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Neonicotinoid insecticide interact with honeybee odorant-binding protein: Implication for olfactory dysfunction

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ABSTRACT

The decline of bee population has caused great concern in recent years. A noticeable factor points to the neonicotinoid insecticides, which remain in the nectar and pollen of plants and impair the olfactory cognition of foraging bees. However, it remains elusive that if and how neonicotinoid insecticides interact with the olfactory system of bees. Herein, we studied the binding interaction between neonicotinoid imidacloprid and ASP2, one odorant-binding protein in eastern bees, *Apis cerana*, by multispectroscopic methods. The results indicate that imidacloprid significantly quenched the intrinsic fluorescence of ASP2 as the static quenching mode, and expanded the conformation of ASP2 measured by the circular dichroism (CD) spectra. The acting force is mainly driven by hydrophobic force based on thermodynamic analysis. Docking analysis predicts a formation of a hydrogen bond, while the corresponding site-directed mutagenesis indicated that the hydrogen bond is not main force here. Moreover, imidacloprid with a sublethal dose (0.8 ng/bee) clearly decreased the binding affinity of ASP2 to a floral volatile, β-ionone, which had been identified to strongly bind with the wild ASP2 before. This study may benefit to evaluate the effect of neonicotinoid insecticides on the olfactory cognitive behavior of bees involved in the crops pollination.

1. Introduction

Up to 75% of crop species used for food depend on insect pollination to some degrees [1]. Honeybees are the major pollinators of crop plants; the decline in honeybee population has caused great concerns in recent years [2–4]. Neonicotinoid insecticides have been investigated as the possible causes of recent declines in pollinator population [5]. Except the partially suspended use of neonicotinoid insecticides, the potential harm of sublethal doses of neonicotinoid insecticides has caused more concerns [6,7] because of its subtle influence on bee behavior including the effect on normal functions [8], the changing foraging, and the decrease in avoiding predatory behavior [9], particularly referring to the impairment of the olfactory associative behavior of adult honeybees [10]. Honeybee olfaction is an important factor for searching flowering crop plants requiring pollination, and the dysfunction of olfaction may cause severe disaster of honeybee colonies [11]. Therefore, it is necessary to understand how neonicotinoid insecticides interact with the olfactory system and affect the odor recognition of honeybees.

Insects have developed very sensitive and sophisticated olfactory systems to detect and correctly recognize different odors or volatiles present in the external ecological environment. In the chemosensory sensilla of insects, the olfactory tissues act like antennae, mainly mediated by some soluble binding proteins, namely, odorant-binding proteins (OBPs), which are small, water-soluble and present at high concentrations in the sensillum lymph of insects surrounding the olfactory receptor neurons [12], OBPs contribute to the sensitivity of the olfactory system by transporting odorants through the sensillar lymph to olfactory receptors [13]. The binding interactions between odors and OBPs may be involved in the first step of the olfactory molecular recognition and signal transduction in insects [14,15]. Because of the current misuse of insecticides such as neonicotinoid pesticides in crop plants, the effect of insecticides on the binding of OBPs to the odors involved in honeybee olfaction discrimination is worthy of investigation.

As a typical social and agricultural insect, honeybees have a developed olfactory ability to maintain their complex social behavior, including hive building, feeding of immature (larvae) bees, particularly as an important pollination facilitator in the production

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of agricultural crops [16]. *Apis cerana*, an original species of the Apis genus in eastern Asia, has a sensitive olfactory system; *A. cerana* is superior to other bee species that pollinate on the strawberry grown in greenhouse in China [17]. In our earlier studies, one typical OBP, ASP2 [18], particularly distributed in the antennae of *A. cerana* worker bees, was characterized with binding affinity for floral volatiles, such as β-ionone [19], and the detailed interaction between β-ionone and ASP2 has been demonstrated [20].

Although neonicotinoid insecticides harm the olfactory associative behavior of adult bees [10], the detailed influence mechanism of neonicotinoid insecticides on bee OBPs has not yet been elucidated. Therefore, in this study, the *in vitro* binding interaction between imidacloprid with ASP2 and the effect of imidacloprid on the binding process of ASP2 to β-ionone were investigated by multispectroscopic methods and docking analysis. This study may help to understand how the neonicotinoid insecticides interact with OBPs, whether they affect the olfactory transmission of OBPs binding to external plant volatiles, and the olfactory dysfunction induced by neonicotinoid insecticides in insects including honeybees in the molecular level.

2. Materials and methods

2.1. Chemicals and reagents

Imidacloprid (the chemical structure is shown in Fig. 1A) and β-ionone (98.0% purity) were purchased from J&K Chemical Ltd. (China), and dissolved in HPLC-grade methanol (Tedia, USA) to prepare 1.0×10^{-3} mol L^{-1} stock solutions, and stored at 4 °C in the dark. All the other solvents and chemicals used in this study were of analytical reagent grade, and Milli-Q water (18.2 MΩ, Millipore, Bedford, MA) was used.

2.2. Preparation of recombinant ASP2 protein

The recombinant ASP2 protein was induced and purified according to the previous method [20]. The purified protein was diluted into 1.0×10^{-6} mol L^{-1} stock solutions in PBS (pH 7.4), and stored at −20 °C until further analysis.

2.3. Fluorescence quenching measurements

The fluorescence spectral data of AcerASP2 with imidacloprid were recorded using a RF-5301 PC spectrofluorimeter (Shimadzu, Japan) with a 1.0 cm quartz cell at different temperatures (290 K and 300 K). The excitation and emission slit widths were set at 5.0 nm, the excitation wavelength was 281 nm, and the emission spectra were recorded between 290 and 550 nm. The maximum emission spectra were observed at 309 nm. An electronic thermostat water bath (9012, PolyScience, USA) was used to precisely control the temperature of the reaction. For the fluorescence quenching spectra, the stock solution of ASP2 (1.0×10^{-6} mol L^{-1}) was titrated with the working solution of imidacloprid (1.0×10^{-3} mol L^{-1}). A sublethal dosage of imidacloprid (30 μL, 1.0×10^{-3} mol L^{-1}) was added into 3 mL ASP2 (1.0×10^{-6} mol L^{-1}) solution beforehand, and then the competitive fluorescence spectra of ASP2-imidacloprid complex and that with β-ionone were recorded to investigate the effect of imidacloprid on the binding affinity of ASP2 to β-ionone.

2.4. UV measurements

The UV absorption spectra of imidacloprid and ASP2 were measured using a Shimadzu UV-1800 UV spectrophotometer (Shimadzu, Japan) in the wavelength range of 220–330 nm with a 1.0 cm quartz cell at room temperature. According to the results of fluorescence quenching, the corresponding ratio between imidacloprid and ASP2 was determined, well and the UV absorption spectra were recorded. All the UV measurements were carried out in PBS buffer (pH 7.4) at room temperature.

2.5. Circular dichroism (CD)

The CD spectra were measured using a CD spectrometer (Jasco-815, Jasco, Japan) with a 1.0 cm path length quartz cuvette. The CD spectra of recombinant ASP2 (1.0×10^{-6} mol L^{-1}) in the presence of imidacloprid were recorded in the range of 190–260 nm at room temperature under constant nitrogen flush. The molar ratios of imidacloprid to ASP2 were varied as 0:1, 3:1, and 5:1. All the observed CD spectra were baseline-subtracted for PBS buffer (pH 7.4), and the results were taken as CD ellipticity. The contents of the secondary conformation forms of ASP2, e.g., α-helix, β-sheet, β-turn, and random coil, were analyzed from the CD spectroscopic data using the online SELCON3 program.

2.6. Molecular docking

The binding docking analysis of ASP2 with imidacloprid was performed by the Molegro Virtual Docker 5.0 (free trial) software. The predicted three-dimensional (3D) crystal structure of ASP2 was obtained from the Swiss-Model Workspace [21] based on the 3D crystal template (entry code, 1tujA) in Protein Data Bank (PDB). Based on the developed grid-based cavity prediction algorithm, the potential binding sites of ASP2 were determined. The best binding pose of ASP2-imidacloprid complex was obtained according to the searching algorithm of MolDock Optimizer and energetic evaluation of the complex with MolDock. The binding pose was then analyzed by Ligplot+ [22] and displayed by Pymol software [23].

2.7. Site-directed mutagenesis

According to the docking results, an alkaline amino acid, Lys51, was predicted to form a unique hydrogen bond of ASP2 with imidacloprid. For the site-directed mutagenesis, the special forward and reverse primers including mutational nucleotide acids (two lowercases, Lys51 to Gly51) were firstly designed as "GCAACTTGGTTGCTTGgg-AGCCTGCGTG" and "ccCAAGCAAC-CAAGTTGCTTCATGTCGG", respectively. Then the "Fast Mutagenesis System (Transgen, China)" kit was used to perform the following site-directed mutational experiment based on the plasmid pET30-ASP2. After sequencing of the mutant ASP2-K51G, the recombinant ASP2m-K51G protein was induced and purified according to the previous method [20]. The binding experiment was operated by using the same method of step 2.3 in this study, and the binding constant of the mutant ASP2m-K51G protein with imidacloprid was finally calculated to compare with that of the wild ASP2 protein with imidacloprid.

3. Result and discussion

3.1. Expression and purification of recombinant ASP2 Protein

The recombinant proteins ASP2 were extracted from the crushed *E. coli*, and then purified by affinity chromatography. As shown in Fig. 1B, recombinant ASP2 was confirmed by SDS-PAGE; its molecular weight was ~23 kD. After the quantification by the Bradford method, recombinant ASP2 was stored in PBS buffer at a concentration of 1.0×10^{-6} mol L^{-1}.