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Rapid and sensitive visual detection of residual pesticides in food using acetylcholinesterase-based disposable membrane chips

Naoki Nagatani [a], Akihiro Takeuchi [b], M. Anwar Hossain [c], Teruko Yuhi [b,d], Tatsuro Endo [e], Kagan Kerman [b], Yuzuru Takamura [b], Eiichi Tamiya [b,*]

[a] Department of Biotechnology and Applied Chemistry, Faculty of Engineering, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan
[b] School of Material Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi City, Ishikawa 923-1292, Japan
[c] Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka 1000, Bangladesh
[d] Japan Science and Technology Agency (JST), Innovation Plaza Ishikawa, 2-13 Asahidai, Nomi City, Ishikawa 923-1211, Japan
[e] Department of Mechano-Micro Engineering, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8502, Japan

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Abstract

The neurotoxic property of organophosphate pesticides due to the inhibition of acetylcholinesterase (AChE) was utilized to develop disposable detection chips for easy and fast detection of the pesticides. An enzymatic assay involving acetylcholinesterase (AChE) was developed using a stable substrate specific to the enzymatic reaction product, thiocholine. The generated thiocholine reacted with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) to produce 5′-mercapto-2′-nitrobenzoic acid, which was measured at 410 nm. Optimum pH, buffer types and concentrations, substrate concentrations and optimum conditions of the color reaction were investigated. The substrate specificity, test interferences were evaluated. Our simple detection system does not require any expensive instruments. Diazinon-oxon (DZN-oxon) could be detected by a visual color change from white to yellow on the surface of the chip. The total amount of time required for the detection of residual DZN-oxon in apple and orange juices was about an hour. This method provides excellent specificity, reproducibility, a wide measurement range and minimal interference from endogenous substances in juice matrices. Since the reagents are stable after preparation, our method would be useful for routine pesticide screening by non-professional end-users.

Keywords: Residual pesticide; Diazinon-oxon (DZN-oxon); Acetylcholinesterase (AChE); 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB); Membrane chip

1. Introduction

Widespread global use of chemical pesticides for crop protection and other household uses have posed a grave threat to human health and environment. The problem is steadily growing and becoming more serious, especially, in the developing countries.

The standard reference methods for the determination of the pesticide residues in real samples are based on gas chromatography (GC) or high performance liquid chromatography (HPLC) (Molina, Honing, & Barcelo, 1994; Sherma & Zweig, 1983). However, these standard methods require a long assay time, complicated operations and a high cost. Day-to-day exposure to pesticides in fresh fruits, vegetables, cereals, drinking water, meat and dairy products and common beverages can cause serious health problems. Pesticides based on organophosphates and carbamates in particular are harmful for human health. In this report, we have used diazinon-oxon (DZN-oxon) as the model pesticide in our device. DZN-oxon, a colorless liquid, is a non-systemic organophosphate insecticide. DZN-oxon has a low persistence in soil with a half-life of two to four weeks.

* Corresponding author. Tel.: +81 761 51 1660: fax: +81 761 51 1665.
E-mail address: tamiya@jaist.ac.jp (E. Tamiya).

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DZN-oxon and many other pesticides inhibit the activity of acetylcholinesterase (AChE), which acts as a key enzyme in the central nervous system (D'Mello, 1993). The inhibitory action of these pesticides is based on their ability to irreversibly modify the catalytic serine residue in AChEs (Massoulie, Pezzementi, Bon, Krejci, & Vallette, 1993). The toxicological effects of DZN are primarily mediated through the effects of its toxic metabolite, DZN-oxon on AChE, which results in accumulation of acetylcholine at neuronal junctions (Poet, Kousba, Dennison, & Timchalk, 2004).

Qualitative and quantitative detection of pesticides based on AChE inhibition assays have been reviewed by several groups (Evtugyn, Budnikov, & Nikolskaya, 1998; Mulchandani, Chen, Mulchandani, Wang, & Rogers, 2001; Velasco-Garcia & Mottram, 2003). These sensors were coupled to a variety of spectrophotometric, (Leon-Gonzales & Townshend, 1990), fluorimetric (Guilbault & Kramer, 1965), piezoelectric (Halámek, Makower, Knösche, Skládal, & Scheller, 2005; Makower, Halamek, Skladal, Kernchen, & Scheller, 2003), potentiometric (Liu et al., 2005; Snejdarkova et al., 2004) and amperometric devices (Nunes, Jeanty, & Marty, 2004).

A novel enzymatic in vitro activation method for phosphorothionates was developed to allow their detection with AChE biosensors (Schulze, Schmid, & Bachmann, 2004). The application of their method to infant food in combination with a disposable electrochemical AChE biosensor enabled the detection of chlorpyrifos and parathion at concentrations down to 20 μg/kg within an overall assay time of 95 min. The sensitivity of electrochemical AChE biosensors for insecticide detection could be increased substantially by engineering AChE B of *Nippostrongylus brasiliensis* (Schulze, Muench, Villatte, Schmid, & Bachmann, 2005). Electrochemical disposable biosensors were used for the detection of organophosphate and carbamate insecticides in foods of animal origin with increased fat contents; such as milk (Zhang et al., 2005). A screen-printed bienzymatic sensor has recently been developed by immobilizing *N. brasiliensis* AChE and cytochrome P450 BM-3 (CYP102-A1) mutant in sol–gel (Waibel, Schulze, Huber, & Bachmann, 2006). The co-immobilization of two enzymes, AChE and P450 BM-3, on one sensor enabled the sensitive detection of parathion with a detection limit of 10 μg/L, which is the pesticide threshold in infant food set up by European Community regulations and about 100 times more sensitive than commonly described for AChE sensors. In this report, we have obtained a detection limit of 0.1 ppm for DZN-oxon using our membrane chips, which would represent a strong alternative to electrochemical devices in terms of simplicity and rapid response that could also be observed by the naked eye.

Recently, an antibody-based gold immunochromatographic assay for easy detection of carbofuran has been reported (Zhou et al., 2004). However, the antibody-based immunosensors are able to detect pesticides of only a particular type. However, many different organophosphate and carbofuran pesticides are currently being used, and the number of their derivatives is increasing every year. Real samples, therefore, may contain many different pesticides belonging to either of the above classes. From the viewpoint of food safety; a rapid and low-cost screening tool to detect the lethal level of the pesticides in food items would have significant practical implications.

Here, we report, the development of a quick and sensitive biosensor for the detection of neurotoxic pesticides in real samples based on the inhibition of AChE. The pesticide residue detection chip does not require any electronic analyzer, and the residual pesticides can be detected even by the naked eye because of the significant color change on the membrane of the chip. Our detection chip is a promising candidate as a preliminary on-field screening device for detecting neurotoxic pesticide residues in real samples.

2. Materials and methods

2.1. Material

Acetylcholinesterase (AChE) from bovine serum and Diazinon oxon (DZN-oxon, (*O*,*O*-diethyl 0-2-isopropyl-6-methyl (pyrimidine-4-yl) phosphorothioate)) standard were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetylthiocholine chloride (ATCh) was purchased from Sigma–Aldrich (Tokyo, Japan). 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) was purchased from Dojindo Laboratories (Kumamoto, Japan). Experiments with real samples were carried out by using apple juice and orange juice, which were obtained from a local supermarket in Japan.

2.2. Apparatus

Qualitative filter paper no. 1 from Advantec Toyo Kaisha, Ltd. (Tokyo, Japan) was used to make the membrane chips in the plastic detection discs supplied by the Shinko Chemical Co., Ltd. (Ishikawa, Japan). The color change of the pesticide residue detection chip was measured by a hand-held photometer (Hokkei Industries, Ishikawa, Japan; BHS-S01) (Kawamura et al., 2005). Micro-plate reader (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) was used for the measurement of the absorbance on a 96-well micro-titer plate.

2.3. Optimization of reaction conditions for AChE inhibition

An organophosphate pesticide, DZN-oxon was used to optimize the AChE activity inhibition. AChE activity was monitored following the coupled reaction of thiocholine (ThC) and DTNB (Fig 1). Each 40 μL of AChE (0.9 U/ml) and 40 μL of DZN-oxon (0.05 ppb–25 ppm) were mixed, and incubated for 2 min to inhibit the enzymatic activity. The mixed solution was then added to 40 μL of ATCh (0.43 μmol/ml), 8 μL of DTNB (10 mmol/ml) and 200 μL of PBS (50 mM, pH 7.4) and incubated for 10 min at room