

Determination of the binding mechanism of cobalt(II) meso-tetraphenyl porphyrin with plant-esterase

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Plant-esterase (EC 3.1.1.X) has received much attention because plant esterase and acetylcholinesterase (AChE) share a similar sensitivity towards organophosphorus (OP) pesticides detection with the same inhibition mechanism. To improve the analytical performance, tetraphenyl metal porphyrin, as an indicator was introduced to combine with plant-esterase. The time of reach equilibrium in PBS solution was shortened after adding plant-esterase by assaying the intensify change of the porphyrin spectrum. Meanwhile, intensify of porphyrin spectrum with plant-esterase was increased compared with that of only the porphyrin spectrum in solution. Tetraphenyl metal porphyrin, such as cobalt(II) meso-tetraphenyl porphyrin, is a mixed reversible inhibitor of plant-esterase from kinetic parameters. The combination ratio of plant-esterase and porphyrin is 2:1. On the other hand, the interaction between CoTPPCL and plant-esterase is the strongest among all tested tetraphenyl metal porphyrin. And the mixed system (CoTPPCL-plant-esterase) showed the best sensitivity towards the tested pesticide. All these results indicated that a complex system composed of tetraphenyl metal porphyrin and plant-esterase was fit for detecting pesticides. They make meaningful guidance on the further design of sensing material in monitoring pesticides.

Keywords: plant-esterase, tetraphenyl metal porphyrin, binding mechanism.

INTRODUCTION

Acetylcholinesterase (AChE) is a highly sensitive bio-recognition element for the inhibition of organophosphorus, carbamate pesticides and is widely studied in the past decades¹. However, the high cost and low accessibility of AChE (extracted from animal blood or tissue) greatly limit its wide application. In recent years, plant esterase (EC 3.1.1.X) as a substitute for AChE has attracted wide attention due to its similar sensitivity towards organophosphorus (OP) pesticides and inhibition mechanism. In addition, the extraction of plant esterase from wheat, soybean, rice, sorghum and other plants is not only cost-effective, but also easy to obtain¹, which enable its great potential in pesticide detection.

However, the study of plant-esterase is not very well-rounded. The nature substrate of plant-esterase is still unsure. The mimic substrate may reduce plant-esterase activity and have negative effects on the sensitivity of the biosensor. Furthermore, it is cumbersome to introduce substrate, chromogenic reagent and stabilizer in the process of enzyme activity assay. Thus, avoiding the enzyme activity assay is an important way to improve sensitivity and shorten detection time. Except for the change of enzyme activity, the structure of enzyme will be change after the detection. Thus, a suitable structure indicator also indirectly reflects the existing pesticide.

Porphyrin is a colored molecule with a high degree of conjugation and displays rich photophysical properties¹. Its absorbance spectrum is sensitive to the change of environment. Changes in acidity², hydrophobicity³ and ion content⁴ can result in a difference of absorbance intensity. Relay on this characteristic, Harmon⁵ chose water-soluble porphyrin as an indicator combined with cholinesterase to detect pesticides. It has been proven that porphyrin compounds are chromogenic reagents which can be indicated the microenvironment changes. In our early study, plant-esterase and monosulfonate tetraphenyl

porphyrin were combined as a complex material. It can be effectively detected dimethyl methyl-phosphonate within 3 or 5 s⁶. Nevertheless, most of the study is related to water-soluble porphyrin. Komatsu⁷ proposed the stability of non-soluble porphyrin in the aqueous phase will be improved after adding protein or enzyme. Non-soluble porphyrin, especially non-water-soluble metal porphyrin, is prone to enter the hydrophobic active site of enzyme and combine to the imidazole of amino acids with coordination bond. Meanwhile, Hou et al⁸ has proven the plant-esterase is a metal ion-dependent enzyme. Thus, tetraphenyl metal porphyrin, the simplest structure of non-water-soluble metal porphyrins is a promising alternative as a chromogenic reagent in our research. Here, we study the interaction between plant-esterase and tetraphenyl metal porphyrin in PBS solution. The complex system is also used to detect carbendazim by its spectrophotometric change.

MATERIALS AND METHODS

Plant-esterase preparation

Wheat flour (Guowei, Anhui) was purchased from the supermarket in Chongqing, China. The wheat flour was made to obtain pure plant-esterase according to the procedure described by Yang et al⁹. In brief, crude plant-esterase was extracted from a solution of wheat flour (1:5 w/w) by using centrifuged at 4000 r/min and 4°C for 10 min. Then it was purified with a PEG1000/NaH₂PO₄ aqueous two-phase system by a two-step extraction. To obtain its powder, the purified plant-esterase underwent dialysis and then was freeze-dried at -50°C.

Enzyme assays

The plant-esterase activity was measured by the method according to the previous report¹⁰. In detail, 0.5 mL of enzyme solution was mixed with 1-naphthyl acetate

(16 mM, 50 μ L) (Yuanju, Shanghai) as a substrate in 1.95 mL phosphate buffer (pH 6.5). After 5 min of incubation at 40°C, 0.5 mL Fast Blue B salt (Yuanju, Shanghai)-SDS solution and 0.1 mL HCl (6 M) was added. Finally, the absorbance was detected at 535 nm using a spectrophotometer (PerkinElmer Instruments, USA). One enzyme activity unit was defined as the amount of enzyme hydrolyzing 1 μ mol of 1-naphthyl acetate per minute under the standard assay conditions.

Effect of plant-esterase on porphyrin

The enzyme was incubated at 25°C with 5 μ M various tetraphenyl metal porphyrin (CoTPPCL, ZnTPP, FeTPPCL, EuTPPCL, InTPP and TPP) respectively. After reactions reached equilibrium, Soret intensities changes of porphyrin in the presence or absences of enzyme in solution were recorded.

Effect of CoTPPCL on plant-esterase

Purified plant-esterase was mixed with various concentrations of Co^{3+} , CoTPPCL, TPP from 2.5 μ M to 15 μ M in PBS, pH 6.5, at 25°C. Then the residual enzyme activity was assayed with the standard method mentioned above after 30 min. Discrete concentrations of CoTPPCL solution (4 to 12 mM) were respectively mixed with discrete concentrations of enzyme solution (5×10^{-8} M to 250×10^{-8} M). After 30 min, the specific activity was determined by the standard method. The enzyme activity was assayed after changing concentrations of the substrate from 0.01 M to 2.0 M. Then the Lineweaver-Burk plot of plant-esterase was described.

Detection of different complex system

The enzyme solution was incubated at 25°C with 5 μ M various tetraphenyl metal porphyrin (CoTPPCL, ZnTPP, FeTPPCL, EuTPPCL, InTPP and TPP) respectively. After reaction equilibrium, different concentrations of carben-dazim (5.23×10^{-6} M to 5.23×10^{-10} M) were mixed with complex systems. According to the spectrum changes, the detection result would be analyzed.

RESULT AND DISCUSSION

Changes of porphyrin spectrum

As shown in Fig. 1, the absorbance strength of porphyrin spectrum was increased with various degrees and peaks of porphyrin spectrum were moved after adding plant-esterase. The detailed changes information was listed in Table 1. There is no change in absorbance wavelength for InTPPCL and EuTPPCL and a significant red-shift of 28 nm for CoTPPCL after the addition of plant esterase. Besides, absorbance strength enhancements were observed with different degrees upon the addition of plant esterase. Among them, CoTPPCL displayed the biggest response of 0.12. In Fig. 2, in the presence of plant-esterase, porphyrin spectrum tends to be stable after 25 min. Comparison by the absence of plant-esterase, porphyrin spectrum keeps down after 25 min. The data were summarized in Table 1.

After reaction with plant-esterase, peaks absorbance of CoTPPCL and FeTPPCL were red-shift. Peaks absorbance of TPP and ZnTPP were blue-shift. While that of EuTPP and InTPP were not changed. Among metal ion above, Zn^{2+} , Fe^{3+} and Co^{3+} were classified as transition metal ions.

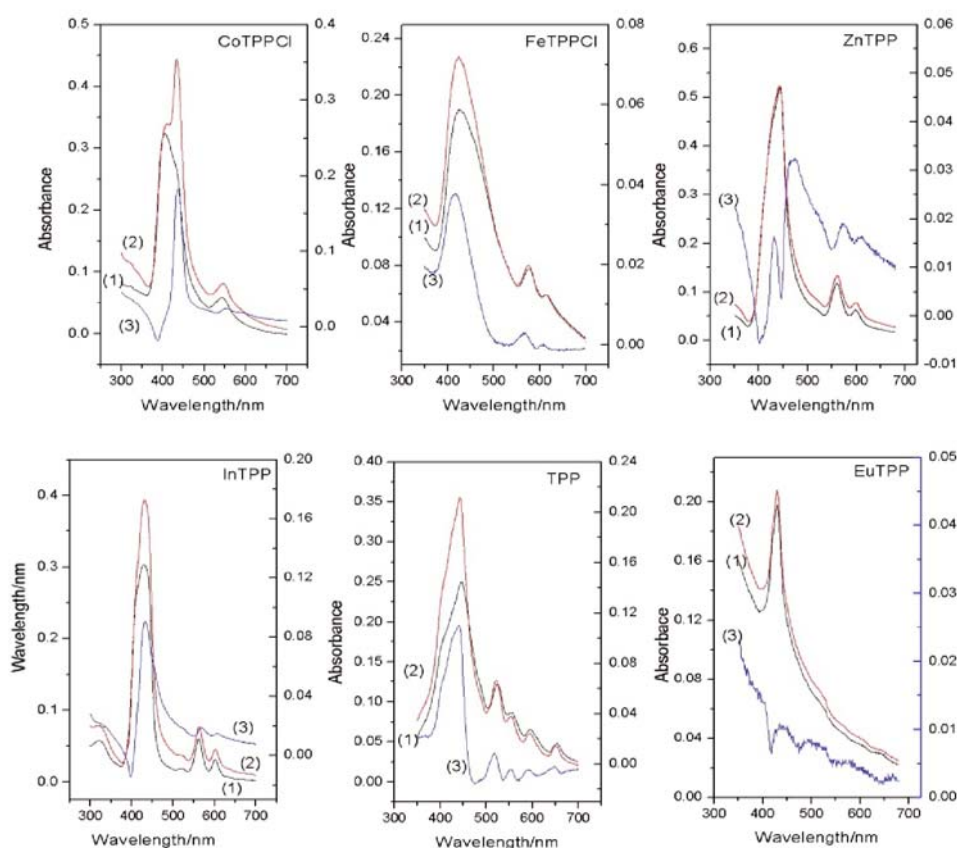


Figure 1. Absorption peak of porphyrin with the presence (Trace 1) and absence (Trace 2) of plant-esterase. Trace 3 represents the difference spectrum Trace 2 minus Trace 1

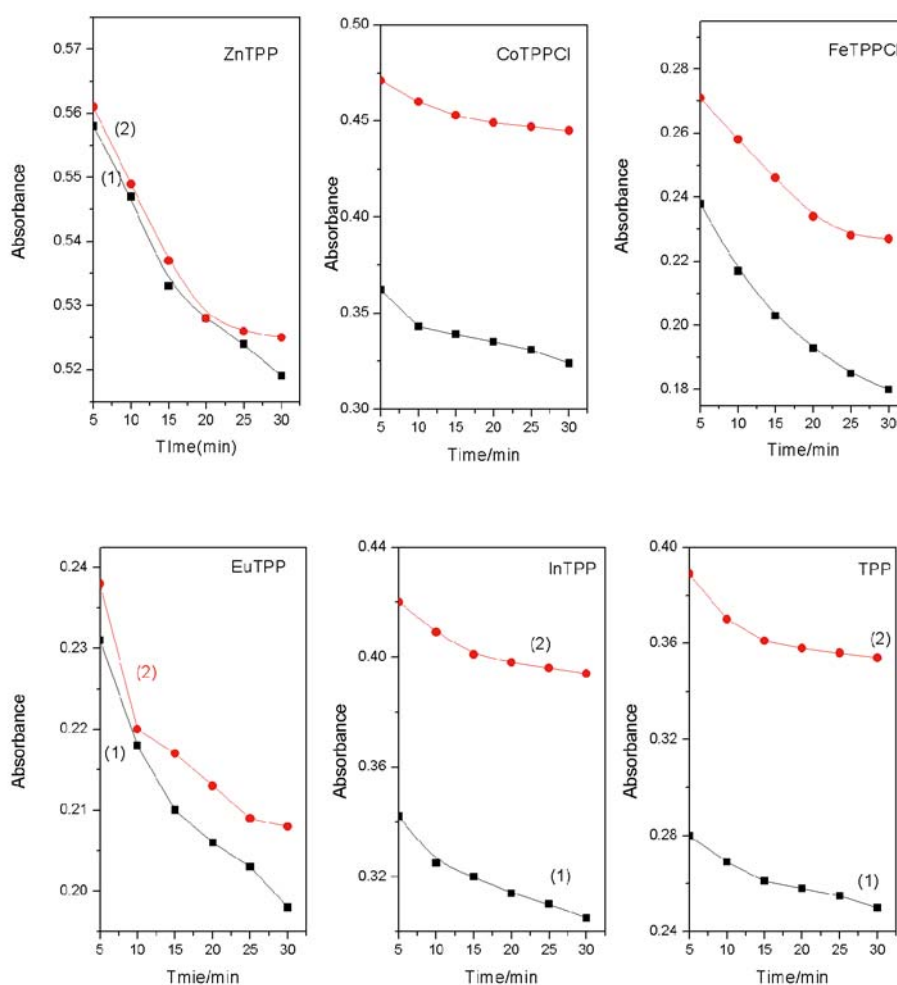


Figure 2. Changes of porphyrin spectrum with the (1) absence and (2) presence of plant-esterase. The absorbance recorded at various wavelength: ZnTPP: 444 nm, 442 nm; CoTPPCL: 406 nm, 434 nm; FeTPPCL: 423 nm, 425 nm; EuTPPCL: 429 nm, 429 nm; InTPPCL: 431 nm, 431 nm; TPP: 446 nm, 444 nm (before: left, after: right)

After reaction with the enzyme, Soret bands were changed. While, Eu^{2+} and In^{2+} were not included in transition metal ions, thus the position of their sort band did not change. Among these changes, the maximum extent of change was CoTPPCL whose atomic radius is largest among referred transition metal ions. It is speculated the size of Co^{3+} is suitable for the cavity of plant-esterase. Thus, the reaction between them is much more severe than others. The nitrogen atoms of amino and the hydroxyl oxygen atom of carboxyl in plant-esterase carry some electronics. These electronics would transfer to the porphyrin. Based on the types of metal ions, the degree of transfer has some differences. The electron-withdrawing ability of trivalent metal ions is much stronger than that of divalent metal ions. When trivalent metal tetraphenyl porphyrin interacts with plant-esterase, electron would flow from porphyrin to enzyme. The electron cloud density of porphyrin would be reduced and its Soret band appeared red-shift. The direction of electrons flow

leads to the opposite result to arise in divalent metal tetraphenyl porphyrin. The spectrum change of TPP and ZnTPP are similar. Because ZnTPP belongs adductoring tetraphenyl metal porphyrin. Its spectroscopic property is similar to tetraphenyl porphyrin.

Another way, the time of tetraphenyl porphyrin reach to balance in PBS was shorten with the presence of plant-esterase. And the peak shape of tetraphenyl porphyrin mixed with plant-esterase is similar to that of tetraphenyl porphyrin in DMF, except for peak position. Thus plant-esterase provides a hydrophobic environment for terrapheynyl porphyrin, and it enhances the stability of hydrophobic porphyrin in aqueous solutions.

Effect on enzyme activity

Impacts of Co^{3+} , CoTPPCL and TPP on the activity of plant-esterase were displayed on Fig. 3. The inhibition of TPP on the enzyme is much more serious than that of CoTPPCL. The concentrations of CoTPPCL and TPP

Table 1. Spectral respond of plant-esterase-metal-porphyrins

	Before the reaction		After the reaction		Shift (nm)	ΔA
	Sort band position (nm)	A	Sort band position (nm)	A_0		
TPP	446	0.250	444	0.355	blue shift 2	0.105
CoTPPCL	406	0.324	434	0.444	red shift 28	0.12
ZnTPP	444	0.519	442	0.525	blue shift 2	0.006
FeTPPCL	423	0.190	425	0.227	red shift 2	0.047
InTPP	431	0.304	431	0.394	0	0.09
EuTPPCL	429	0.198	429	0.208	0	0.01

are 1.5×10^{-6} M, relative activities are 48.9% and 43.8% respectively. While Co^{3+} at the same range of concentration acted as activators in increasing the activity of plant-esterase. Especially when the concentration of Co^{3+} reached 7.5×10^{-6} M, activation intensify is the most obvious. Thus, from the data of enzyme activity, the

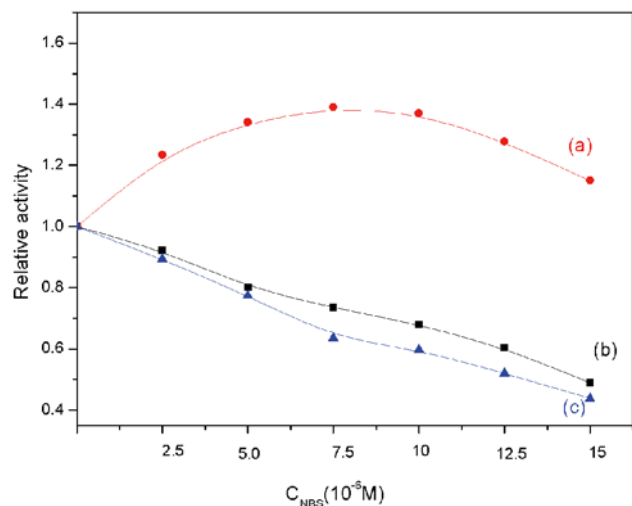


Figure 3. The influence of (a) Co^{3+} , (b) CoTPPCL, (c) TPP on the activity of plant-esterase

effect of CoTPPCL on plant-esterase seems to combine the effects of both TPP and Co^{3+} .

Then specific activities of different concentrations enzyme are shown in Fig 4. With concentrations of plant-esterase increased, the specific activity of each sample increased. No matter which concentration of plant-esterase, values of specific activities are nearly through the origin. Further, with a decrease of CoTPPCL concentrations, slopes of line were decreased. It illustrates that decreasing the activity of plant-esterase caused by increasing CoTPPCL is rooted from inhibition of enzyme

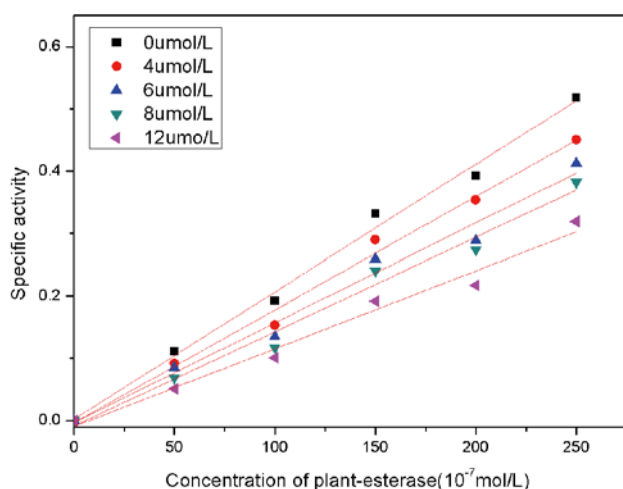


Figure 4. Effects of different enzyme concentrations on its activity

activity, rather than decreasing enzyme concentrations. Thus, CoTPPCL is a reversible inhibitor of plant-esterase.

At last, Lineweaver-Burk plot was used to exploit the type of reversible inhibition. From equations in Fig. 5, after the reaction with CoTPPCL, the value of K_m was increased and the value of V_{max} was decreased. It means

that CoTPPCL can combine with plant-esterase no matter whether the substrate is present or not. Thus, CoTPPCL is a mixed reversible inhibitor of plant-esterase. This conclusion is also used to illustrate the change of porphyrin spectrum. Porphyrins would lead the conformational

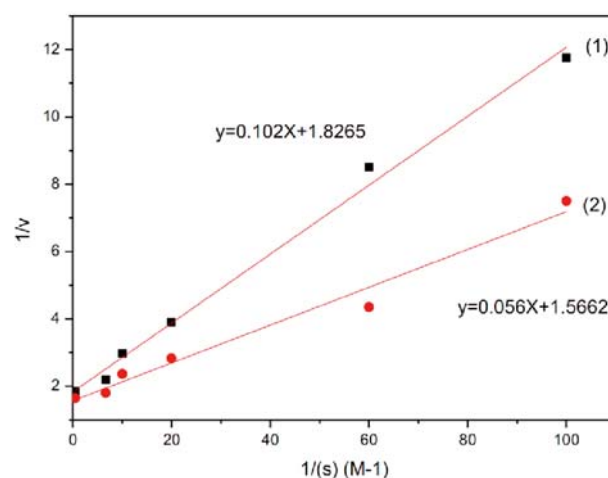


Figure 5. The Lineweaver-Burk plot of plant-esterase (1) the presence of CoTPPCL (2) the absence of CoTPPCL

changes of plant-esterase¹¹, while plant-esterase would have an impact on electronic distribution of porphyrin.

Interaction between plant-esterase and CoTPPCL

The spectrophotometric studies on increasing CoTPPCL concentration showed in Fig 6. With different concentrations of CoTPPCL, intensity of plant-esterase spectrum is different. The binding affinity constant K was calculated from the change of plant-esterase at 208 nm using the relation¹²:

$$A_0/\Delta A = A_0/\Delta A_{max} + A_0/\Delta A_{max} \cdot 1/K \cdot 1/L_t$$

Where $\Delta A = A_0 - A$, A_0 and A represent the absorption intensities of plant-esterase at 208 nm in the absence and presence of CoTPPCL concentration (L_t) $\cdot \Delta A_{max}$ is

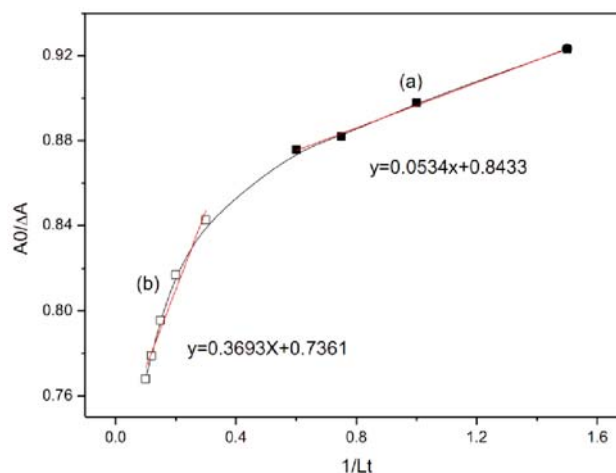


Figure 6. Estimation of the binding affinity constant in the interaction of Plant-esterase with CoTPPCL (a) The concentration of plant-esterase from 0.67 μM to 1.67 μM (b) The concentration of plant-esterase from 3.3 μM to 10 μM

the maximum change of absorption value. The slopes of plots provide affinity constant, which are summarized in Table 2.

The possible numbers of binding sites were also calculated from the plot of $1/(1-\theta)$ versus L_t/θ (Fig. 4) for CoTPPCL-plant-esterase interaction following the relation:

$$1/1 - \theta = KL_t/\theta - K \cdot P \cdot A_t$$

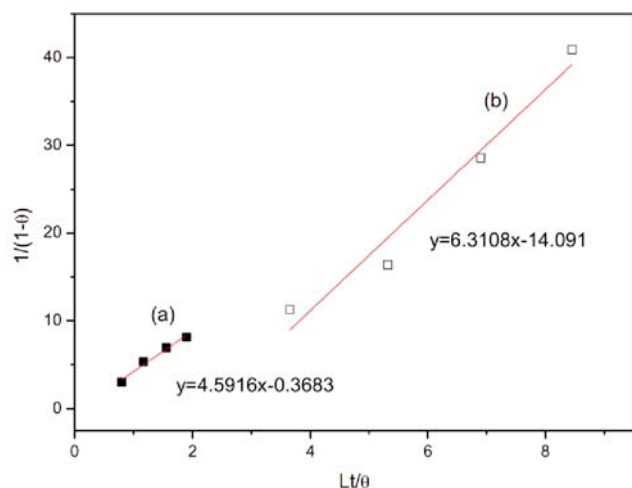


Figure 7. Estimation of the possible number of binding sites involved in the interaction of Plant-esterase with CoTPPCL (a) The concentration of plant-esterase from 0.67 μM to 1.67 μM (b) The concentration of plant-esterase from 3.3 μM to 10 μM

Table 2. Estimated values of binding affinity constant (K) and possible number of binding sites (P) for binding of plant-esterase with CoTPPCL

CoTPPCL concentration (μM)	Binding Affinity Constant $K(\times 10^5 \text{M}^{-1})$	Number of Binding Sites (P)
0.67–1.67	15.79	0.08
3.3–10	1.99	2.23

where $\theta = \Delta A / \Delta A_{\text{max}}$, p = plant-esterase concentration

The calculated number of binding sites was summarized in Table 2

Detection results from different complex system

The spectral changes of CoTPPCL-plant-esterase in the absence and presence of carbendazim ($5.23 \times 10^{-7} \text{M}$) was shown in Fig. 8. The absorption intensity in Soret band of CoTPPCL was increased after reaction with carbendazim. The original balance of CoTPPCL-plant-esterase was broken by adding pesticide. The absorption intensity in Q band of CoTPPCL was decreased after detection with carbendazim. The symmetry of porphyrin molecule was increased, suggesting that the number of free porphyrin molecules was increased in solution. When the concentration changes of CoTPPCL from $8.3 \times 10^{-8} \text{M}$ to $5.23 \times 10^{-7} \text{M}$, the absorbance value of CoTPPCL

Table 3. Detection effect and spectral response of plant esterases-porphyrin against carbendazim

Porphyrins	Detection limit (M)	Precision	Sensitivity	Peak	Difference spectrum			
					ΔA	Trough	$\Delta A'$	
CoTPPCL	5.23×10^{-10}		0.008	600	428 nm	0.028	447 nm	-0.055
ZnTPP	5.23×10^{-9}		0.003	190	428 nm	0.099	448 nm	-0.076
CuTPP	5.23×10^{-8}		0.002	41.47	411 nm	-0.018	438 nm	-0.2642
FeTPPCL	5.23×10^{-8}		0.005	137	-	-	417 nm	-0.0312
MnTPP	5.23×10^{-8}		0.003	7.80	473 nm	0.031	-	-
TPP	5.23×10^{-8}		0.007	24.21	-	-	442 nm	-0.057

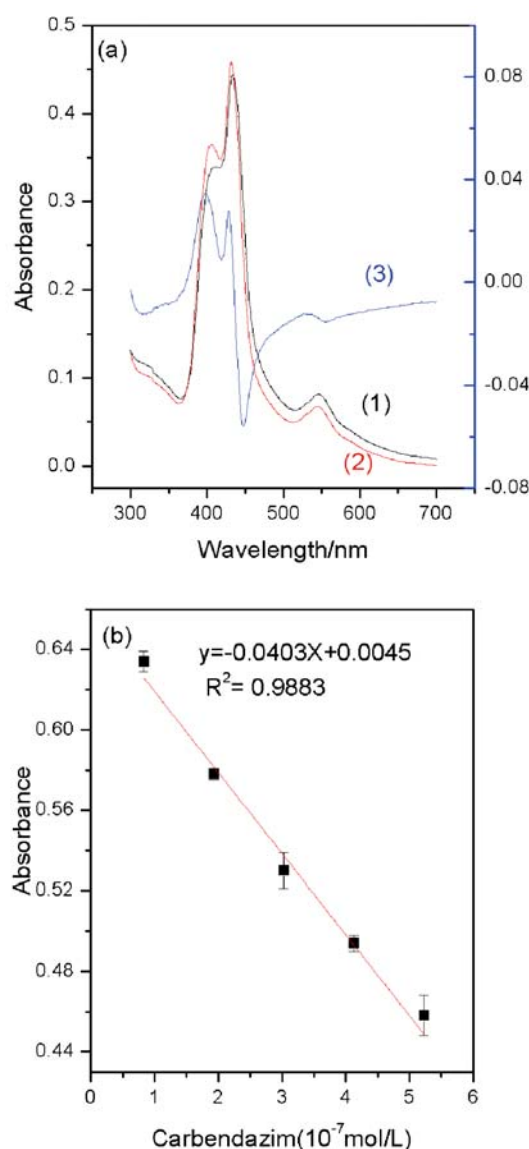


Figure 8. (a) Reaction between plant-esterase-CoTPPCL and $5.23 \times 10^{-7} \text{mol/L}$ carbendazim. (b) Dependence on carbendazim concentration ($8.3 \times 10^{-8} \text{mol/L}$ – $5.23 \times 10^{-7} \text{mol/L}$) of the 432 nm absorbance

in Soret band has linear relationship ($R^2 = 0.9883$) with the concentration of carbendazim.

The same tests did for changing different kinds of tetraphenyl metal porphyrin (ZnTPP, FeTPPCL, EuTPPCL, InTPP and TPP). The results were summarized in Table 3. Complex systems consisted of InTPP and EuTPP respectively are not very suited for the detection carbendazim. Because the absorbance value of both of them nearly did not change with the concentration change of carbendazim. Among them, CoTPPCL-plant-esterase complex shows the best sensitivity towards carbendazim. Combined with the conclusion from Fig. 1, the stronger

intensity of action between porphyrin and esterase, the better detection performances would be obtained.

CONCLUSION

We explored the interaction between tetraphenyl metal porphyrin and plant-esterase. Plant-esterase plays the role of stabilizing and enhancing the strength porphyrin spectrum in PBS. It provides a hydrophobic environment for porphyrin. At the same time, tetraphenyl metal porphyrin, such as cobalt tetraphenyl porphyrin is a mixed reversible inhibitor of plant-esterase. The binding site of plant-esterase with CoTPPCL is nearly equal to 2. Furthermore, the spectrum of complex solution displayed a promising potential for pesticides detection quantitatively.

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CONFLICT OF INTEREST STATEMENT

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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