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# Selection of phage-displayed peptides for the detection of imidacloprid in water and soil

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ABSTRACT

Imidacloprid is the most widely used neonicotinoid insecticide in the world and shows widespread environment and human exposures. A phage clone designated L7-1 that selectively binds to imidacloprid was selected from a commercial phage display library containing linear 7-mer randomized amino acid residues. Using the clone L7-1, a competitive enzyme-linked immunosorbent assay (ELISA) for imidacloprid was developed. The half-maximum signal inhibition concentration ($IC_{50}$) and the limit of detection (LOD) of the phage ELISA for imidacloprid were 96 and 2.3 ng $ml^{-1}$, respectively. This phage ELISA showed relatively low cross-reactivity with all of the tested compounds structurally similar to imidacloprid, less than 2% with the exception of 6-chloronicotinic acid, a metabolite of imidacloprid that showed 11.5%. The average recoveries of the phage ELISA for imidacloprid in water and soil samples were in the ranges of 74.6 to 86.3% and 72.5 to 93.6%, respectively. The results of the competitive phage ELISA for imidacloprid in the fortified samples agreed well with those of a high-performance liquid chromatography (HPLC) method. The simple phage-displayed peptide technology has been proven to be a convenient and efficient method for the development of an alternative format of ELISA for small molecules.



Imidacloprid is the most widely used neonicotinoid insecticide in agriculture for the control of sucking insects such as aphids, whiteflies, leaf and plant hoppers, thrips, some microlepidoptera, and a number of coleopteran insects. This systemic insecticide can be applied using a variety of techniques such as soil injection, tree injection, application to the skin of the plant, broadcast foliar spray, ground application as a granular or liquid formulation, or pesticide-coated seed treatment [1].

Imidacloprid acts on the central nervous system of insects with much lower toxicity to mammals [2], but numerous concerns exist about the use of the neonicotinoids. In animals, exposure to a high dose of imidacloprid may be associated with degenerative changes in thymus, bone marrow, and pancreas [1]. A study conducted in rats suggests that imidacloprid may adversely affect brain development [3]. Recent research suggests that widespread agricultural use of imidacloprid and other pesticides may be contributing to honey bee colony collapse disorder, the decline of honey bee colonies in Europe and North America observed recently [4,5]. The European Union (EU)[1] Commission published the Commission Implementing Regulation (EU) 485/2013, which contains restriction on the use of neonicotinoids, including imidacloprid, clothianidin, and thiamethoxam, on certain bee attractive crops in the EU region [6]. Imidacloprid also poses a potentially high acute risk for insectivorous birds. A study of insectivorous bird population trends in the Netherlands revealed that temporal and spatial patterns of the bird population decline appeared only after the introduction of imidacloprid to the Netherlands, during the mid-1990s, and that this correlation is not linked to any other land-use factor [7].

The main routes of dissipation of imidacloprid in the environment are aqueous photolysis and plant uptake [8]. When not exposed to light, imidacloprid breaks down slowly in water and soil and, thus, has the potential to persist in the environment.

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[1] *Abbreviations used:* EU, European Union; mAb, monoclonal antibody; pAb, polyclonal antibody; HRP, horseradish peroxidase; BSA, bovine serum albumin; PEG, polyethylene glycol; TMB, 3,3′,5,5′-tetramethylbenzidine; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; $IC_{50}$, half-maximum signal inhibition concentration; LOD, limit of detection; CR, cross-reactivity; HPLC, high-performance liquid chromatography; pfu, plaque-forming units.

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Based on its high water solubility (0.5–0.6 g $L^{-1}$) and persistence, both the U.S. Environmental Protection Agency and the Pest Management Regulatory Agency in Canada consider imidacloprid to have a high potential to run off into surface water and to leach into groundwater; thus, they warn not to apply it in areas where soils are permeable, particularly where the water table is shallow [8,9].

Liquid chromatography-based techniques have been widely used in determining imidacloprid in various samples [10–13]. As an alternative, immunoassays have been developed for imidacloprid for environmental detection with high sensitivity, selectivity, and throughput [14–18]. In the development of immunoassays for small-molecule compounds like insecticides, the preparation of the immunoreagents, such as haptens, monoclonal antibodies (mAbs), and polyclonal antibodies (pAbs), can be challenging and time-consuming [19–21]. Phage-displayed peptide libraries have been used to select peptides that bind various molecular targets, offering an alternate method for the easy and rapid development of immunoassay [22–24]. Phage displaying is a selection technique in which a library of peptides or proteins are expressed as a fusion with coat proteins of a filamentous bacteriophage such as M13, resulting in display of the fused protein on the surface of the virion [25]. Phages can be produced rapidly and economically in large quantities and are very stable and resistant to degradation by organic solvents [26], proteases [27], and heat [28]. Based on these advantages, we hypothesized that the peptide ligands displayed on phage surface might provide attractive alternatives for the conventional antigen-binding antibodies for pesticide detection.

There are many successful examples of screening phage display libraries against large molecules such as antibodies, receptors, and enzymes. However, because small molecules have less binding surface and are difficult to immobilize, application of the technology directly for small molecules can be challenging. A few studies have successfully employed peptide display to select binders for small molecules, for example, fluorophores [29], 2,4,6-trinitrotoluene [30], microcystin-LR [31], and paclitaxel (Taxol) [32]. We recently presented a panning strategy for the integrated selection of phage-borne peptides binding to either a particular ligand–antibody complex or a ligand-free antibody, leading to the development of assays for the targeted ligand 2,2′,4,4′-tetrabromodiphenyl ether (BDE47) [33]. To explore the possibility of expanding the scope of this technology to environmental monitoring, in the current study we used commercially available random peptide libraries to screen peptide ligands specific for imidacloprid, providing an economical and convenient method to isolate specific peptides directly binding small molecules. To the best of our knowledge, this is the first report of phage selections for binding to an insecticide. Phage particles selected for imidacloprid binding were then characterized and tested for their utility in the analysis of imidacloprid in water and soil.

## Materials and methods

### *Reagents and materials*

All reagents were of analytical grade unless specified otherwise. Reference standards of imidacloprid, thiamethoxam, acetamiprid, clothianidin, and dinotefuran were purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture (Beijing, China). A linear heptapeptide library (Ph.D.-7) and a loop-constrained heptapeptide library (Ph.D.-C7C), both expressing pentavalent peptides on the minor coat protein pIII, were purchased from New England Biolabs (Beijing, China). Mouse anti-M13 phage mAb–horseradish peroxidase (HRP) conjugate was purchased from GE Healthcare (Piscataway, NJ, USA). Reagents purchased from Sigma–Aldrich (St. Louis, MO, USA) were bovine serum albumin (BSA), polyethylene glycol (PEG) 8000, Tween 20, 6-chloronicotinic acid, and 3,3′,5,5′-tetramethylbenzidine (TMB). Other reagents were purchased from Beijing Chemical Reagent Company (Beijing, China). Imidacloprid hapten (IMI) (Fig. 1) was synthesized and conjugated to the carrier protein BSA in our previous study [14].

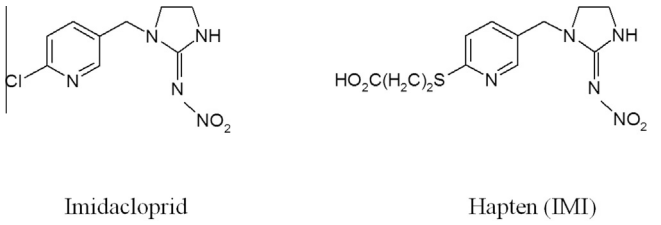
Enzyme-linked immunosorbent assays (ELISAs) were performed on 96-well microtiter plates (Nunc–Immuno plates, MaxiSorp surface, Roskilde, Denmark). Absorbance values were read with a microplate reader (Wellscan MK3, Labsystems Dragon, Finland).

### *Selection of peptides*

The conjugate IMI–BSA was used as binding templates to select peptides specific for imidacloprid from the random peptide libraries Ph.D.-C7C and Ph.D.-7. The selection principles were summarized as follows. Four wells of a microtiter plate were coated with IMI–BSA (20 μg $ml^{-1}$) in 100 μl of phosphate-buffered saline (PBS: 0.01 mol $L^{-1}$ phosphate, 0.137 mol $L^{-1}$ NaCl, and 3 mmol $L^{-1}$ KCl, pH 7.4) overnight at 4 °C. After blocking with 3% BSA in PBS for 2 h at ambient temperature, an aliquot of 100 μl of a phage library in PBS containing 1% BSA and 5% methanol was added to the wells of the plate. The plate was incubated for 2 h at ambient temperature and then washed 10 times with PBST (PBS containing 0.05% Tween 20) immediately. Bound phages in the wells were eluted by incubation with PBS containing 1000 ng $ml^{-1}$ imidacloprid (100 μl per well) for 1 h at ambient temperature. The eluted phage solutions were transferred onto wells coated with 3% BSA alone to eliminate the nonspecific binding phages by incubating for 1 h at ambient temperature. The unbound phage solution (300 μl) was added to exponentially growing *Escherichia coli* ER2738 culture (20 ml, $OD_{600}$ = 0.5) and incubated at 37 °C with shaking for 5 h. The phage culture was centrifuged at 12,000*g* for 10 min, and the resulting supernatant was mixed with 1/6 volume of a PEG solution (20% [w/v] PEG 8000 and 2.5 M NaCl) and stored at 4 °C overnight. The phages were precipitated by centrifuging at 12,000*g* for 15 min. Phage pellet was resuspended with 1 ml of sterile PBS and precipitated again using the above PEG solution by incubating on ice for 1 h. After centrifugation, the phages were resuspended with 200 μl of sterile PBS and then titrated in *E. coli* ER2738. This entire panning procedure was then repeated three times except that the concentrations of IMI–BSA used for the second, third, and fourth pannings were 10, 5, and 2.5 μg $ml^{-1}$, respectively. After the final round of panning, phage isolates were obtained by culturing phage clones from titration plates, and they were screened for their binding ability to IMI–BSA by a competitive phage ELISA in the absence or presence of imidacloprid (0 or 1000 ng $ml^{-1}$).

### *Isolation and sequencing of phage DNA*

The positive clones specific for imidacloprid were sequenced as follows. Individual phage plaques were picked from agar plates




Imidacloprid Hapten (IMI)

**Fig.1.** Structures of imidacloprid and its hapten.