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Development of a monoclonal antibody-based immunoaffinity chromatography and a sensitive immunoassay for detection of spinosyn A in milk, fruits, and vegetables

Jianqiang Lan[a,b], Hongwei Zhao[b,**], Xiaotuo Jin[b], Huai Guan[a], Yuan Song[c], Yongmei Fan[b], Xiaoping Diao[b], Baomin Wang[d], Qian Han[a,b,*]

[a] Key Laboratory of Tropical Biological Resources of Ministry of Education, Hainan University, Haikou, Hainan, 570228, China
[b] Institute of Tropical Agriculture and Forestry, Hainan University, Haikou, 570228, China
[c] College of Biological Sciences, China Agricultural University, Beijing, 100193, China
[d] College of Agronomy and Biotechnology, China Agricultural University, Beijing, 100193, China

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ABSTRACT

Spinosad has been extensively used for insect control in agricultural crops. In the present study, a novel succinic derivative of spinosyn A as the hapten was synthesized, and an indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed using a new monoclonal antibody (designed as mAb3E6) specifically against spinosyn A. A mAb3E6-based immunoaffinity column was developed for enrichment extraction of spinosyn A and reduction of matrix interference. The half maximum inhibitory concentration and limit of detection (LOD) of the icELISA were 4.11 ng/mL and 0.63 ng/mL, respectively. The icELISA had average fortified recoveries from 78.1% to 103.2% and the validation results correlated well with high-performance liquid chromatography. The mAb3E6 showed cross reactivity of 80.4%, 75.7% and less than 0.1% with spinosyns B, D and other macrolides, respectively. Interactions of the antibody and macrolides were further analyzed by molecular docking. The developed immunoaffinity chromatography-assisted icELISA is potentially a sensitive and convenient tool for monitoring spinosad residues in milk, fruits, and vegetables.

1. Introduction

Spinosad, a mixture of spinosyns A (85%) and D (15%), is a naturally fermentation-derived insecticide from *Saccharopolyspora spinosa* (Kirst, 2010), and acts through interaction with the nicotinic acetylcholine receptor (nAChR) (Salgado, 1998; Salgado, Sheets, Watson, & Schmidt, 1998; Somers, Nguyen, Lumb, Batterham, & Perry, 2015), which possesses a different mode of action with the majority of commercial macrolides (Salgado, Sparks, Gilbert, & Gill, 2010). Since registered for cotton insect control in 1997, spinosad has been extensively used on a variety of crops for the control of insect pests (Bacci, Lupi, Savoldelli, & Rossaro, 2016), such as soybeans, cabbage and fruits. Besides, spinosad has been used for the control of ectoparasites such as tick and lice on cattle. United States Environmental Protection Agency (EPA) had concluded that spinosyns A and D are the major residues for tolerances in water, soil as well as in crops, and its metabolites such as spinosyn B, spinosyn K and N-demethyl spinosyn D were identified as minor degradates (Cleveland et al., 2002; Hale & Portwood, 1996; Rutherford, Gardner, West, Robb, & Dolder, 2000). Maximum residue limits (MRLs) for spinosad on cotton seed, Chinese cabbage and eggplant in China are proposed as 0.01, 2 and 0.5 mg/kg, respectively (Gao, Dong, Zhang, & Chen, 2007). The Joint FAO/WHO Meeting on Pesticide Residues (JMMR) has set the MRL for spinosad in milk and grape at 1 and 0.5 mg/kg, respectively (Joint FAO/WHO Meeting on Pesticide Residues, 2004).

Conventional methods have been developed for the determination of spinosad, such as high-performance liquid chromatography (HPLC) with ultraviolet detector (Adak & Mukherjee, 2016; West, 1997; West et al., 2000) and HPLC–mass spectrometry (HPLC-MS) (Arienzo, Cataldo, & Ferrara, 2013; Benincasa, Perri, Iannotta, & Scalercio, 2011; Dasenaki, Bletsou, Hanafi, & Thomaidis, 2016; Schwedler, Thomas, & Yeh, 2000). These methods are accurate and reliable but time-consuming, especially the related equipments are complicated and expensive. Immunoassays are sensitive, high-throughput, selective,

* Corresponding author.
** Corresponding author.
E-mail addresses: hwzhao@hainu.edu.cn (H. Zhao), qianhan@hainu.edu.cn (Q. Han).

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convenient and low cost. However, only a few immunoassays have been developed for the rapid monitoring of spinosad, such as fluorescent excitation transfer immunoassay and enzyme-linked immunosorbent assay (ELISA) (Lee, Walt, & Nugent, 1999; Young et al., 2000). Immunoaffinity chromatography is commonly used for the determination of residue analysis, which can easily remove matrix interference and selectively enrich the target compounds. However, to our knowledge, neither report had described how to synthesize a spinosad hapten, nor an immunoaffinity column developed for the detection of spinosyn A.

In the present study, a protocol for spinosyn A hapten synthesis was reported. The obtained hapten was coupled to keyhole limpet hemocyanin (KLH) and ovalbumin (OVA), as immunogen and coating antigen, respectively. A monoclonal antibody (mAb) against spinosyn A was obtained and further used to construct a spinosyn A immunoaffinity column-assisted indirect competitive ELISA (icELISA) for the determination of spinosad in milk, Chinese cabbage, grape, long bean, mango and java apple. HPLC validation was performed to evaluate the credibility of icELISA method. The interactions between the antibody and the spinosyn analogs were further evaluated using molecular docking based on the antibody 3D homology model.

2. Materials and methods

2.1. Reagents and apparatuses

Spinosad and other insecticides were purchased from J & K scientific Ltd (Beijing, China). OVA, KLH, 4-dimethylaminopyridine (DMAP), succinic anhydride, anhydrous N, N-dimethylformamide (DMF), N-hydroxysuccinimide (NHS), N,N-dicyclohexylcarbodiimide (DCC), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), 3, 3′, 5, 5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO), 8-azaguanine, hypoxanthine-aminopterin-thymidine (HAT), hypoxanthine-thymidine (HT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Goat anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP) was purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Dulbecco's Modified Eagle's Media (DMEM) and fetal calf serum were obtained from Gibco BRL (Carlsbad, CA, USA). All other reagents were from Beijing Chemical Reagents Company (Beijing, China). Cell culture plates and 96-well polystyrene microtiter plates were purchased from Costar (Corning, NY, USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA).

BALB/c mice were obtained from Guangdong Medical Laboratory Animal Center (Foshan, China). Sp2/0 cell lines were obtained from China Agricultural University (Beijing, China).

2.2. Synthesis of the hapten

Spinosyn A was re-crystalized from the crude spinosad sample according to Mei's study (Mei, Liu, Ma, & Xie, 2013), and the purity of spinosyn A was > 95%. The synthetic scheme of the new spinosyn A hapten was showed in Fig. 1. C-17 Pseudoaglycone of Spinosyn A (C-17 PSA) was obtained by the instruction of Creemer et al. (Creemer, Kirst, & Paschal, 1998) with some modifications. To a solution of 72 mg of C-17 PSA in 2 mL dichloromethane (DCM) was added 37 mg of succinic anhydride and 14.9 mg of DMAP, the reaction solution was stirred for 48 h at room temperature (RT). The C-17 Succinyl PSA (C-17 PSAS) was further purified by silica gel chromatography using a solvent system of ethyl acetate: DCM: acetic acid (60:40:1), a product of 60 mg C-17 PSAS (M-H: 689.2) was finally obtained. The NMR data for C-17 PSAS is shown below:

^{1}H NMR (500 MHz, CD_3OD) δ7.09 (s, 1H), 5.93 (d, J = 9.8 Hz, 1H), 5.86 (dt, J = 9.8, 2.8 Hz, 1H), 5.03–4.95 (m, 1H), 4.87 (d, J = 1.7 Hz, 2H), 4.70–4.61 (m, 1H), 4.34 (dd, J = 12.7, 7.1 Hz, 1H), 3.60–3.52 (m, 3H), 3.52 (s, 3H), 3.48–3.44 (m, 9H), 3.43 (t, J = 3.3 Hz, 1H), 3.35 (s, 6H), 3.08 (dt, J = 18.8, 7.2 Hz, 2H), 2.98–2.96 (m, 1H), 2.91–2.83 (m, 1H), 2.64–2.58 (m, 4H), 2.47 (dd, J = 13.4, 3.2 Hz, 1H), 2.41–2.30 (m, 1H), 2.22–2.12 (m, 1H), 2.04–1.93 (m, 1H), 1.66–1.27 (m, 11H), 1.24 (d, J = 6.2 Hz, 3H), 1.09 (d, J = 6.7 Hz, 3H), 0.99–0.88 (m, 1H), 0.83 (t, J = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ203.44, 175.96, 173.93,173.92, 150.37, 145.06, 130.66, 129.74, 97.06, 83.55, 82.52, 78.63, 77.96, 77.91, 76.29, 69.10, 61.14, 59.15, 57.72, 51.06, 49.85, 47.46, 46.72, 42.87, 42.55, 38.54, 37.44, 35.01, 33.58, 31.11, 30.27, 29.84, 29.28, 22.34, 18.13, 16.45, 9.62.

2.3. C-17 PSAS-protein conjugate preparation

Before the hapten was conjugated to protein carriers, its carboxyl group was activated to form an N-hydroxysuccinimide ester. Typically, 10 mg hapten was added to 1.1-fold molar excess of NHS and DCC in 0.2 mL DMF. The mixture was magnetically stirred overnight at RT and centrifuged at 8000 g for 5 min to discard the precipitate. Approximately 0.2 mL of supernant of the activated hapten was dropwise added to a protein solution (20 mg of KLH in 2 mL coating buffer or 26 mg of OVA in 3 mL coating buffer) (Coating buffer: 14.2 mM Na_2CO_3 and 35.8 mM $NaHCO_3$, pH 9.6) with stirring at RT. The solution was allowed to react overnight. The obtained conjugates (C-17 PSAS-KLH as immunogen and C-17 PSAS-OVA as coating antigen) were then dialyzed against 3 L of PBS (1.5 mM KH_2PO_4, 8.3 mM Na_2HPO_4 $12H_2O$ and 154 mM NaCl, pH 7.5) for 3–4 days with 3 times of changes of dialysate each day. The concentration of conjugate was determined by Bradford method (Bradford, 1976). The obtained solutions were diluted to 1 mg/mL in the PBS buffer and stored at −20 °C. The effect of different coupling ratios of coating antigen was evaluated by conjugating 3.45–13.8 mg C-17 PSAS with 11 mg OVA in 2 mL coating buffer. The number of hapten attached to carrier proteins was calculated according to the UV–vis spectra (Barbarakis & Bachas, 1991), couple ratio = $[\varepsilon_{(conjugates)} - \varepsilon_{(carrier\ protein)}]/\varepsilon_{(hapten)}$, ε represents for the absorbance coefficient.

2.4. Monoclonal antibody production

The protocol was similar with the previous report (Zhao et al., 2006) with some modifications. Five BalB/c mice were immunized with C-17 PSAS-KLH conjugate. First injection was performed intraperitoneally by giving 0.1 mg of immunogen emulsified with 0.2 mL complete Freund's adjuvant. Subsequent injection was subcutaneously given at 2-week intervals emulsified with incomplete Freund's adjuvant. After 3 times of immunization, icELISA was performed to screen one mouse that gave the best antibody titers and specificity. A booster immunization was performed with 0.1 mg immunogen without adjuvant 4 days before cell fusion. The spleen cells were fused with myeloma cells Sp2/0 at the ratio of 10: 1. After 7 days in culture, the positive hybridoma was screened by icELISA, and cloned by limiting dilution method. During the selection, two standards (Spinosyn A and C-17 PSA) were used to choose a monoclonal cell line that specifically recognized spinosyn A. The monoclonal cell line (mAb3E6) showing the greatest titer and sensitivity was used for the production of ascites.

2.5. Prototcol of the icELISA

The protocol was developed according to a previous report with some modifications (Fu et al., 2018; Zhao et al., 2011). The microtiter plate (96-wells) was coated with 200 μL C-17 PSAS-OVA conjugate diluted in coating buffer at 37 °C for 3 h. The plate was washed with PBST (0.1% (v/v) Tween-20 diluted in PBS) for 3 times, and blocked by non-fat milk in PBS for 30 min. After washed, 100 μL of standard or analytes in PBSTG (0.1% (w/v) gelatin in PBST) and 100 μL mAb were added together to allow to compete each other for 1 h. Then, 200 μL per well of goat anti-mouse IgG-HRP diluted in PBSTG at 1:1000 was added. After incubation at 37 °C for 0.5 h, the plate was washed and 200 μL of TMB substrate solution was added to each well. The reaction was

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