Study on the Inclusion Complexes of Flumorph and Dimethomorph with β-Cyclodextrin to Improve Fungicide Formulation

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Summary: The β-Cyclodextrin (β-CD) complexes of Flumorph and Dimethomorph have been investigated quantitatively by UV measurement. The results showed that the 1:1 inclusion complexes were formed and the formation constants reduced with elevation of temperature. Thermodynamic parameters of the inclusion reaction (ΔH、ΔS、ΔG) were all negative, which suggested that the interaction process between β-CD and the two fungicides were spontaneous, driven by entropy changes of inclusion reaction. Two-dimensional (2D) ROESY NMR experiments were performed to identify the binding geometry of inclusion complex. Both of *Z* and *E* isomers of Flumorph formed complexes with β-CD, while only the *Z*-isomer of Dimethomorph formed complex with β-CD, and the phenyl ring containing fluorine or chlorine atom entered into the cavity of β-CD through the wider rim. Phase-solubility experiments demonstrated that the water-solubility of the two fungicides enhanced in the presence of β-CD. The preliminary bioassay testified that the complexes improved the fungicidal activity. The present study provided a promising outlook for developing water-based formulation of Flumorph and Dimethomorph, increasing its efficacy and improving its formulation.

Introduction

Research and development trend of pesticide formulation is the environment friendly formulation. The advantages of the water-based pesticide formulation are highly safety, green, low-cost and without organic solution evaporation. Thus the formulation has attracted growing interest to the investigators. However, there are many difficulties in developing water-based pesticide formulation, due to their poor water-solubility and molecular structure diversity. One of the potential solutions is using cyclodextrins (CDs).

CDs are cyclic oligosaccharides containing six (α-CDs), seven (β-CDs), and eight (γ-CDs) glucose units, having an internal highly hydrophobic hollow cavity and a hydrophilic surrounding walls. In aqueous solution and in solid state, the CDs molecules are able to include a variety of guest molecules in their hydrophilic internal cavity, which can improve physical, chemical and biological properties of the guest molecules [1-3]. Owing to these advantages, CDs have been widely used in many fields, such as pharmaceutical science, food manufacturing, and cosmetics industries [4-7]. Recently, CDs have aroused considerable interest in agriculture field. Many synthetic pesticides can form inclusion complexes with CDs, offering several advantages over conventional formulations: such as enhancing stability of volatile or unstable pesticides against the degradation effects of oxidation, visible or UV light [8, 9], controlling the release rate and increasing effects on the degradation of poisonous pesticides in environment [10, 11], improving pesticides absorption and inhibiting their leaching in soils [12, 13], increasing solubility, wettability, and bioavailability of highly water-insoluble pesticides [14-19]. Among all the advantages, the latter aspect is particularly important, because it allows more effective penetration of the active compound. In addition, CDs are natural compounds, which can be decomposed by many plant related bacteria and soil microbes [20]. It is predicted that the application of CDs to pesticide formulations will have a rapid development in the future [21].

Flumorph and Dimethomorph, both represent cinnamamide fungicides as shown in scheme 1, have excellent preventive efficacy on Oomycete disease of plant [22, 23]. But they have poor water solubility. Herein we report the characterization of two inclusion complexes, formed by Flumorph and Dimethomorph with β-CD

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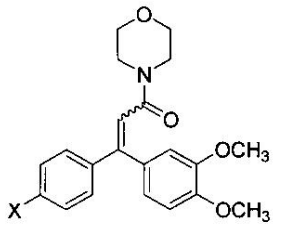


Scheme 1: Chemical Structure of Flumorph and Dimethomorph.

through UV-spectrophotometry, thermal analysis, two-dimensional NMR spectroscopy, phase-solubility experiment and fungicidal activity assays, with an aim to improve the water-solubility and bioavailability of Flumorph and Dimethomorph. It's an elementary study for developing water-based formulation that provides a more rational, safe, effective and low-cost use of these fungicides.

Results and Discussion

Absorption Spectra Studies

The UV-vis adsorptions of Flumorph and Dimethomorph in different concentrations of β-CD are shown in Fig. 1. The absorption intensities of pesticides increases gradually with the increasing concentrations of β-CD, which illustrates the formation of the inclusion complexes. However, the biggest absorption peak (203 nm) is not changed, it is speculated that the inclusion reactions are driven by force produced from intermolecular action. The behavior of the increasing absorption intensities with increasing concentration of β-CD has been attributed by electron shift of guest molecular induced by high electron intensities of β-CD cavity.

In inclusion reaction, the binding stoichiometry and binding constant are important parameters characterized the structure of inclusion complex. When the inclusion complex has a 1:1 stoichiometry, the Ks value and stoichiometric ratio can be detemined according to the Benesi-Hildebrand equation (Eq. (1)) [24].

$$\frac{[G]_0}{\Delta A} = \frac{1}{\Delta\varepsilon \bullet K_s} \bullet \frac{1}{[CD]_0} + \frac{1}{\Delta\varepsilon} \qquad \text{Eq. (1)}$$

Here ΔA is the difference between the absorption of pesticide in the presence and absence of β-CD, $\Delta\varepsilon$ is the difference between the molar absorption Co-efficient of pesticide and inclusion complex. $[G]_0$ and $[CD]_0$ are the initial concentration of the pesticides and β-CD, respectively.

Fig. 2 shows the double reciprocal plot of $1/\Delta A$ versus 1/ [CD]. These plots exhibit good linearity, suggesting that 1:1 inclusion complexes are formed. The inclusion constants Ks can be defined from the intercept and slop values.

Temperature is an important parameter in thermodynamic study. As seen in Fig. 2, the Ks decreases by increasing of temperature, suggesting that the inclusion reaction between the two pesticides and β-CD were an exothermic process. Therefore, the

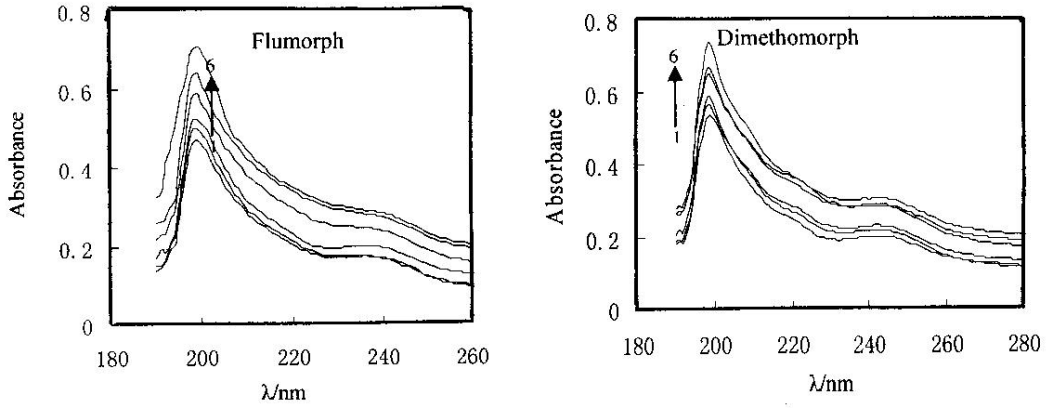


Fig. 1: UV spectroscopy of Flumorph and Dimethomorph in the presence of β-CD (298 K). $C_{guest} = 1.0 \times 10^{-5}$ mol/L; $C_{\beta\text{-CD}}$ 1~6: 0, 0.5, 1, 3, 5, 10×10^{-3} mol/L.

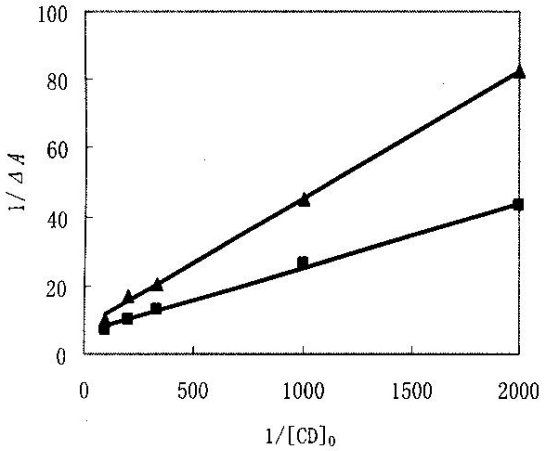


Fig. 2: The linear fitting of the inclusion complexes of Flumorph and Dimethomorph with β-CD. ▲Flumorph y = 0.0375x + 7.7847 R^2 = 0.9982 ■ Dimethomorph y = 0.0189x + 6.1323 R^2 = 0.9973.

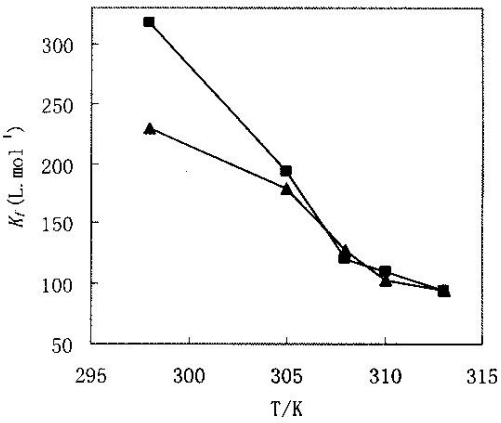


Fig. 3: Effect of temperature on Ks. ▲Flumorph ■Dimethomorph

inclusion complex was suitable to be prepared at lower temperature. The reason may be that the increasing of thermal behavior of guest molecular trigged by the elevation of temperature is not in favor of guest entering the cavity of β-CD.

The thermodynamic parameters involved in the complex formation can be applied to explain the binding force. The linear relationship between lnKs and the inverse of the absolute temperature (1/T) are shown in Fig. 5. The values of enthalpy changes (ΔH) and entropy changes (ΔS) can be abstained from the Van't Hoff equation (Eq. (2)), depending on the variation of the stability constants with temperature. The Gibbs free energy change (ΔG) at different temperatures can be calculated from the Ks by Eq. (3). All the results are listed in Table-1.

$$\ln Ks = -\frac{\Delta H}{R} \bullet \frac{1}{T} + \frac{\Delta S}{R} \qquad \text{Eq. (2)}$$

$$\Delta G = - RT\ln Ks \qquad \text{Eq. (3)}$$

As can be seen from Table-1, ΔG are negative, which indicate that the inclusion process proceeds spontaneously at the range of experimental temperature. ΔH and ΔS are also negative, implying that the inclusion complex formation is exothermic and the process was controlled the change of enthalpy. The relatively low negative ΔH is attributed to some typical low energy interactions, including hydrophobic interactions, the increase of Vander Waals interactions of intermolecular and the formation of hydrogen bonds, etc. While the negative ΔS is from limiting of β-CD cavity to the freedom of shift and rotation of guest molecule.

Two-Dimensional (2D) NMR Studies

2D ROESY NMR has been recognized as one of an indispensable method for identifying the geometry of the inclusion complex. ROESY cross-peaks are indicative of specific proximity relationships between adjacent protons. There is several key cross-peaks between the protons of Flumorph and β-CD in the 2D NMR spectrum (Fig. 5a), indicating that the Flumorph molecule is included in the cavity of β-CD. Peaks A gives the NOE correlation between olefin proton of *E*-isomer and protons of aromatic ring containing methoxy in Flumorph molecule, and peak B exhibit correlation signals between olefin proton of *Z*-isomer and protons of aromatic ring containing F in Flumorph

Table-1: The thermodynamics parameters of the inclusion reaction (pH=6.86).

	ΔG (kJ.mol^{-1})					ΔH	ΔS
Inclusion Complexes	298K	303K	308K	310K	313K	(kJ·mol^{-1})	(J·mol^{-1}·k^{-1})
β-CD-Flumorph	- 13.47	- 13.07	- 12.41	- 11.95	- 11.83	- 48.57	- 117.58
β-CD-Dimethomorph	- 14.27	- 13.49	- 12.47	- 12.30	- 12.03	- 49.33	- 117.31

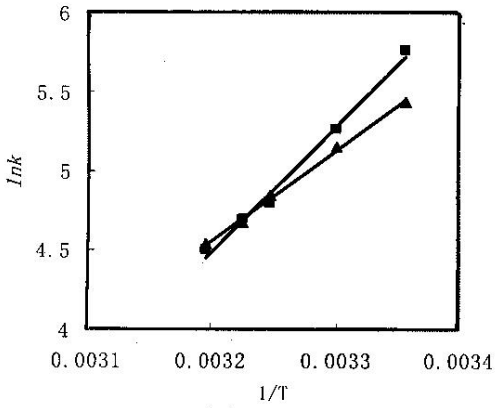
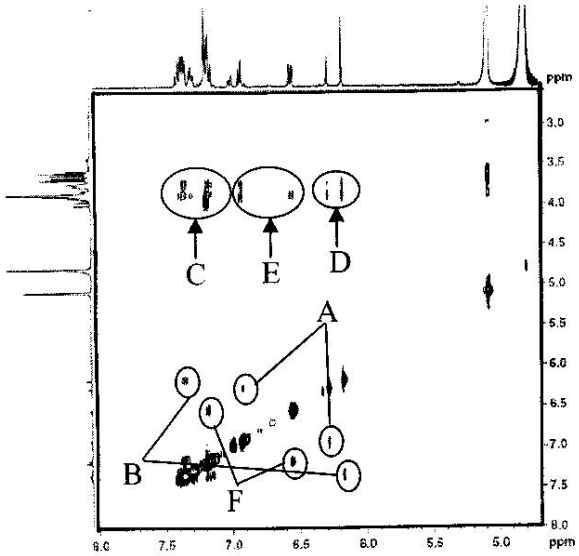


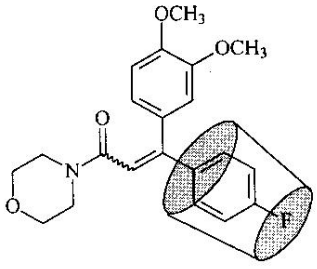
Fig. 4: The relationship between LnKs and 1/T
▲Flumorph y = 5675.8x - 13.6 R^2 = 0.9954
■ Dimethomorph y = 7936.4x - 20.907 R^2 = 0.9913

molecule. The results indicate that both *Z* and *E* Flumorph molecules formed inclusion complex with *β*-CD. Peaks C display clear correlation between the H-3/H-5 protons of *β*-CD inner cavity and protons of phenyl ring with F atom in compound Flumorph-*β*-CD, which means that the aromatic ring containing F enter into the *β*-CD cavity. Peaks D show the weak correlation signals between olefin proton of *Z* and *E* Flumorph and H-3 protons of *β*-CD, Peaks *E* present the weak NOE correlation between the protons of phenyl ring with methoxy and the interior protons of *β*-CD, suggesting that the phenyl ring with methoxy should not be included into *β*-CD cavity. In addition, peaks F exhibit the NOE correlation among protons of methoxy phenyl ring. Since H-3 protons are located near the wider rim of CD, and the H-5 protons near the narrow rim, the possible structure of the inclusion complex of Flumorph with *β*-CD can be deduced as Fig. 5b.

Fig. 6a shows the ROESY spectrum of the *β*-CD complex of Dimethomorph. Different from the Flumorph / *β*-CD system, the NOE correlation between olefin protons of Dimethomorph and H-3 protons of *β*-CD is only one cross-peak (peak A), giving the information that only one isomer of Dimethomorph forms inclusion complex with *β*-CD. The result is confirmed by peaks B, which obviously display the NOE correlation between olefin proton of *Z* isomer and protons of phenyl ring containing Cl atom. Peaks D distinctly display the NOE correlation between protons of phenyl ring with Cl atom and the H-3/H-5 protons of *β*-CD, implying that phenyl ring with Cl atom enter into *β*-CD cavity. Therefore, we deduce the possible binding model illustrated in Fig.6b. In addition, Peaks C show correlation signals

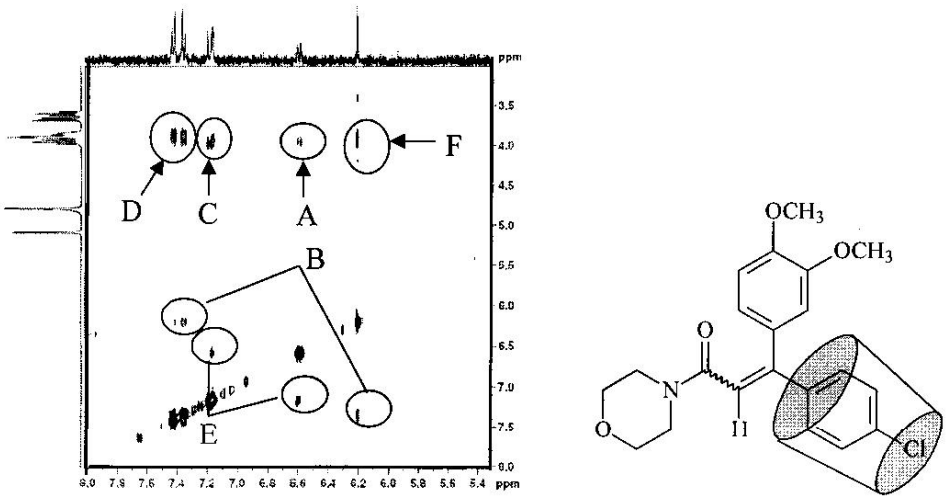


(a)



(b)

Fig. 5: (a) 1H ROESY spectrum (400 MHz) of Flumorph and *β*-CD inclusion complex in D_2O. Continuous-wave irradiation (B_1=2800Hz) was used during a mixing time of 6.0 s. (b) The possible geometry of the inclusion complex of Flumorph and *β*-CD.



(a) (b)

Fig. 6: (a) ^{1}H ROESY spectrum (400 MHz) of the Dimethomorph and β-CD inclusion complex in D_2O. Continuous-wave irradiation (B_1=2800Hz) was used during a mixing time of 6.0 s. (b) The possible geometry of the inclusion complex of Dimethomorph and β-CD.

between protons of phenyl ring with methoxy and H-3 protons of β-CD. Peaks E exhibit the NOE correlation of protons interaction of phenyl ring with methoxy, and Peaks F represents the correlation signals between olefin protons and morpholine ring protons. Fig. 5 and 6 showed that the volume of the phenyl group is the key factor to determine whether they could enter into the cavity of β-CD.

Water-Solubility and Fungicidal Activity

The effect of β-CD on the water-solubililty of Flumorph and Dimethomorph were measured by the phase solubility method. As can be seen in Table-2, the water solubility of the two fungicides gradully enhanced with the increase of the concentration of β-CD. The water solubility was increased from 0.79 mM to 2.39 mM in the presens of 6 mM β-CD for flumorph, While to Dimethomorph, the water solubility was increased from 0.48 mM to 0.97 mM in the presens of 6 mM β-CD. There is a 3-fold and 2-fold increase for Flumorph and Dimethomorph, respectively.

The fungicidal activities of Flumorph, Dimethomorph and their complexes with β-CD against Phytophthora capsici *in vitro* are assayed according to our previously reported method [25]. The results revealed that the EC_{50} values of the inclusion complexes, Flumorph-β-CD and Dimethomorph-β-CD, against Phytophthora capsici are 1.185 $\mu g \cdot mL^{-1}$ and 0.305 $\mu g \cdot mL^{-1}$, respectively. Whereas the EC_{50} values of Flumorph and Dimethomorph alone against Phytophthora capsici are 1.74 $\mu g \cdot mL^{-1}$ and 0.44 $\mu g \cdot mL^{-1}$. This demonstrates that the formation of complexes of the two cinnamamide fungicides with β-CD is helpful to improve their fungicidal activities.

Experimental

Materials

β-CD (MW = 1134) was obtained from Shanghai Chemical Reagents Co. Ltd. (Shanghai, China), Flumorph and Dimethomorph were provided by Jiangshu Frey Chemical Co. Ltd. (Jiangshu,

Table-2: Solubility of Flumorph and Dimethomorph in different β-CD solutions.

Cβ-CD (mM)	0	1	2	3	4	5	6
CFlumorph (mM)	0.79	1.47	1.62	1.82	2.00	2.19	2.39
CDimethomorph (mM)	0.48	0.66	0.70	0.76	0.80	0.83	0. 97

China). The other chemicals were of analytical grade, and the water used throughout the study was the milli-Q water.

β-CD was dissolved in water and prepared to 1.0×10^{-2} mol•L^{-1} stock solutions. Flumorph and Dimethomorph were dissolved in methanol and prepared to 1.0×10^{-3} mol•L^{-1} stock solution, respectively.

Apparatus

Absorption spectra were measured on a TU-1901 spectrophotometer (Puxi Instrument Company, Beijing, China). Two-dimensional NMR spectra were recorded on a Bruker Avance-400 spectrometer in D_2O solution.

Preparation of Solid Complexes

The mixtures of crystalline-β-CD (1.1 g) and pesticides (Flumorph, 0.37 g; Dimethomorph, 0.39 g) were thoroughly grounded under normal humidity, respectively. Then added 10 mL water to the mortar and stirred. After centrifuging 10 min., the solutions were stored in refrigerator for 12 h. Finally, the precipitates formed were filtered across a glass filter (G4) and dried in vacuum desiccators over P_2O_5 to constant weight.

Phase-Solubility Measurements

A fixed amount of pesticide, exceeding its solubility, was added to 10 mL capped tubes containing different concentration of β-CD (0, 0.3×10^{-2}, 0.6×10^{-2}, 0.9×10^{-2}, 1.0×10^{-2}, 1.2×10^{-2}, 1.5×10^{-2} mol•L^{-1}). The tubes were sealed to avoid changes due to evaporate and shaken for 3 days in a thermostatic bath at 25.0 ± 0.01 °C. After the equilibrium was reached, the solution was centrifuged, diluted and the concentration of pesticide was spectrophotometrically determined. Experiments were carried out in triplicate, and solubility data were averaged.

Acknowledgment

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