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Assisted inhibition effect of acetylcholinesterase with *n*-octylphosphonic acid and application in high sensitive detection of organophosphorous pesticides by matrix-assisted laser desorption/ionization Fourier transform mass spectrometry

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ABSTRACT

A simple and practical approach to improve the sensitivity of acetylcholinesterase (AChE)-inhibited method has been developed for monitoring organophosphorous (OP) pesticide residues. In this work, matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) was used to detect AChE activity. Due to its good salt-tolerance and low sample consumption, MALDI-FTMS facilitates rapid and high-throughput screening of OP pesticides. Here we describe a new method to obtain low detection limits via employing external reagents. Among candidate compounds, *n*-octylphosphonic acid (*n*-Octyl-PA) displays assistant effect to enhance AChE inhibition by OP pesticides. In presence of *n*-Octyl-PA, the percentages of AChE inhibition still kept correlation with OP pesticide concentrations. The detection limits were improved significantly even by 10^2–10^3 folds in comparison with conventional enzyme-inhibited methods. Different detection limits of OP pesticides with different toxicities were as low as 0.005 $\mu g\,L^{-1}$ for high toxic pesticides and 0.05 $\mu g\,L^{-1}$ for low toxic pesticides. Besides, the reliability of results from this method to analyze cowpea samples had been demonstrated by liquid-chromatography tandem mass spectrometry (LC–MS/MS). The application of this commercial available assistant agent shows great promise to detect OP compounds in complicated biological matrix and broadens the mind for high sensitivity detection of OP pesticide residues in agricultural products.

1. Introduction

The family of pesticides with diverse groups has been rapidly expanding throughout recent decades. Meanwhile, the extensive use of these toxic compounds has also brought about serious problems: the risks from pesticide residues continue to be a great threat to human health [1]. For instance, the emergence of the toxic cowpeas in Hainan province of China in 2009 had caused widespread concern. It was reported that cowpeas grown in Hainan province were contaminated by poisonous pesticides. In this scandal, the arch-criminal was a class of organophosphorous (OP) compounds. These OP pesticides could inhibit the activity of acetylcholinesterase (AChE) and engendered the continuous growth of acetylcholine (ACh) until killing the organisms [2–4]. To prevent such incidents from recurring, fast, sensitive and high efficient screening techniques are still highly acquired for responding the OP pesticides.

Up to the present time, many methods have been applied to the separation and identification of pesticides, including thin-layer chromatography, capillary electrophoresis, spectrophotometric assay, etc. [5–8]. With the development of ionization modes and detector techniques, mass spectrometry has been playing an essential role in pesticides analysis [9,10], especially coupled with gas chromatography (GC) or high performance liquid chromatography (HPLC) [11–14].

Other than screening of OP compounds, several approaches involving the current research about enzyme have also been reported, such as the measurement of pesticide metabolites [15,16] and the monitoring of phosphorylated adducts [17]. Although these methods are accurate and sensitive, they still have weakness in rapid detection. Besides, more attention has been focused on the measurement of enzyme activity. AChE inhibition by OP pesticides takes place via a chemical reaction in which the serine hydroxyl (OH) in the enzyme active site is phosphorylated [18–20]. The irreversible phosphorylated AChE is no longer able to catalyze the hydrolysis of substrate. Therefore, the level of AChE inhibition can be used to indicate the amounts of OPs. In order to measure the enzyme activity, numerous methods have been developed, including microfluorometric determination [21], optical oxygen sensing [22], eukaryotic expression [23], and enzyme biosensor assays [24,25]. Generally, through comparing the different conversion rates of unknown sample and blank sample,

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whether OP pesticide has caused serious inhibiting action can be judged.

In recent years, mass spectrometry has also attracted great attention in studying enzyme activities and kinetics as well as screening enzyme inhibitor [26–30]. With various ionization techniques to monitor the contents of substrate and product, mass spectrometry offers great facilities for studying enzyme system [31–34]. Desorption/ionization mass spectrometry-based enzymatic assays have also been reported widely [35–40]. Our laboratory had previously applied matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) to monitor enzyme reaction and screen enzyme inhibitor [41,42]. MALDI-FTMS has obvious advantages to analyze enzyme inhibitor in real samples, attributed to its high speed, salt tolerance and low sample consumption. These advantages allow us to reveal the levels of OP pesticide residues via detection of AChE activity.

Although MALDI-FTMS has the capacity of rapid and high-throughput analysis of OP pesticides, the great challenge still lies in reducing the detection limits. Previously, Marty et al. have reported that the mutants of dmAChE would vary sensitivity of AChE to methamidophos [43]. However, there are difficulties in obtaining appropriate mutants of AChE at present. With the aim of increasing the sensitivity of detecting OP pesticides in a simple and available way, we studied many chemical compounds (such as glucose, polyethyleneglycol-6000) about their effect on AChE inhibition. Finally, our research concentrated on a class of *n*-alkylphosphonic acids (*n*-Alkyl-PA) and considered them as the potential assistant candidates to enhance the inhibition effect of OPs. The change of AChE activity and inhibitions in presence of various *n*-Alkyl-PA compounds had been investigated. Among these *n*-Alkyl-PA reagents, *n*-octylphosphonic acid (*n*-Octyl-PA) increased AChE inhibitions, which was thought to be assistant effect on the interaction between OPs and AChE in enzyme-inhibited procedure. With employment of *n*-Octyl-PA, the high enzyme inhibitions were still positively correlated with pesticides concentrations and the detection sensitivity had been improved significantly.

Moreover, this approach was applied to analyze the extracted solutions from three kinds of cowpea samples. It turned out that two ordinary cowpeas showed high inhibition to AChE, whereas organic cowpea caused no significant inhibition. These results are in accord with those of standard LC–MS/MS method [44], and confirmed the reliability of the assistance approach with *n*-Octyl-PA. This method is anticipated to open up perspectives for rapid and sensitive screening of OP pesticides, especially for assessing and predicting the risks from unknown OP compounds in agriculture products.

2. Experimental

2.1. Reagents and materials

Acetylcholinesterase from electrophorus electricus (Type VI-S, EC 3.1.1.7, 426 U mg^{-1}), acetylthiocholine iodide, 2,5-dihydroxybenzoic acid (DHB) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were purchased from Sigma Chemical Co. (St Louis, MO). All the organophosphorus pesticides were from Shanghai Institute for Food and Drug Control (Shanghai, China). The *n*-alkylphosphonic acids (*n*-hexylphosphonic acid (*n*-Hexyl-PA), *n*-octylphosphonic acid (*n*-Octyl-PA), *n*-decylphosphonic acid (*n*-Decyl-PA), *n*-dodecylphosphonic acid (*n*-Dodecyl-PA), *n*-hexadecylphosphonic acid (*n*-Hexadecyl-PA)) were from Strem Chemicals, Inc. (Newburyport, MA 01950, USA). The ultrapure water used in this paper was purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Methanol and acetonitrile were of HPLC-grade quality purchased from Merk (Darmstadt, Germany). Polyethylene glycol-200 (PEG-200), PEG-400, hydrochloric acid (HCl), trifluoroacetic acid (CF_3COOH), glycerol were from Shanghai Chemical Reagent Corporation (Shanghai, China).

2.2. Materials preparation

AChE solutions were prepared and diluted by 50 mM Tris–HCl buffer (pH 8.0) to the final concentration 5 U mL^{-1}, and were preserved at −20 °C in dark. The substrate acetylthiocholine (ATCh) solutions (5 mM) were prepared with Tris–HCl buffer freshly. All of the OP pesticides and *n*-Alkyl-PA solutions were made by fresh ultrapure water and stored at −20 °C. The *n*-Alkyl-PA reagents (0.2 mM) were neutralized with NaOH solution to pH 7.0–8.0. The matrix solution was prepared daily as follows: DHB solid (50 mg) was dissolved with methanol (500 μL), adding 1% CF_3COOH; and then, the DHB solution was mixed with a glycerol/water solution (60/40, vol/vol).

2.3. Cowpea samples extraction

The two ordinary cowpea staffs were collected from vegetable base of production in Shanghai city. The organic cowpea was from Shanghai Organic Agriculture Co., Ltd. All of the cowpeas were extracted in reference to standard method [44]. 1.0 g sample was weighed in a 10 mL glass centrifuge tube. 5 mL acetone, 0.1 g NaCl, and 0.4 g $MgCl_2$ were added, and the samples were agitated and extracted with a blender for 1 min. The extracted solutions (1 mL) were centrifuged for 5 min at 10 000 rpm. The supernatants were divided into two equivalent sets with one-half being analyzed by LC–MS/MS and the other as potential inhibitor. The supernatant (200 μL) was dried via nitrogen blow-down techniques and then were dissolved in equal volume of water. The final aqueous solutions were used in enzyme reaction as inhibitor.

2.4. Measurement of AChE and reaction conditions

All of the reactions were carried out in eppendorf plastic tubes. AChE solution (10 μL), along with 10 μL of *n*-Alkyl-PA or water (control group), incubated for 30 min at 37 °C temperature controlled oven. Then 10 μL of inhibitors (standard pesticides solutions or cowpea aqueous solutions) were added and incubated for 30 min at 37 °C with 10 μL of ultrapure water as control group. In the end, the substrate (ATCh, 5 mM, 10 μL) participated in the reaction for 20 min at 37 °C until the acetonitrile (30 μL) was added to the mixture to quench the reaction.

2.5. MALDI-FTMS conditions and analysis

Experiments were conducted using an Ionspec 4.7 T HisRes MALDI-FTMS (Ionspec, Irvine, CA, USA). The external Ionspec MALDI ion source used an air-cooled Nd:YAG laser (355 nm, New Wave Research, Fremont, CA) with a gradient filter for adjusting the UV-laser power. Ions, generated from a MALDI source, were transferred via a quadruple ion guide to the capacitive coupled closed cylindrical cell. The intensity of MALDI-laser irradiation was varied between 25% and 35% as needed. The laser irradiation pulse time was set at 50 ms. For low-mass region, the quadruple guide had an applied voltage of 30 V (base to peak) at a frequency of 725 kHz. The mass spectrometry was calibrated with PEG-200 or PEG-400 for each test. The acquisition mass-to-charge (*m/z*) range was 80–250.

The matrix solution (60 μL) was added to each final reaction system and mixed completely. Then, the mixture (2 μL) was deposited on the stainless steel target. In vacuum, these wet sample spots were getting dried slowly to produce a microcrystalline layer which