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Analytical Methods

Development of a sensitive monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay for analysing chlorantraniliprole residues

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ABSTRACT

Chlorantraniliprole (CAP) is a new anthranilic diamide insecticide acting with high efficacy on ryanodine receptors of most of the species within the *Lepidoptera* order and has been used worldwide. To monitor its residue in food and environmental samples, we developed an indirect competitive enzyme-linked immunosorbent assay (icELISA), based on monoclonal antibodies. The established icELISA possesses a concentration of producing 50% inhibition (IC_{50}) of 1.60 ng/mL. In addition, the assay had the average recoveries of 79–119% when CAP was fortified in tap water, soil, spinach (*Spinacia oleracea*), and rape (*Brassica campestris*). Moreover, the icELISA results of spinach samples were confirmed by high performance liquid chromatography–mass spectrometry. Overall, we demonstrate that the developed immunoassay could be used as the quick and convenient determination method of CAP residues in environmental and agricultural samples.

1. Introduction

Chlorantraniliprole (CAP), 3-bromo-N-[4-chloro-2-methyl-6-[(methylamino)carbonyl] phenyl]-1-(3-chloro-2-pyridinyl)-1H-pyrazole-5-carboxamide, is a new environmentally friendly insecticide with high efficacy and low toxicity. It was developed and launched commercially by DuPont Crop Protection in 2007. CAP exerts exceptionally insecticidal activity against important agricultural pests, such as lepidopteran insects as well as *Coleoptera*, *Diptera*, *Isoptera*, and *Hemiptera* pests, by activating insect ryanodine receptors (RyRs). RyRs are ion channels that play a critical role in muscle function by modulating the release of calcium, which is a significant secondary messenger in a cell. CAP causes uncontrolled release and depletion of internal calcium, thereby preventing further muscle contraction. Insects treated with CAP exhibit rapid cessation of feeding, lethargy, regurgitation, and muscle paralysis, all of which ultimately lead to death (Cordova et al., 2006; Coronado, Morrissette, Sukhareva, & Vaughan, 1994; Lahm, Cordova, & Barry, 2009). Because of its excellent performance, CAP has been applied as an agricultural insecticide to control a variety of insect pests in some agricultural crops and turf. Meanwhile, the levels of CAP residues in food or environment are strictly controlled by regulatory authorities. For example, a limit value of 0.02 μg/g was set for CAP in rice by the U.S. Environmental Protection Agency (EPA, 2011, 2012), whereas the Health Canada Pest Management Regulatory Agency prescribed 0.05 μg/g as the maximum residue limit of CAP in meat for human consumption. Therefore, to manage the efficient application of CAP, as well as monitor its misuse and control its residues in food and environment, it is significant to develop a quick and convenient determination method.

The CAP levels in environmental and food samples are often analysed with high performance liquid chromatography (HPLC) methods (Dong et al., 2011; Kar, Mandal, & Singh, 2012; Zhang et al., 2011). Specifically, the amount of CAP residues in fruits and vegetables could be determined by HPLC-tandem mass spectrometric ion-switching, with a limit of detection and limit of quantification of 0.8 μg/kg and 1.6 μg/kg, respectively (Caboni et al., 2008). However, the reported methods require expensive instruments and time-consuming sample treatments.

Immunoassays such as enzyme-linked immunosorbent assays (ELISA) is a biochemical test based on specific action between antigen and antibody, and have been developed as a kind of rapid and

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cost effective techniques because they do not require complicated cleanup procedure and expensive instruments, and are able to monitor plenty of samples simultaneously. These features convert ELISAs into very powerful tools for pesticide residue analysis (Moreno, Abad, & Montoya, 2001). No rapid and ease assay for monitoring CAP like enzyme-linked immunosorbent assay (ELISA) was reported up to date.

The objective of this present study was to develop a sensitive indirect competitive enzyme-linked immunosorbent assay (icELISA), which was based on monoclonal antibodies (mAbs), to quickly analyse and monitor CAP residues in environmental and agricultural samples.

2. Materials and methods

2.1. Materials

2-(3-Bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-8-methyl-4H-benzo[d][1,3]oxazin-4-one, CAP, cyantraniliprole (Cyazypyr), and flubendiamide (FLU) were synthesised according to the published methods and characterisation by ^{1}H NMR (Chai, He, Wang, Li, & Liu, 2010; Chai, Peng, Li, Zhang, & Liu, 2009; Lahm et al., 2007; Taylor, 2009; Tsubata, Tohnishi, Kodama, & Seo, 2007; Wang, Zhang, & Wu, 2010). FLU structural analogues were synthesised as described in our previous study (Liu et al., 2010). Reagents purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO) included bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvant, hypoxanthine, aminopterin and thymidine (HAT), horseradish peroxidase-conjugated goat anti-mouse IgG, *N*-hydroxysuccinimide, *N*,*N*′-dicyclohexylcarbodiimide, *o*-phenylenediamine (OPD), and polyoxyethylene sorbitan monolaurate (Tween-20). Cell culture media (Dulbecco's Modified Eagle's Medium) and fetal bovine serum were obtained from Gibco BRL (Paisley, Scotland).

Phosphate-buffered saline (PBS; 0.01 M, pH 7.4), carbonate-buffered saline (CBS; 0.05 M, pH 9.6), PBS containing 0.1% Tween-20 (PBST), PBS containing 0.1% Tween-20 and 0.5% gelatin (PBSTG), and substrate solution (4 μL of 30% H_2O_2 added to 10 mL of citrate–phosphate buffer containing 2 mg/mL OPD) were used. All reagents and solvents were analytical grade.

2.2. Instruments

High-resolution mass spectrometry (HRMS) were recorded with a Q-TOF 6530 mass spectrometer in electrospray ionisation mode (Agilent, Santa Clara, CA). Cell culture plates and 96-well plates were from Corning Costar (Oneonta, NY). An automated plate washer (Wellwash 4 MK2), a microplate reader (Multiskan MK3), and a direct heat CO_2 incubator (311) were from Thermo (Vantaa, Finland). An electric heating constant temperature incubator was manufactured by Tianjin Zhonghuan Experiment Electric Stove Co. Ltd. (Tianjin, China).

2.3. Hapten synthesis

Haptens were synthesised according to the synthetic route illustrated in Fig. 1. Briefly, a mixture of 25 mg (0.24 mmol) of γ-amino-n-butyric acid, 18 mg (0.45 mmol) of sodium hydroxide, and 100 mg (0.22 mmol) of 2-(3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-8-methyl-4H-benzo[d][1,3]oxazin-4-one was dissolved in 5 mL of *N*,*N*-dimethyl formamide in a 25-mL glass flask by stirring at room temperature for 12 h. The reaction mixture was poured into 15 mL of water, adjusted to pH 3 using 2 N HCl, and then extracted thrice with ethyl acetate (5 mL each). Combined extracts were washed thrice with water (5 mL each), dried over anhydrous sodium sulphate, and concentrated to dryness in a vacuum rotary evaporator. Residues were recrystallised in a mixture of ethyl ether and n-hexane (3:1, v/v) to obtain the CAP hapten (CAPA) as a white solid (89 mg; yield 72%), which was characterised by ^{1}H NMR, ^{13}C NMR, and HRMS.

^{1}H NMR (DMSO): 1.64 (m, CH_2, 2H), 2.16 (s, CH_3, 3H), 2.24 (t, CH_2, 2H), 3.14 (m, CH_2, 2H), 7.34–8.49 (m, aromatic-H, 6H), 8.35 (t, $CONHCH_2$, 1H), 10.25 (s, CONH, 1H), 12.07 (s, COOH, 1H); ^{13}C NMR (DMSO):17.8, 24.4, 31.2,40.2, 110.7, 125.5, 126.7, 126.9, 128.0, 131.0, 131.2, 131.6, 136.2, 138.8, 139.3, 139.5, 147.2, 148.5, 155.6, 165.8, 174.3. HRMS:*m*/*z* calcd for $C_{21}H_{19}BrCl_2N_5O_4$ ($M+H^+$) 553.9992, found 553.9973.

2.4. Preparation of CAP–protein conjugates

CAPA was conjugated with BSA and OVA to produce an immunogen (CAP–BSA) and coating antigen (CAP–OVA), respectively, following the carbodiimide method (Cui et al., 2011). Conjugates were dialyzed against 3 L of PBS (×6) over 72 h at 4 °C. After dialysis, CAP–BSA and CAP–OVA were stored at −20 °C. The UV–vis spectrum was used to confirm the structures of the final conjugates, according to the reported method (Zhang et al., 2007).

2.5. Immunization protocol, monoclonal antibody production and purification, and establishment of conventional icELISA

Six female Balb/c mice (7-week-old) were immunized with the immunogen CAP–BSA. The protocols of immunization, fusion, antibody production and purification, and establishment of a conventional icELISA were the same as those described previously (Cui et al., 2011).

2.6. ELISA optimisation

Experimental parameters, including organic solvent, ionic strength, and buffer pH, were studied sequentially to improve the sensitivity of the immunoassay. Evaluation of the immunoassay was based on IC_{50} (the concentration of producing 50%

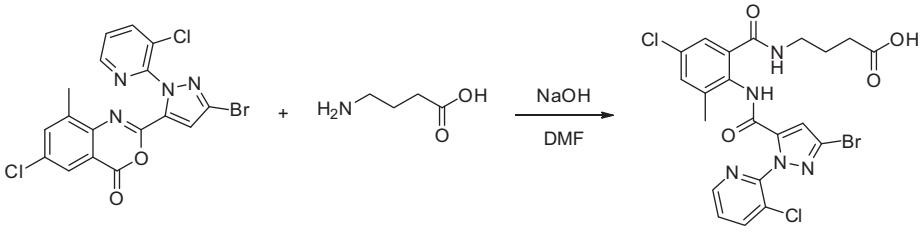

Fig. 1. Synthetic route for hapten.