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Competitive and noncompetitive phage immunoassays for the determination of benzothiostrobin

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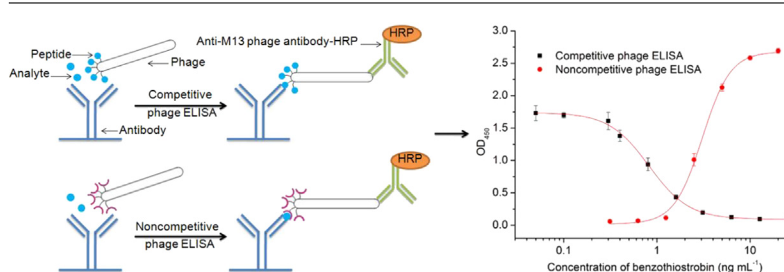
HIGHLIGHTS

- Benzothiostrobin peptidomimetics have been isolated.
- Anti-benzothiostrobin immunocomplex phage-displayed peptides have been obtained.
- Competitive and noncompetitive phage ELISAs have been developed.
- The IC_{50} and SC_{50} of the phage ELISAs are 0.94 and 2.27 ng mL^{-1}, respectively.
- The phage ELISAs have better performance than conventional ELISA.

GRAPHICAL ABSTRACT



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ABSTRACT

Twenty-three phage-displayed peptides that specifically bind to an anti-benzothiostrobin monoclonal antibody (mAb) in the absence or presence of benzothiostrobin were isolated from a cyclic 8-residue peptide phage library. Competitive and noncompetitive phage enzyme linked immunosorbent assays (ELISAs) for benzothiostrobin were developed by using a clone C3-3 specific to the benzothiostrobin-free mAb and a clone N6-18 specific to the benzothiostrobin immunocomplex, respectively. Under the optimal conditions, the half maximal inhibition concentration (IC_{50}) of the competitive phage ELISA and the concentration of analyte producing 50% saturation of the signal (SC_{50}) of the noncompetitive phage ELISA for benzothiostrobin were 0.94 and 2.27 ng mL^{-1}, respectively. The noncompetitive phage ELISA showed higher selectivity compared to the competitive. Recoveries of the competitive and the noncompetitive phage ELISAs for benzothiostrobin in cucumber, tomato, pear and rice samples were 67.6−119.6% and 70.4−125.0%, respectively. The amounts of benzothiostrobin in the containing incurred residues samples detected by the two types of phage ELISAs were significantly correlated with that detected by high-performance liquid chromatography (HPLC).

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1. Introduction

Immunoassays are practical analytical tools for detecting pesticides because of their well-known advantages of simplicity, low cost and large parallel-processing capacity [1,2]. Pesticides are low-molecular-weight chemicals that are buried in the antibody

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binding pocket after binding, so they are usually detected using a competitive format. In this format, a competitor (a competing hapten conjugated with a carrier protein, enzyme, fluorophore, etc.) that competes with the analyte for binding to the antibody is a necessary reagent [3]. Generally, the sensitivity of the immunoassay can be significantly improved by using heterologous competing haptens, but the chemical synthesis of a series of competing haptens depending on the analyte may be difficult, expensive and potentially hazardous [4,5]. Besides, some immunoassays have cross-reactivity with analogues of the target because of the analogues can also bind to the antibody [6,7]. These limitations hinder the widespread use and application of immunoassay in detection of pesticide residues.

The phage display technique offers attractive materials to develop alternate immunoassays that may enhance assay performance by increasing sensitivity and selectivity [8]. Phage-displayed peptide libraries have been a powerful tool for isolating peptides that specifically bind to analyte-free antibodies (peptidomimetics) or analyte-bound antibodies (anti-immunocomplex peptides) [9]. Analyte peptidomimetics can be used as surrogate competing haptens to accelerate the development of heterologous immunoassays. Some investigators have successfully obtained analyte peptidomimetics that can be used as a competing hapten for low-molecular-weight compounds from phage display peptide libraries, and the analyte peptidomimetics show better sensitivity than chemical synthesis of homologous and heterologous competing haptens [3,9–18]. Anti-immunocomplex phage peptides can specifically recognize an analyte-bound antibody to enhance the affinity and selectivity of the primary antibody because of the formation of a ternary complex, which translates into an improved noncompetitive immunoassay for a low-molecular-weight compound [8]. Although only eight low-molecular-weight compounds (brominated diphenyl ether 47, clomazone, malachite green, leucomalachite green, gibberellin, 2,2′,4,4′-tetrabromodiphenyl ether, phenoxybenzoic acid, molinate and atrazine) have been successfully detected by noncompetitive immunoassays based on anti-immunocomplex phage peptides, each presented better performance than the corresponding conventional competitive immunoassays [8,9,19–24]. Additionally, real-time polymerase chain reaction and loop-mediated isothermal amplification have been successfully used to detect low-molecular-weight chemicals by using phage-displayed peptides because of the unique characteristic that phage-displayed peptides can be connected to nucleic acids (single stranded DNA) [3,25,26].

Benzothiostrobin, a fungicide developed by the Central China Normal University, is a novel strobilurin fungicide that exhibits highly efficient control of most fungal diseases in crops [27,28], and patent applications for preparation and use of benzothiostrobin have submitted in China, the United States, Europe, and worldwide (Pub. No.: CN 102302012 B, CN 101379967 A, CN 101268780 B, US 2010/0292285 A1, WO 2007/073637 A1, WO 2009/135407 A1). In our previous study, a homologous competitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of benzothiostrobin. The half maximal inhibition concentration (IC_{50}) and the limit of detection (LOD, IC_{10}) were 7.55 and 0.428 ng mL^{-1}, respectively. The assay had cross-reactivity (CR) of 0.34% with pyraclostrobin [29].

In this study, the phage-displayed peptides that specifically recognize anti-benzothiostrobin monoclonal antibody (mAb) $4E_8$ in the absence or presence of benzothiostrobin were isolated from a cyclic 8-amino-acid random peptide library. Both competitive and noncompetitive phage ELISAs for benzothiostrobin based on the isolated phage-diplayed peptides were developed and compared with the conventional ELISA reported earlier [29]. The developed phage ELISAs were validated by high-performance liquid chromatography (HPLC) in the analysis of the samples containing incurred residues.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade unless specified otherwise. Benzothiostrobin (99.02%) was obtained from the Central China Normal University (Wuhan, China). The pesticide standards used for cross-reactivity studies were purchased from Dr Ehrenstorfer GmbH (Germany). Bovine serum albumin (BSA), tetramethylbenzidine (TMB), isopropyl-β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl- β-D-galactoside (Xgal), and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Mouse anti-M13 monoclonal antibody-horse radish peroxidase (HRP) conjugate was purchased from GE Healthcare (Piscataway, NJ). *Escherichia coli* ER2738 was purchased from New England Biolabs (Ipswich, MA). The mAb $4E_8$ and the cyclic 8-amino-acid random peptide library (the transducing units (TU) and titer of the library were 5.52×10^9 pfu μg^{-1} and 3.4×10^{13} pfu mL^{-1}, respectively) were developed previously [15,29].

2.2. Biopanning

Three wells of each microtiter plate were coated with purified $4E_8$ mAb (10 μg mL^{-1}) in 100 μL of phosphate-buffered saline (PBS) by overnight incubation at 4 °C. Nonspecific binding was blocked by incubation with 300 μL of PBS containing 3% bovine serum albumin (BSA) for 1.5 h at 37 °C. To eliminate nonspecific binding of phage to BSA, another plate coated with 100 μL of 3% BSA in PBS was used for preabsorption. The phage library (10^{11} phage) diluted with PBS was first added to the preabsorption plate and incubated at room temperature for 1 h. Then, the supernatant was transferred to the plate coated with $4E_8$ mAb and incubated with shaking at room temperature for 1 h. The wells were washed 10 times with PBS containing 0.1% (v/v) Tween 20 (PBST), and 100 μL of benzothiostrobin (10 μg mL^{-1} in PBS) was added to each well with shaking for 1 h to compete the binding phage from the coating antibody (peptidomimetics panning). The phage library that had been used for peptidomimetic panning was transferred into other wells of the mAb-coated plate that had been preincubated with 10 μg mL^{-1} benzothiostrobin and washed five times with PBST, followed by incubation at room temperature for 1 h. The bound phage was eluted with 100 μL of 0.1 mol L^{-1} glycine-HCl (pH 2.2) per well for 15 min and neutralized with 13 μL of 1 mol L^{-1} Tris–HCl (pH 9.1) (anti-immunocomplex phage peptides panning). The elution solutions were then collected and used to infect *E. coli* ER2738 for amplification and titration. The amplified phages were used for a subsequent round of panning. In the second and third rounds of panning, the concentration of coating antibody was reduced to 5 and 2.5 μg mL^{-1}, while the concentration of benzothiostrobin was reduced to 1 and 0.1 μg mL^{-1}, respectively.

After three rounds of panning, 180 μL of ER2738 cell culture (mid-log phase, $OD_{600} = 0.5$ AU) was mixed with 10 μL of diluted phage eluates. The infected cells were transferred to culture tubes containing 5 mL 45 °C top agar and poured on an LB/IPTG/Xgal plate. The plates were incubated overnight at 37 °C. A total of 48 clones for peptidomimetics or anti-immunocomplex phage peptides were picked, transferred to diluted ER2738 culture and grown at 37 °C with shaking for 4.5 h. Cells were pelleted by centrifugation at 10000 rpm for 10 min and the supernatants were collected for phage ELISAs. The positive clones as described above were further amplified and used for phage DNA isolation as introduced in the

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