*).

In this study, the CP−β-CD inclusion complex was prepared by a kneading method and identified by ultraviolet (UV), differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FT-IR), and X-ray powder diffraction (XRD) techniques. Then, a modified accelerated storage stability study was carried out to determine the change in storage stability of CP when it was encapsulated with β-CD. In addition, activity and toxicity of CP with or without β-CD were evaluated by in vitro acetylcholinesterase inhibitory assay and acute aquatic toxicity assay, respectively. The aim of our study is to develop a new pesticide formulation that improves stabilization without any disadvantageous effects of its bioefficacy on organisms.

MATERIALS AND METHODS

Chemicals. CP (*O*,*S*-dimethyl(2,2,2-trichloro-1-hydroxyethyl)phosphoramidothioate) with purity >98% was a gift by Wuhan Zhongxin Chemical Engineering Co. Ltd. (Wuhan, China). β-CD was purchased from Shanghai Bio Life Science & Technology Co. Ltd., and recrystallized in distilled water before being used. Acetylcholinesterase of *Electrophorus electricus* (*E. electicus*, EE-AChE type V-S) was purchased from Sigma Chemicals (St. Louis, MO). Other solvents or chemicals were of HPLC or analytical grade.

Preparation of β-CD Complexes. The molar ratios of CP over β-CD were established at 5:1 to 1:1 to enhance the loading efficiency of CP. A kneading method was carried out to prepare the inclusion complexes. Briefly, 1 mmol (1.135 g) of β-CD and a small quantity of water were added to a mortar and mixed together. A quantity of 1−5 mmol (0.288–1.442 g) of CP dissolved in 25 mL of acetone was slowly added to the mortar and kneaded for 1 h. During this process, aliquots of acetone were added to the mixture to maintain a suitable consistency. The product was then air-dried and washed with acetonitrile (3 × 5 mL) to remove the uncomplexed CP. Finally, the complex was dried at 30 °C for 3 h.

Quantification of CP. Quantities of pure CP and CP content in its CD inclusion complexes were measured on a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan) equipped with a PU-2089 quaternary gradient pump, a mobile-phase vacuum degasser, an AS-1559 autosampler with a 100 μL loop, a CO-2060 column temperature control compartment, a variable-wavelength UV-2075 detector, and an LC-Net II/ADC data collector. The concrete operating conditions were as follows: Shimadzu Shim-pack VP-ODS C_{18} column (150 mm × 4.6 mm, 5 μm); mobile phase, H_2O/acetonitrile = 80/20 (v/v); flow rate, 1 mL/min; detection wavelength, 200 nm; and temperature, 25 °C. Samples for the HPLC assay were extracted with methanol by sonication for 2 min and filtered through a 0.45 μm pore membrane filter.

Determination of Stoichiometry by UV Spectra. Absorption spectra measurements were carried out with a Jasco V550 spectrophotometer (Tokyo, Japan). The concentration of CP was held constant at 0.5 mM. Then, an appropriate amount of β-CD was added with the final concentrations varied from 0 to 2 mM. Both CP and β-CD were dissolved in distilled water. The absorption spectra measurement was taken after 1 h.

DSC. Thermal analysis was carried out with a TA Instruments Q100 differential scanning calorimeter (New Castle, DE), using 10 °C/min scanning rate. The sample weight was about 4–5 mg. Baseline optimization was performed before each run.

FT-IR. FT-IR spectra were recorded from 4000 to 400 cm^{-1}, using a Nicolet 6700 FT-IR spectrophotometer on samples prepared as KBr disks.

XRD. The X-ray powder diffraction patterns were obtained from a D/max-rA X-ray diffractometer (Rigaku). The samples were irradiated with monochromatized Cu Kα radiation and mounted on a sample holder and scanned with a step size of 0.02 °C between $2\theta = 3$ and 50 °C. The voltage and current were 50 kV and 80 mA, respectively.

Thermal Stability Profile. A modified accelerated storage stability study (*19*) was used to identify the changes in stability of CP when it was inclusion complexed with β-CD. In a typical case, CP and CP−β-CD complexes predetermined with equimolar CP were incubated at 54 ± 2 °C in solid state. Weighted amounts of samples were taken out at the scheduled intervals, dissolved in methanol, filtered through a 0.45 μm pore membrane filter, and measured using HPLC. This experiment was repeated 3 times during a period of 3 months.

Acetylcholinesterase Inhibitory Potency (in Vitro). The inhibitory potency of CP and its complexes with β-CD were evaluated against EE-AChE by calculating the concentration of inhibitor leading to half-inhibition of enzyme activity (IC_{50}). In a typical experiment, the solutions of free CP and CP−β-CD complex were prepared in phosphate buffer (pH 8.0). Each of five test tubes containing properly diluted enzyme solutions (180 μL) was treated in the phosphate buffer (pH 8.0) with 20 μL of free CP or CP−β-CD solutions at various concentrations that inhibited enzymatic activity by 10–90%. Meanwhile, control samples were also prepared including 180 μL enzyme solutions and 20 μL phosphate buffer (pH 8.0). The mixtures were incubated at 37 °C for 30 min. Then, 20 μL of the AChE-inhibitor solution (or AChE-control solution) was taken to measure the residual activity of AChE according to a modified Ellman's method fully described in our previous study (*20*). All of the above tests and measurements were performed in four replicates.

IC_{50} was calculated by the logit transition model using the following equations (*21*):

$$\text{logit} = \ln \frac{I}{100 - I} \quad (1)$$

$$\lg[C] = A + B\,\text{logit} \quad (2)$$

Here I and [C] represent the percent of inhibition on AChE activity and the corresponding concentration of the inhibitor, respectively. A and B are two constants. When logit = zero, the corresponding [C] = IC_{50}.

Acute Aquatic Toxicity (in Vivo). The median lethal concentrations (LC_{50}) for CP and its complex with β-CD were performed against a

Table 1. Loading Efficiency of CP at Various Mixing Ratios

CP:β-CD molar ratio		
in the reaction mixture	in the complex	loading efficiency[a] (%)
1:1	0.14:1	14.0
2:1	0.31:1	15.6
3:1	0.48:1	16.2
4:1	0.69:1	17.3
5:1	0.70:1	14.0

[a] CP loading efficiency (%) = (total amount of CP encapsulated into β-CD/total amount of CP used in the preparation) × 100.

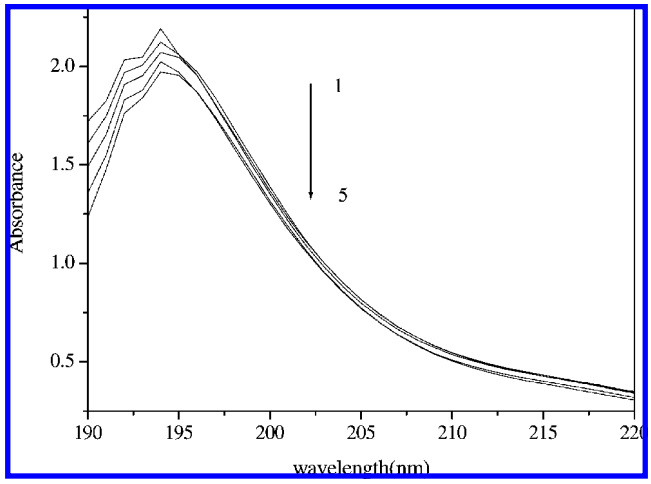

Figure 2. Absorption spectra of CP (0.5 mM) in distilled water containing β-CD. Concentrations of β-CD: (1) 0, (2) 0.5, (3) 1.0, (4) 1.5, and (5) 2.0 mM.

standard bioindicator *Daphnia magna* (*D. magna*). Stock organisms were originally obtained from the Chinese Academy of Protection and Medical Science (Beijing, China). A previous study has described the methods to culture the organisms and perform the detail test (*20*). Four replicates for each treatment were prepared. The mortality of daphnids of all vials was monitored at 24 h intervals for the 48 h exposure period.

RESULTS AND DISCUSSION

Loading Efficiency of CP. The loading efficiency of CP was various at the mixing ratio of 1.0–5.0 for CP over β-CD. Relations of the CP: β-CD molar ratio between the reagent mixture and the product complex are shown in **Table 1**. It is found that the latter gradually increased with increasing mixing ratio, reaching a probable saturated value at the mixing ratio at 4:1 for CP over β-CD. All the CP:β-CD molar ratios lower than 1 of the kneaded products suggests that eventually "empty" β-CD might be present because of the incomplete inclusion. The maximum loading efficiency of CP was also observed at 4:1 CP:β-CD mixing ratio (**Table 1**), and the corresponding value was 17.3%. One probable reason for this relative lower loading efficiency is the high hydrophilicity of CP and its weaker exchange ability with water in β-CD. On the basis of the above reasons, we chose the product synthesized at the 4:1 CP:β-CD mixing ratio to study its structural determination, thermal stability, and bioefficacy.

Characterization and Identification. The UV absorption spectral changes of CP titrated with β-CD are shown in **Figure 2**. Spectra were first performed by of a set of aqueous solutions containing 0.5 mM CP and different concentrations of β-CD with concentrations ranging from 0 to 0.2 mM. Because of the absorbance of β-CD from 190 to 230 nm, spectra of a second set of aqueous solutions containing the same β-CD concentrations but without CP were also conducted. The spectra in **Figure 2** ultimately were the subtraction of the spectra from set 2 from those of set 1 for removal of contribution of β-CD from cospectra. As seen from **Figure 2**, although the absorption maximum remains unchanged, addition of β-CD causes a visible decrease of intensity which indicates that CP forms complexes with β-CD in the experimental conditions.

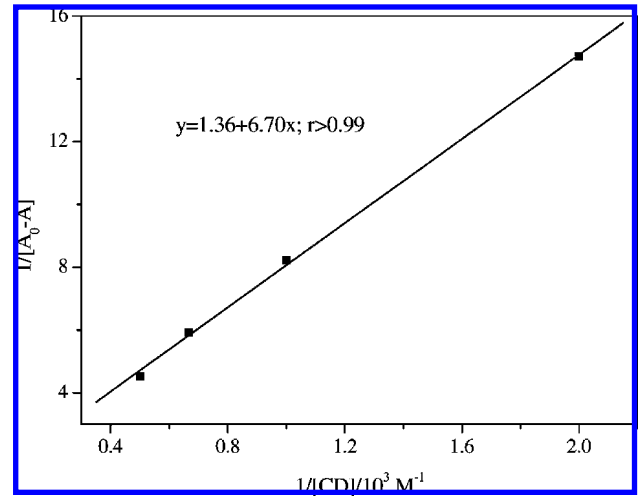

Figure 3. Double reciprocal plot for the determination of the inclusion constant of the CP−β-CD complexed system at 194 nm.

The inclusion constant, which represents the inclusion capacity, was also determined in the study. The double reciprocal method (*22*) was used to estimate the inclusion constant of the complex. Good linearity of the 1:1 complex suggests that a 1:1 CP−β-CD complex has formed (**Figure 3**). Thus, the inclusion constant can be obtained from the Benesi−Hildebrand equation (*22, 23*):

$$\frac{1}{A-A_0}=\frac{1}{a}+\frac{1}{aK[\mathrm{CD}]_0}$$

Here, A, A_0, a, K, and $[\mathrm{CD}]_0$ are the absorbance of CP in the presence of CD, that in the absence of CD, a constant, the inclusion constant for the formation of the 1:1 CP−β-CD inclusion complex, and the initial concentration of β-CD, respectively. The inclusion constant K can be calculated by the ratio of intercept over slope. The determined K value for β-CD is 203.0 M^{-1}, implying feeblish inclusion happened between the CP and β-CD in the water (*24*). Various noncovalent interactions between host and guest, such as dipole–dipole, hydrophobic, electrostatic, van der Waals, and hydrogen-bonding interaction, cooperatively contribute to the inclusion process (*2*). Because of complexity in the intermolecular forces, it is difficult to exactly elucidate any structural features of CP or β-CD that correlated with this result. However, dipolar or hydrogen bonding interactions may mainly influence the stability of the complex owing to the great polarity of CP.

DSC. The thermal method is widely used to characterize CDs and their inclusion complexes. It can provide evidence for evaluating the inclusion process by any differences in the number and/or position of peaks between the physical mixture and the putative inclusion compound (*25*). **Figure 4** illustrates the DSC curves of the pure CP, pure β-CD, the mechanical mixture of CP:β-CD (at 4:1 mixing ratio), and the solid complex. The DSC curve of CP (**Figure 4a**) shows a characteristic endothermic fusion peak at 78 °C. The DSC thermogram of β-CD (**Figure 4b**) gives a broader endothermic peak in the range of 80–120 °C, considered to be a dehydration process. The DSC thermogram of the physical mixture (**Figure 4c**) is nearly the overlap of the curves corresponding to β-CD and CP which

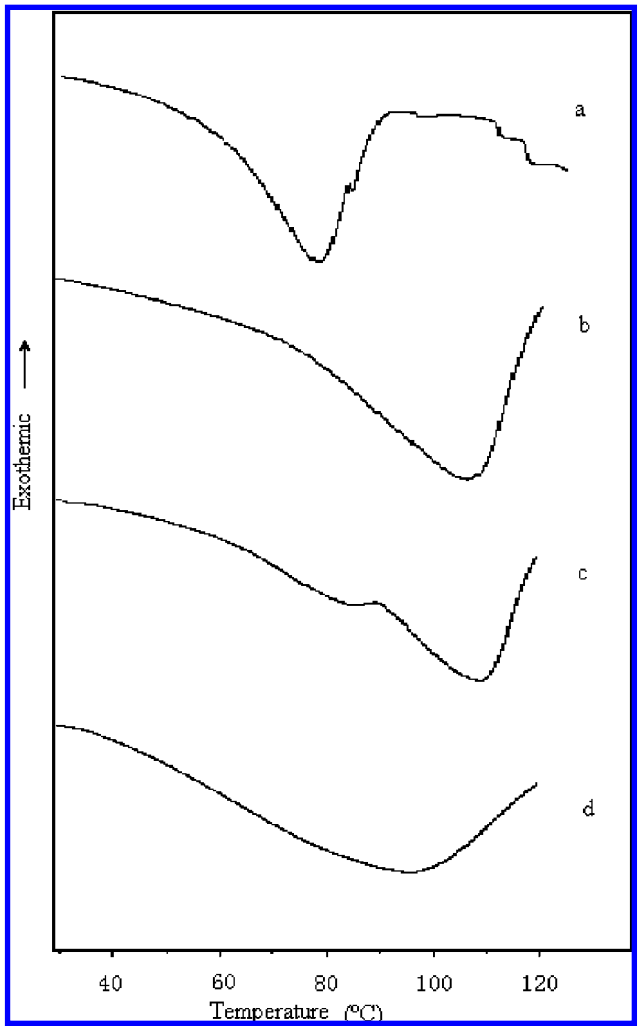

Figure 4. DSC thermograms of CP (**a**), β-CD (**b**), and 4:1 CP−β-CD systems: physical mixture (**c**) and kneaded sample (**d**).

indicates the absence of interaction between both components. For the CP−β-CD complex (**Figure 4d**), only an endothermic peak at 97 °C corresponding to the dehydration of β-CD is observed, causing the CP endothermic effect to disappear. In other words, the endothermic peak corresponding to the dehydration peak is displaced to lower temperatures (from 108 to 97 °C). A similar result was also found in the study of Villaverde and his co-workers, detecting the endothermic peak of β-CD descending from 150 to 120 °C when included with herbicide norflurazon using the vacuum evaporation process (*26*). This phenomenon indicates that the bindings between the hydration water and the β-CD are weaker, and it is probably due to the interaction with pesticide molecules that is taking place in the cavity of the cyclodextrin.

FT-IR. The complexation between CP and β-CD was further evidenced by the FT-IR data in **Figure 5**. Not all changes of the stretching frequency of CP can be observed when it was encapsulated with β-CD, because parts of the characteristic bands of CP were overlapped with those of β-CD. However, three bands of CP appearing at 1426, 1210, and 804 cm^{-1}, which were respectively assigned to the C−N, P═O, and C−Cl stretching can be definitely distinguished from the bands of β-CD both in the diagrams of the physical mixture and the inclusion complex. The most significant difference between the spectra of the inclusion complex and the mechanical mixture was the disappearance of the obsorbance band at 804 cm^{-1}, deducing that the -CCl_3 group of CP was possibly included into

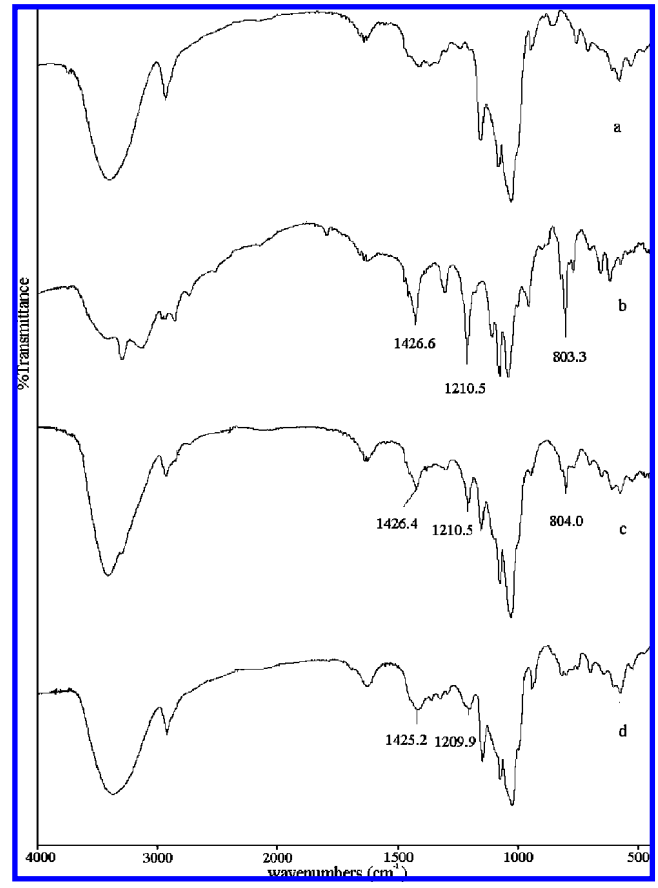

Figure 5. FT-IR spectra of β-CD (**a**), CP (**b**), and 4:1 CP−β-CD systems: physical mixture (**c**) and kneaded sample (**d**).

the cavity of β-CD. Furthermore, the band characteristic of the C−N stretching vibration was shifted from 1426.6 cm^{-1} to 1425.2 cm^{-1}. This suggests that hydrogen bonding between the N atom of CP and the hydroxyl of β-CD may also contribute to the formation of complexes.

XRD. Additional evidence of the complexation was derived from the X-ray powder diffraction shown in **Figure 6**. Sharp peaks over the diffraction angles indicate the crystal nature of β-CD (**Figure 6a**) and CP (**Figure 6b**). In contrast, the diffraction diagrams obtained by the kneading method (**Figure 6c**) exhibit a dramatic decrease in the number and intensity of diffraction peaks, suggesting formation of a new amorphous inclusion complex. A similarly great reduction of the particle size was also observed in other studies using the kneading method, reflecting a loss of crystallinity or amorphization of the samples (*26*).

Improvement of Thermal Stability. The inclusion of a guest molecule in the cyclodextrin cavity has been identified to improve the storage stability both in solution and solid state (*27, 28*). It is also successfully applied in various industries, particularly in pharmaceutics *4, 29, 30*), to improve their stability and be able to make these compounds easily available. In our study, we just chose the changes in the thermal degradation by an accelerated storage stability analysis which is considered to be a useful indication of product stability (*31*). Residues of CP and CP−β-CD inclusion complex are listed in **Table 2**. The results show that the content of CP in its inclusion complex was almost unchanged during a period of 7 day incubation. When referring to the pure CP, about one-third of the active molecule disappeared. In the case of 14 day incubation, residue of the pure CP was only about 10.2%, while that of the encapsulated CP was 75.3%. In other words, the degradation

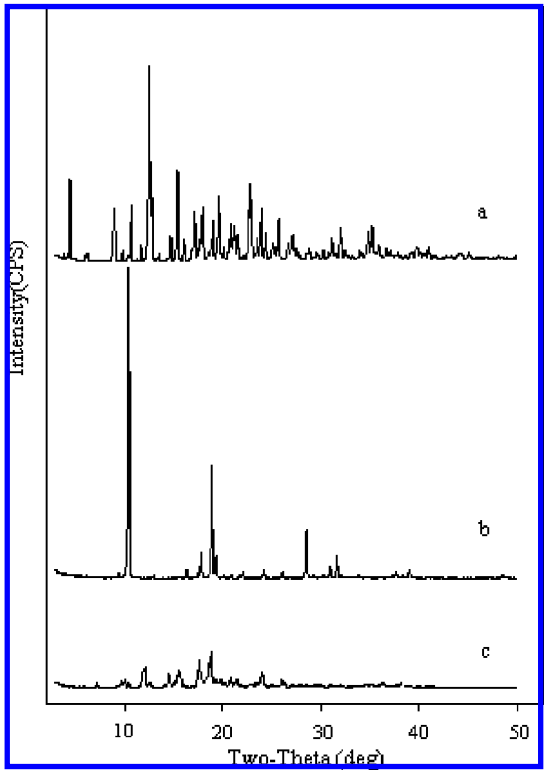

Figure 6. X-ray diffraction pattern of β-CD (**a**), CP (**b**), and their complex (**c**).

Table 2. Percent of the Active Compound Remained in the Accelerated Storage Stability Studies (%)

compound	0 day[a]	7 days[a]	14 days[a]
CP	100.0 ± 0.0	67.2 ± 6.5	10.2 ± 1.7
CP−β-CD complex[b]	100.0 ± 0.0	98.8 ± 3.8	75.3 ± 6.2

[a] All values are means ± SDs of the mean (n = 3). [b] The mixing ratio of CP:β-CD = 4:1.

rate of CP in 14 days was slowed by a factor of 3.6 when it was inclusion-complexed with β-CD, which intensely developed the possibility of its application.

Bioefficacy of the Inclusion Complex. For a successful pesticide, high activity and low toxicity are two indispensable factors for its register and application. When the pesticides are inclusion-complexed with CDs, their activity and/or toxicity might be influenced because of a change of the solubility or inclusion of positions of the guests which bind with the organisms (*32, 33*). It has been known for many years that the mechanism of the acute toxicity of OPs both for pharmacology and toxicology is inhibition of a serine esterase acetylcholinesterase, with the resulting excess acetylcholine accumulation leading to symptoms of cholinergive excess. Although AChE from target or nontarget species have different sensitivities, they have similar structure in the general active site gorged with the residues covalent and/or noncovalent combining with the OPs having appropriate steric orientation and electronic distribution. Thus, if the steric orientation and/or electronic distribution of CP are interfered with by its inclusion complex with β-CD, the toxic potency of CP against AChE will be changed regardless of the origins of this target enzyme. In this study, we chose the target enzyme from *E. electricus* instead of the target animals to show the differences in activity of CP with or without β-CD. Meanwhile, we established a standard aquatic toxicity assay using *D. magna* to indicate any changes in toxicity of CP when it was inclusion-complexed with β-CD. The effects of β-CD toward CP on activity against AChE and toxicity to *D. magna* are depicted in **Table 3**. The *t*-test reveals that no significant differences between CP and its complex with β-CD exist in both IC_{50} of AChE and LC_{50} of *D. magna*. The results gained above may attribute to the following factors. First, β-CD has little toxicity alone as the reported results (*34, 35*). Second, the same concentrations of CP in each treatment with CP alone or with the inclusion complexes were used. Furthermore, the steric orientation and nucleophilic ligand of CP may not be influenced. Then, many biological activities, especially absorption, metabolism, transfer, and accumulation that affect the toxicity in vivo may also not be changed by the inclusion process. It can be concluded that both the activity and toxicity of the CP−β-CD complex were not influenced compared with that of CP alone, which also offers useful and exciting information for improving the application of CP with β-CD.

Table 3. Bioefficacy of the Pure CP and CP−β-CD Complex against AChE in Vitro and *Daphnia magna* in Vivo

	IC_{50} of AChE (M)[a,c]	LC_{50} of *Daphnia magna* (M)[a,d]
CP	7.25 ± 0.37	1.31 ± 0.12
CP−β-CD complex[b]	7.52 ± 0.48	1.01 ± 0.08

[a] All values are means ± SDs of the mean (n = 4). [b] The mixing ratio of CP:β-CD = 4:1. [c] IC_{50} in 30 min. [d] LC_{50} in 48 h.

Conclusions. CP−β-CD inclusion complexes were successfully prepared by a kneading method with a 1:1 stoichiometry ratio and an inclusion constant of 203.0 M^{-1}. When the encapsulated CP was formed, great improvement in thermal stability happened without any obvious influences on activity and toxicity of the guest. The promising results with β-CD imply that the gained new inclusion complex can be used as the active ingredients of various formulation additives and give the possibility of enhancing and widening the usage of CP.

ABBREVIATIONS USED

AChE, acetylcholinesterase; CD, cyclodextrin; CP, chloramidophos; DCPE, *a*-(diethoxyphosphinoximino)dicyclopropylmethane; DSC, differential scanning calorimetry; EE-AChE, acetylcholinesterase of *Electrophorus electricus*; FT-IR, Fourier transform infrared spectroscopy; OPs, organophosphorus pesticides; UV, ultraviolet; XRD, X-ray powder diffraction.

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