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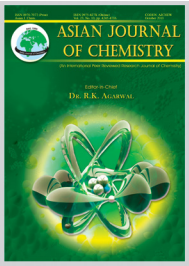

2,6-Dimethylbenzoquinone as a New Enzymatic Spectrophotometric Indicator for the Assessment of the Organophosphate Impact on Cholinesterase Biomarker of Duck

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Changes in biomarkers of a duck serum from stressed environments represent a reliable tool in evaluating toxious effect of cropland biogeocenose. A new and alternative enzymatic spectrophotometric indicator, 2,6-dimethylbenzoquinone (2,6-DMBQ) was explored to assess the organophosphate impact on cholinesterase (ChE) from duck serum. The enzymatic activity could be assessed by measuring the absorbance decrease at 257 nm wavelength and the decrease of enzymatic activity is related with the inhibiton of parathion methyl on cholinesterase.

Key Words: 2,6-Dimethylbenzoquinone, Organophosphate, Cholinesterase, Biomarker, Buck blood serum.

INTRODUCTION

Organophosphates (OPs) are extensively used in modern agricultural production that they contribute significantly to the contamination of the environment[1]. Biomarkers are biochemical, molecular, cellular or physiological changes related to exposure or effects of environmental impact. During the recent years, biomarkers have become an increasingly important tool in assessing the contaminant exposure on resident biota[2-4]. The cholinesterase (ChE) is considered as a suitable biomarker for detecting exposure to and effects of organophosphate poisoning[5]. Due to organophosphates irreversibly inhibition cholinesterase, exposure can also be assessed by measuring the decrease in cholinesterase activity. There are different methods for determination cholinesterase[6,7], however, the most frequent is the method based on the hydrolysis of thiocholine esters and following detection of free SH-group of the released thiocholine. The principle of this method is measurement of the rate of production of thiocholine as the substrate acetylthiocholine (ATCh) is hydrolyzed by the cholinesterase present in the sample. Thiocholine reacts with the chromophore 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) to produce a yellow 5-thio-2-nitrobenzoic acid and the rate of colour production is measured at 412 nm wavelength by a spectrophotometer.

To evaluate the pollution of cropland biogeocenose, the blood serum cholinesterase of a duck was used as biomarker of early effects here. A new and alternative enzymatic spectrophotometric indicator, 2,6-dimethylbenzoquinone (2,6-DMBQ) was explored to assess the organophosphates impact on cholinesterase from duck serum.

EXPERIMENTAL

Acetylthiocholine (ATCh) chloride was obtained from Sigma (St. Louis, USA), the parathion methyl from Dr Ehrenstorfer GmbH (Augsburg, Germany) and 2,6-dimethylbenzoquinone from aladdin-reagent (shanghai, China). All other chemicals from commercial sources were of analytical grade. Ultra-pure water was prepared from Milli-Q. The parathion methyl was dissolved in acetone to obtain the desired concentration. Phosphate buffer solution (PBS, pH 7.5) contains 50 mM Na_2HPO_4 and 50 mM KH_2PO_4. The duck serum with cholinesterase was separated and collected after the whole blood was allowed to clot for 0.5 h at 37 ºC.

For spectrophotometric measurements, a Nicolet Evolution 500 UV/VIS spectrometer (Thermo Spectronic, USA) was used.

Spectrophotometric study of cholinesterase activity and its inhibition by parathion methyl: cholinesterase activities were assayed spectrophotometrically according to the dapted Ellman's method[8]. This method is based on the coupled enzyme reaction of acetylthiocholine as the specific substrate for cholinesterase and 2,6-dimethylbenzoquinone as indicator for the enzyme reaction. Hereby acetylthiocholine is hydrolyzed by cholinesterase, producing thiocholine and acetic acid. Subsequently the reaction of thiocholine with the 2,6-dimethylbenzoquinone leads to the decrease of absorbance at 257 nm wavelength.

For cholinesterase activity measurement, 700 µL of 50 mM PBS (pH 7.5), 50 µL duck serum and 150 µL of 1.2 mM acetylthiocholine were mixed in a cuvette at water pot at 37 ºC for 0.5 h. Then the cuvette was taken into 0 ºC ice water to stop enzymatic reaction. Finally, 100 µL of 2.5 mM 2,6-dimethylbenzoquinone was added, mixed enough to allow a 3 min reaction. Sample absorbance A_1 was measured immediately at 257 nm wavelength. A blank absorbance A_0 was read at 257 nm with the absence of only acetylthiocholine. The cholinesterase activity was calculated according to the decrease of absorbance at 257 nm wavelength and was expressed: $\Delta A_0 = A_0 - A_1$.

The inhibition of parathion methyl was determined by incubating cholinesterase and parathion methyl firstly before enzyme reaction. The parathion methyl solution in acetone was dried at 37 ºC in centrifuge tubes where the duck serum was added to be inhibited for a desired duration. The degree of inhibition was calculated as the relative decrease of ΔA using the formula:

Inhibition % = $[(\Delta A_0-\Delta A_i) /\Delta A_0] \times 100 = (1-\Delta A_i /\Delta A_0) \times 100$

where, ΔA_0 is the cholinesterase activity without parathion methyl inhibiton and ΔA_i is the one with parathion methyl inhibition.

RESULTS AND DISCUSSION

The stability of 2,6-dimethylbenzoquinone was evaluated firstly. Three samples of new prepared 0.12 mM 2,6-dimethylbenzoquinone were kept at the room temperature away from light (Fig. 1a), 4 ºC (Fig. 1b) and -20 ºC (not shown) in the refrigerator, respectively. It was found that 2,6-dimethylbenzoquinone was relatively stable by observing the absorbance decrease of 2,6-dimethylbenzoquinone at 257 nm during the period of 15 h. As shown in Fig. 1, the absorbance of sample preserved at the room temperature away from light decreases only 2.6 % after 15 h and that at 4 ºC in the refrigerator does only 2.18 %. No obvious absorbance decrease was observed for the sample frozen -20 ºC (not shown) in the refrigerator. Therefore, the prepared 2,6-dimethylbenzoquinone solution is stable enough to be used for assay application.

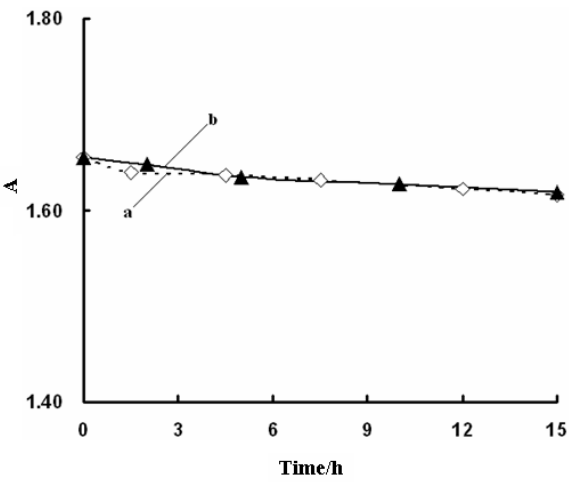
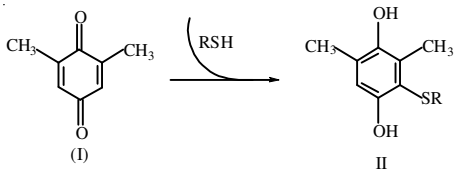


Fig. 1. Absorbance shift of 2,6-DMBQ at 257 nm preserved at the room temperature away from light (a) and 4 °C (b) in the refrigerator, respectively

Fig. 2 shows the spectral absorbance curves of single or coexistent components of enzyme catalytical system. Obviously, acetylthiocholine has a strong absorbance at about 230 nm (g), duck serum at about 280 nm (f) and 2,6-dimethylbenzoquinone at 257 nm (b). The peak value of Fig. 2a at 257 nm is higher than that of Fig. 2b due to the coexistence of 2,6-dimethylbenzoquinone and duck serum. After 0.5 h catalytical reaction of acetylthiocholine by cholinesterase followed by immediately adding 2,6-dimethylbenzoquinone into the reacted solution, the peak at 257 nm decreases. It means that a reaction between 2,6-dimethylbenzoquinone and produced thiocholine happens. Nucleophilic addition of the thiol species to the quinoid structure (I) leads to the production of a chemically reduced adduct (II), which is similar with the reports from other groups[9-11]. The possible reaction mechanism is as following:



(I) II

2,6-Dimethylbenzoquinone

According to the study of spectral absorbance, the peak value at 257 nm keeps stable within the 3 min after 2,6-dimethylbenzoquinone was added to react with the produced thiocholine. After 2,6-dimethylbenzoquinone is allowed to react with thiocholine for 15 min, a new peak at 265 nm is observed due to the possible side reaction (not shown), which is similar with the previous report from the other groups[12, 13]. According to their observation, there are possible intermediates and the reaction between thiol and quinone does not follow the simple process. However, the absorbance keeps nearly unchanged within the first 3 min after 2,6-dimethylbenzoquinone added. It means that the reacted products keep stable during a short term and it is enough to read UV data immediately.

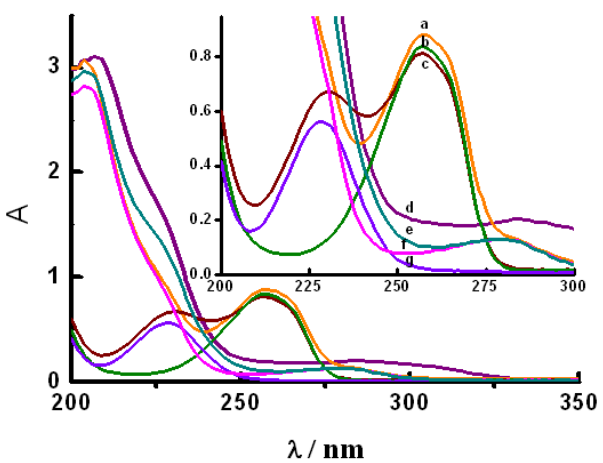


Fig. 2. Spectral absorbance curves of single or coexistent components of enzyme catalytical system. a) duck serum and 2,6-DMBQ; b) 2,6-DMBQ; c) ATCh and 2,6-DMBQ; d) duck serum and ATCh to react 0.5 h at 37 ºC and then 2,6-DMBQ added; e) duck serum and ATCh; f) duck serum; g) ATCh. Duck serum: 50 µL; 2,6-DMBQ: 100 µL 2.5 mM; ATCh: 150 µL 1.2 mM; PBS (pH 7.5): 700 µL 50 mM; Pure water added to obtain the whole volume of 4 mL

Fig. 3 shows the calibration plots of absorbance *versus* acetylthiocholine concentration with the enzymatic reaction time 0.5 h. With the increasing concentration of acetylthiocholine the absorbance ΔA increases linearly in the range of 0.05-0.2 mM and then reaches a plateau value, which means a typical Michaelis-Menten process. As shown in the Lineweaver -Burk plot (inset in Fig. 3), the apparent Michaelis-Menten constant (K_m) value was given to be 0.074 mM.

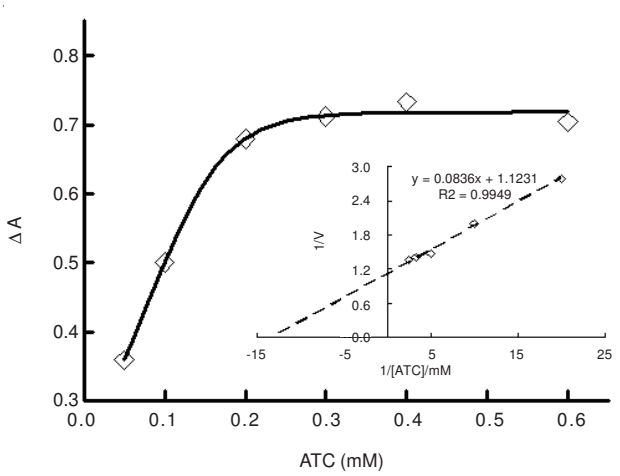


Fig. 3. Calibration plot of absorbance *versus* ATCh concentration with the enzymatic reaction time 0.5 h. Inset: Lineweaver-Burk plot representing reciprocals of the initial enzyme velocity *vs*. ATCh concentration

The effect of pH on cholinesterase activity was evaluated from 4.5 to 8.0 (Fig. 4). A known amount of duck serum was added into a centrifugal cannulation, which contained an aliquot of 150 μL of substrate at different pHs (0.05 M, phosphate buffer solution). 100 μL 2.5 mM 2,6-dimethylbenzoquinone was finally added. The highest enzymatic activity is observed at pH 7.5 and a rapid decrease at pH 6 or 8. In the context, phosphate buffer at pH 7.5 was chosen for further experiments.

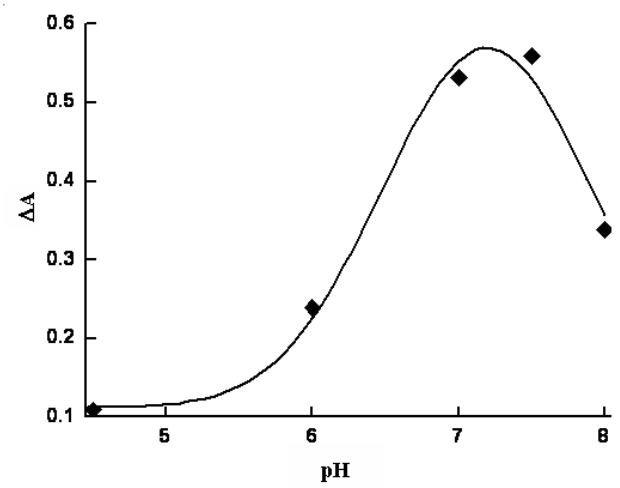


Fig. 4. Effect of different pHs of phosphate buffer solution on cholinesterase activity

The parathion methyl was employed to study the organophosphates inhibition on cholinesterase from duck serum activity and a study of the incubation time with the inhibitor was performed between 0 and 20 min. It is usually accepted that for the case of irreversible inhibition, a low detection limit could be obtained by increasing the inhibitor incubation time, but for reversible inhibition, a longer incubation time does not lead to an increase in the degree of inhibition. Nearly 80 % inhibition rate is observed for 5 min incubation time and the highest inhibition rate for 10 min (not shown). The inhibition rate does not increase with time after 10 min. Here the mechanism of parathion methyl inhibition was proved to be irreversible on cholinesterase by the dependence of the degree of inhibition on the incubation time.

Following an incubation time of 10 min, the inhibition curve of cholinesterase to different concentration of parathion methyl is given in Fig. 5. The obtained plot is sigmoid as expected. The inhibition of cholinesterase activity is increasing with the concentration of parathion methyl ranging from 0.01 to 100 μg/mL. 1 μg/mL parathion methyl shows an about 28 % cholinesterase inhibition and 10 μg/mL does a 54 % inhibition, while 100 μg/mL parathion methyl nearly does 95 % inhibition. Observed from Fig. 5, over the concentration of 0.1 μg/mL, parathion methyl is able to be detected obviously and a tremendous break appears in the percentage of inhibition range from 0.1 μg/mL to 100 μg/mL, especially the percentage of inhibition almost reaches to 95 % when the concentration is at 100 μg/mL. Above the concentration, percentage of the inhibition begins to achieve saturation.

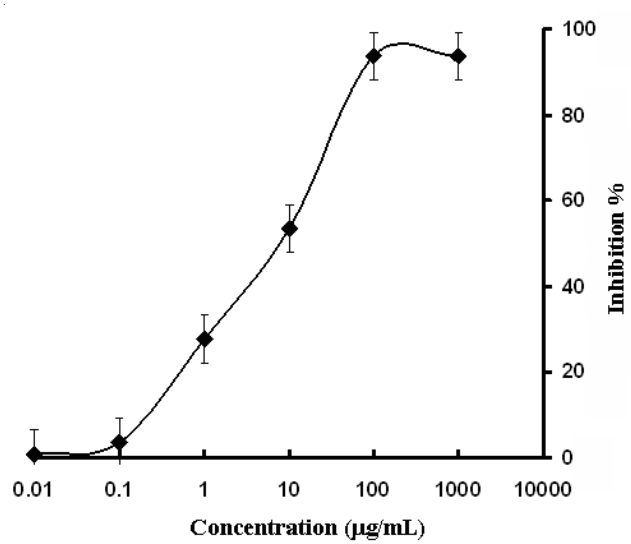


Fig. 5. Inhibition curve of the cholinesterase to different concentration of parathion methyl (n = 3)

Conclusion

It is reported that 2,6-dimethylbenzoquinone as an alternative enzymatic spectrophotometric indicator was used to evaluate the organophosphate impact on cholinesterase biomarker of duck serum.

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