International Journal of Biological Macromolecules 162 (2020) 1862–1868

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Assessments on the molecular toxic mechanisms of fipronil and neonicotinoids with glutathione transferase Phi8

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ARTICLE INFO

Article history:
Received 10 June 2020
Received in revised form 6 August 2020
Accepted 6 August 2020
Available online 10 August 2020

Keywords:
Fipronil and neonicotinoids
AtGSTF8
Toxic assessment

ABSTRACT

As the most widely used pesticides, fipronils and neonicotinoids exhibit harmful effects to many species including crops mainly via the oxidative damages. However, the potential toxic mechanisms of these pesticides to plants remain unclear. In this work, glutathione *S*-transferase Phi8 was employed as the biomarker to assess the adverse oxidative effects of these two kinds of pesticides. The structural changes and binding characteristics of AtGSTF8 with the pesticides were investigated by multispectral techniques and the latest generation neonicotinoid dinotefuran exhibited the most evident effects on the structure of AtGSTF8. Then dinotefuran displayed weak binding ability to AtGSTF8 comparing with fipronil and clothianidin based on the bio-layer interferometry technique. Besides, the glutathione *S*-transferase activities of AtGSTF8 were decreased upon binding with these two kinds of pesticides but dinotefuran displayed minor effect on the enzyme activity. At last, dinotefuran and clothianidin were presumed to locate on the molecular surface of AtGSTF8, while fipronil was predicted to insert into the cavity of AtGSTF8 which was adjacent to the active G-site based on the molecular docking results. The molecular investigations on the toxic mechanisms would help to evaluate the harmful effects of these two kinds of prevalent pesticides to plants.

1. Introduction

With the rapid development of agriculture, neonicotinoids and fipronils have become the two most widely used pesticides in the past two decades due to their broad-spectrum, low toxicity and high specificity [1–3]. Although fipronils were reported to be strictly regulated on usages in pest control, they still were detected in agricultural surface waters and most of them were remain in soil. Fipronil has been found to block insect GABA receptor (Rdl), while neonicotinoids act on nicotinic acetylcholine receptors (nAChRs), leading to persistent excitability for insects until death [1,4–6]. Along with the rapid development and widespread applications, neonicotinoid insecticides have come through three stages. The first-generation of neonicotinoids exhibit the excellent toxic effects on some pests but with low selectivity resulted in the loss of biodiversity, which has aroused widespread concern in society [7,8]. Compared with the first-generation of neonicotinoids, the second-generation neonicotinoid insecticides including clothianidin are characteristic of the higher specificity to most of pests, but the toxicity to bees limits their extensive applications [1,9,10]. In light of the limitations of the first two stages of neonicotinoids, the latest developed neonicotinoid insecticide dinotefuran exhibits the broader spectrum and lower toxicity resulted in the worldwide applications rapidly [11,12]. However, due to its persistent over usage in agricultural production the emerging widespread concern has been currently raised evidently [13]. At present, the effective components of neonicotinoid insecticides were reported to be detected in many kinds of crops treated with nicotinic acid [14]. However, as the two most widely used pesticides, the adverse effects of fipronils and neonicotinoids on plants are still uncertain at present.

Given that ubiquitous pesticides would be released into the soil along with the wide usages and accumulation, plants may face these two kinds of pesticides challenges during their lifespan [15,16]. Nevertheless, most research regarding the adverse effects of these pesticides mainly focused on the human and animal health, relatively little is known about the response of plants to pesticides exposure [4,17]. As a matter of fact, the abusage of pesticides would lead to the excessive generation of reactive oxygen species (ROS) and then endanger the organisms [16–18]. In the meanwhile, the activity of ROS scavenging enzymes will decreases due to the exposure under oxidative stress, which affects the normal metabolism of cells [16]. In addition, the accumulation of ROS can also lead to membrane lipid peroxidation and serious damage to the cell membrane system [17]. To protect against oxidative damages, plants have developed the effective antioxidant defense system including enzymatic and non-enzymatic antioxidants [17,19]. As the core component of glutathione antioxidant system, glutathione *S*-transferases (GSTs) play important roles in scavenging the damages of ROS and removing toxins. GSTs catalyzed one of the vital steps in the

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https://doi.org/10.1016/j.ijbiomac.2020.08.061
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detoxification of xenobiotics through catalyzing the binding of reduced GSH with various toxic substrates to produce water-soluble products, thus glutathione *S*-transferases appeared to be a potential biomarker to assess ROS induced cell injuries produced by contaminants [20,21]. Based on the sequence homology and structural similarity, GSTs in plants can be divided into five categories: phi, tau, theta, zeta and lambda [22]. AtGSTF8, the glutathione *S*-transferase Phi8 class in *Arabidopsis thaliana*, was reported to be specific to plants and the gene expression could be induced by defense signals, certain chemical stresses and pathogens [23,24]. Therefore, AtGSTF8 was presumed as an important biomarker to evaluate ROS induced cell damage due to the sensitive response to oxidative stress [24].

In view of the profusely distribution in the natural environment and a potential threat to plants of these two kinds of most widely used pesticides, it is of great significance to employ AtGSTF8 as an important biomarker to evaluate the toxic effects of these pesticides on plants at the molecular level. In this study, the structural changes of AtGSTF8 conformation upon binding with the three pesticides were investigated by multispectral methods firstly. Then the differences of the binding affinity about AtGSTF8 with pesticides were further characterized by Bio-Layer Interferometry (BLI). Besides, the glutathione S-transferase activity changes of AtGSTF8 upon binding with these two kinds of pesticides were also determined to evaluate the hazardous effects of them. At last, the molecular docking experiments were performed to perceive the binding differences of AtGSTF8 with these two kinds of pesticides. This work can help to evaluate the different damages of these pesticides to plants through exploring on the binding mechanism of AtGSTF8 with them at molecular level.

2. Materials and methods

2.1. Materials

Fipronil, clothianidin and dinotefuran were purchased from Ark Pharm, Inc. (USA), whose structures were shown in Fig. 1. All the chemicals in this work were analytical purity and ultra-pure water was used throughout the experiment. The preparation of AtGSTF8 was performed as described previously [25].

2.2. Fluorescence spectrometry

The fluorescence measurements were carried out by F-4600 fluorescence spectrometer (Hitachi, Japan) with a buffer involving 50 mM HEPES (pH 7.5), 50 mM NaCl and 3 mM DTT. The excitation wavelength was set at 280 nm and the fluorescence spectra of AtGSTF8 were collected from 290 to 450 nm. The excitation and emission slits were set at 5 nm.

Synchronous fluorescence spectra of AtGSTF8 were recorded with fixed $\Delta\lambda = 60$ nm for tryptophan residues and $\Delta\lambda = 15$ nm for tyrosine residues, respectively.

2.3. Ultraviolet-visible absorption spectra analysis

The ultraviolet-visible absorption spectra of AtGSTF8 in the presence and absence of three pesticides were determined by UV-3900 spectrophotometer (Hitachi, Japan) with the scanning range from 190 nm to 450 nm. The same concentration of pesticide without AtGSTF8 was used as a reference during the measurement.

2.4. Spectrometric determination of circular dichroism

The circular dichroism (CD) spectra of AtGSTF8 with and without these insecticides were collected by Chirascan Circular dichroism spectrometer (Applied Photophysics, England) ranging from 190 nm to 260 nm. Each spectrum was measured three times and the content of secondary structure was calculated by CD-Pro software.

2.5. Glutathione S-transferase activity measurement

The glutathione *S*-transferase family can catalyze the binding of GSH with 1-chloro-2,4-dinitrobenzene (CDNB). In this study, the absorbance of remaining GSH at 412 nm was measured by ultraviolet spectrophotometer (TU-1810, Beijing) using CDNB as substrates to reflect the activity changes of GST. Each measurement was performed in triplicate.

2.6. Bio-layer interferometry

A typical bio-layer interferometry (BLI) assay involves four essential steps including an immobilization of the ligand, equilibrium step, association of the analyte and a dissociation step [26]. Affinity of the complex of AtGSTF8 with three pesticides was determined by Octet RED96e instrument (ForteBio Inc., Menlo Park, USA) based on the biolayer interference technique with a buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl and 1% absolute ethanol at 30 °C. The anti-GST biosensors were pre-balanced in the same buffer for 10 min before the experiments. Fipronil and neonicotinoid insecticides including clothianidin and dinotefuran, were diluted in the same buffer solution and used as analytes. The experimental data were fitted globally by the Octet data analysis software 10.0, and the kinetic and affinity parameters were obtained.

2.7. Molecular docking

To investigate the molecular binding mechanism of the two kinds of pesticides with AtGSTF8, molecular docking was performed through AutoDock 4.2.0 program [27,28]. The tertiary structure of AtGSTF8 was generated by the iterative threading assemble refinement (I-TASSER) server based on homology modeling [29]. The predicted structures with high C-score and TM-score were analyzed and selected. The three-dimensional structures of pesticides were constructed in Sybyl×1.1 (Tripos Inc., St. Louis, USA) and the energy minimized conformations were obtained through MMFF94 force field. Polar hydrogens

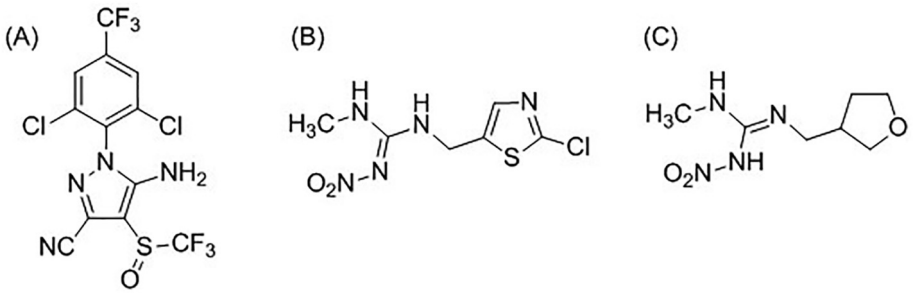

Fig. 1. The molecular structures of fipronil (A), clothianidin (B) and dinotefuran (C).

were added and the partial charges were assigned to the structure model before docking. Rotations and torsions for the ligands were assigned using AutoDock Tools. During the docking process, a grid box with dimensions of 126 Å × 126 Å × 126 Å and a grid spacing of 0.375 Å was employed. The Lamarchian genetic algorithm (LGA) was used to obtain the binding conformations and a total of 100 runs were performed during docking simulation. The conformation with energetic optimum was selected for further analysis.

3. Results and discussion

3.1. Fluorescence intensity comparisons

With wide applications of fipronils and neonicotinoids, the release and accumulation has aroused widespread concerns [14,15]. Currently, most of researches are mainly focused on the negative impacts on human beings and animals [1,13]. However the toxic effects on the agricultural environment, especially for glutathione antioxidant system, have rarely been reported. Endogenous fluorescence has been widely used to explore the structural changes of proteins, particularly from the alterations of fluorescence intensity [30]. Based on the analysis of fluorescence intensity, the binding information of the protein with compounds can be obtained, such as binding constant, binding forces, quenching mechanism, etc. [30,31]. As shown in Fig. 2, the fluorescence intensity of AtGSTF8 was quenched significantly in the presence of these two kinds of pesticides respectively. Besides, dinotefuran was observed to exhibit more evident ability to quench the fluorescence of AtGSTF8 comparing with fipronil and clothianidin. Therefore, it can be inferred that the three pesticides may exert some effects on the structure of AtGSTF8, especially for dinotefuran. Fluorescence quenching of the protein is usually attributed to a variety of molecular interactions including excited-state reactions, energy transfer, ground-state complex formation and collisional quenching [32]. Therefore, it will be essential to elucidate the binding properties of AtGSTF8 with these pesticides to further evaluate the toxic mechanism produced by pesticides exposure own to glutathione antioxidant system.

3.2. Interaction characterizations of AtGSTF8 with fipronil and neonicotinoids by bio-layer interferometry

As a label-free and real-time optical analysis technique, bio-layer interferometry can be used to measure the intermolecular interactions [33]. In this work, AtGSTF8 was firstly immobilized onto the anti-GST sensors and then the whole association and dissociation processes of AtGSTF8 with the three pesticides were recorded in real time using different concentrations of the pesticides as analytes. The kinetic rate constants (K_a, K_d) and affinity constants (K_D) were listed in Table 1. The magnitude of K_D is generally used to compare the binding strength of different types of reactions [34], and the affinity with the range of 10^{-4}–10^{-7} M is representative for the binding with small molecules (molecular weight < 2 kDa). As illustrated in Fig. 3, the affinity values of fipronil and clothianidin with AtGSTF8 in the order of 10^{-6} M indicated a relative moderate interaction, but the latest generation of neonicotinic pesticide dinotefuran displayed extremely weaker binding affinity with AtGSTF8 clearly deviated from clothianidin and fipronil. Deduced from Table 1, the differences of binding affinity among the three pesticides were resulted from the evidently distinct association constants although the dissociation constants were exhibited in the same order of magnitude. In contrast to other functional proteins from human with the binding constant of 10^{-4} M between HSA and clothianidin and 10^{-5} M between the chemosensory protein (CSP) and fipronil, the reported binding affinities are presumed to be weaker than AtGSTF8 with fipronil and clothianidin described in this work [35]. However, the reported binding constants speculatively deduced from the mechanism of fluorescence quenching and binding constants in this work measured by the BLI analysis technique are not comparable due to the different determination methods. Therefore, fipronil and neonicotinoids including clothianidin and dinotefuran were exhibited to combine with AtGSTF8 based on the BLI technique throughout the whole measurements, and among of which dinotefuran displayed relatively weak binding affinity with AtGSTF8 and was presumed to impose weak impacts on the plants damage.

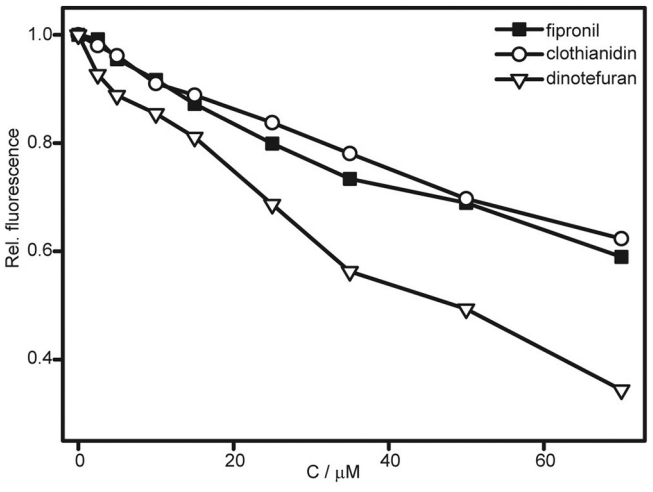

Fig. 2. Effects of three pesticides on the fluorescence intensity of AtGSTF8. c(AtGSTF8) = 5.0×10^{-7} mol/L and c (fipronil, clothianidin, dinotefuran) = 0, 2.5, 5, 10, 15, 25, 35, 50 and 70 μM.

3.3. Conformational analysis of AtGSTF8 under exposure of fipronil and neonicotinoids

To evaluate the structural and conformational changes of AtGSTF8 in the presence of the three pesticides, UV–vis absorption spectra, synchronous fluorescence and CD spectra were investigated.

3.3.1. Ultraviolet-visible absorption spectra

Ultraviolet-visible absorption spectroscopy technique can be used to investigate the structural changes of proteins and the complex formation [36]. As shown in Fig. 4, two absorption peaks with varying concentrations of the pesticides were observed. The strong absorption peak at about 230 nm is ascribed to the π → π* electronic transitions of peptide backbone structure C═O, which reflects the protein skeleton conformations [37]. The other weak absorption peak located around 280 nm is originated from conjugated double bonds of aromatic amino acids [32]. From Fig. 4, the intensity of the strong peak decreased clearly with an apparent red shift in the presence of the three pesticides separately due to the loosening and unfolding of the protein skeleton. In addition, the absorption peak around 280 nm decreased with the addition of neonicotinoids including clothianidin and dinotefuran indicating the hydrophobic microenvironments of aromatic amino acids increased (Fig. 4B and C), whereas the peak intensity of AtGSTF8 with fipronil increased in concentration dependent manner from Fig. 4A, which indicated that fipronil could enhances the microenvironmental hydrophilicity of aromatic amino acids.

Table 1
Kinetic and thermodynamic parameters of the interactions between AtGSTF8 and fipronil and neonicotinoids including clothianadin and dinotefuran.

Analytes	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (M)
Fipronil	3.8×10^3	2.74×10^{-2}	7.22×10^{-6}
Clothianadin	1.46×10^4	3.58×10^{-2}	2.45×10^{-6}
Dinotefuran	5.57×10^2	6.93×10^{-2}	1.24×10^{-4}

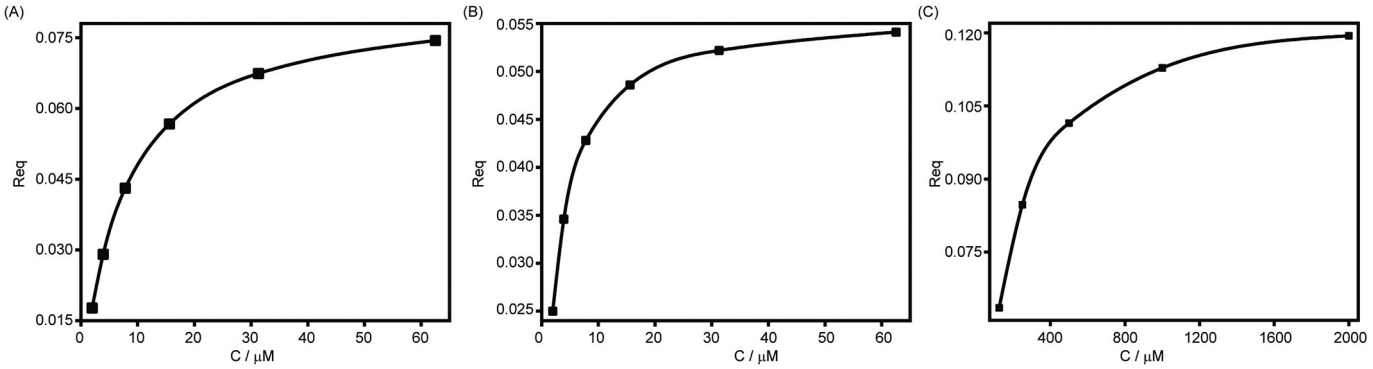

Fig. 3. Steady-state kinetics of AtGSTF8 with fipronil (A), clothianidin (B) and dinotefuran (C).

3.3.2. Synchrotron fluorescence

The synchrotron fluorescence spectroscopy can provide characteristic information about microenvironmental structural variations around the tryptophan and tyrosine residues by scanning the excitation and emission wavelengths simultaneously [38]. The changes of maximum emission wavelength of the synchronous fluorescence spectra generally reflect the polar variations of the aromatic residues [39]. The red shift means the more hydrophilicity around the aromatic residues, while the blue shift implies the increase of hydrophobicity [31,40]. As illustrated in Fig. 5, the fluorescence intensity of both tryptophan and tyrosine residues decreased gradually with the separate addition of fipronil, clothianidin and dinotefuran to AtGSTF8. Furthermore, from Fig. 5A and C the emission peaks of tryptophan residues of AtGSTF8 in the presence of fipronil and clothianidin exhibited slight blue shift, indicating the increment of hydrophobicity around the tryptophan residues. In contrast, significant red shift could be observed for the tryptophan residues of AtGSTF8 as shown in Fig. 5E, resulted from the more hydrophilic microenvironment along with addition of dinotefuran. Besides, all the õemission peaks of tyrosine residues of AtGSTF8 with three pesticides exhibit a slight red shift due to the decrease of hydrophobicity and conformational changes of the protein as illustrated in Fig. 5.

3.3.3. Circular dichroism

Circular dichroism (CD) is universally considered to be a conventional method to detect the secondary structural changes of the protein upon interaction with micromolecules [41,42]. In this work, circular dichroism measurements were also essential to explore the effects of fipronil and neonicotinoids on the secondary structure of AtGSTF8. As shown in Fig. 6, there are two negative peaks at about 208 and 222 nm, representing the α-helix [43]. With the addition of fipronil and clothianidin, the negative peak intensity at 208 nm decreased and a slight blue shift was observed, while the negative peak at 222 nm did not alter apparently owing to the enhancement of hydrophobic interaction of AtGSTF8 and the energy increase of π-π* electron transition [37,44]. In contrast, the negative peak intensity at 208 nm of AtGSTF8 with dinotefuran decreased with red shift. Furthermore, the α-helix contents of AtGSTF8 decreased from 17% to 12.1%, 9.1% and 7.5% with addition of fipronil, clothianidin and dinotefuran, respectively, indicating adverse effects of the three pesticides on the secondary structure of AtGSTF8 and the more significant impact of dinotefuran on AtGSTF8 in comparison with another two pesticides.

3.4. Assessment of GST activity

Certain structures of proteins are closely related to their specific functions, so the normal physiological functions of proteins will also change if their structures are altered [25,45]. Based on the series of spectroscopic results as mentioned above, the structure of AtGSTF8 was varied along with addition of fipronil, clothianidin and dinotefuran, respectively. Therefore, it could be deduced that fipronil and neonicotinoids also have different effects on the activity of AtGSTF8. As shown in Fig. 7, the relative activities of AtGSTF8 without pesticides were regarded as 100%. With the increase of pesticide concentration, the relative activity of AtGSTF8 declined to 65%, 75% and 80% of the initial level with gradual addition of fipronil, clothianidin and dinotefuran, respectively. Hence, the activity of the glutathione S-transferase was affected based on the structural alterations of AtGSTF8, but the activity variation of AtGSTF8 in the presence of dinotefuran was not proportional to the evident influence on the structure for AtGSTF8.

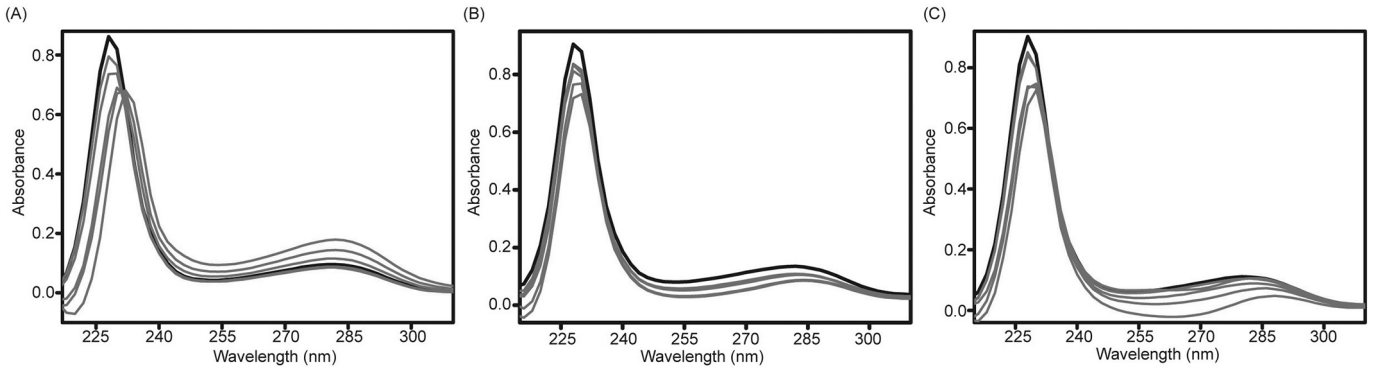

Fig. 4. Ultraviolet-visible absorption spectra of 10 μM AtGSTF8 with gradual increasing concentrations of fipronil (A), clothianidin (B), and dinotefuran (C). c(fipronil, clothianidin, dinotefuran) = 0, 5, 15, 30, 50, 70 and 100 μM.

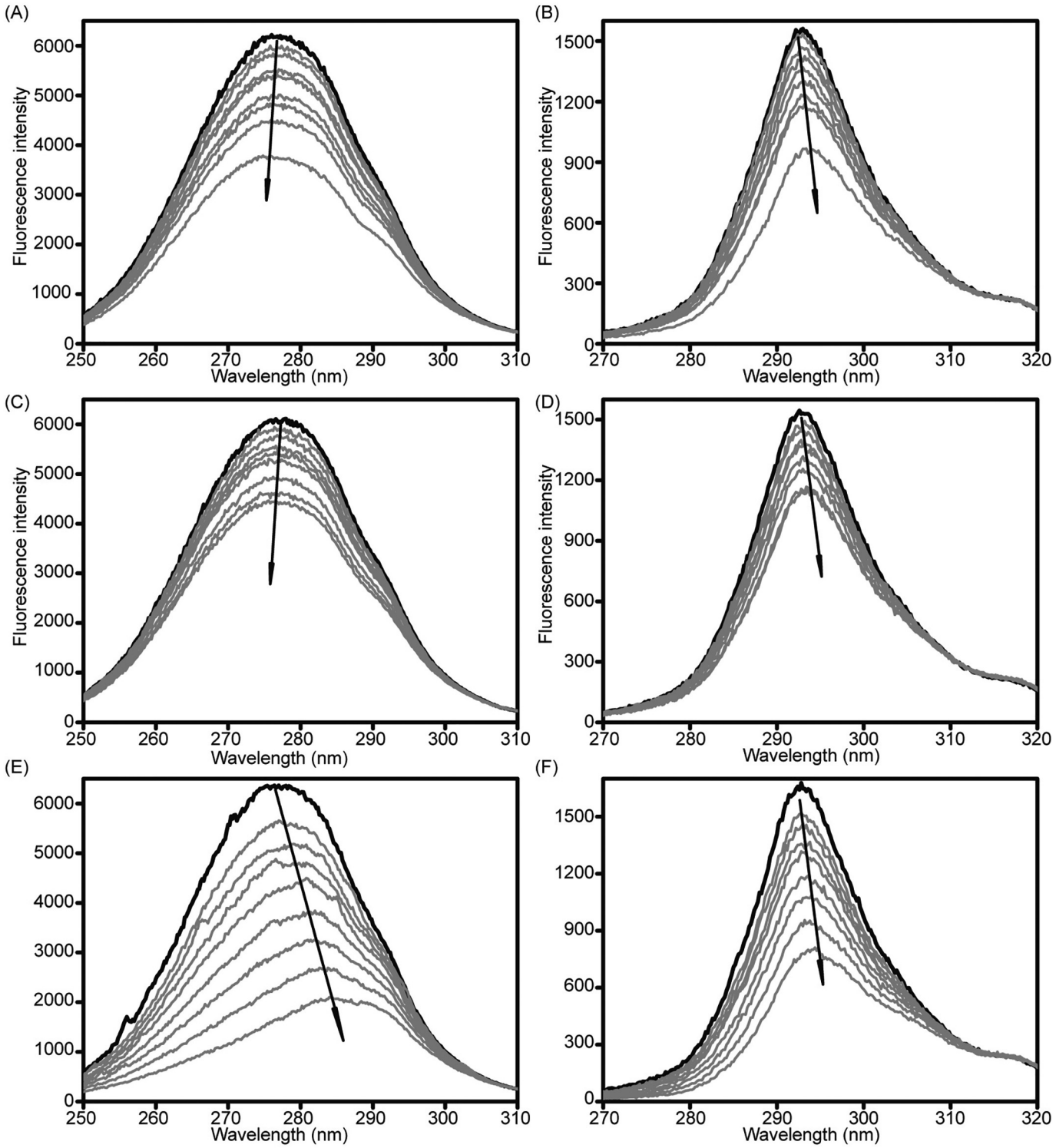

Fig. 5. Synchronous fluorescence spectra of AtGSTF8 at $\Delta\lambda = 60$ nm (A, C, E) and $\Delta\lambda = 15$ nm (B, D, F) at variable concentrations of fipronil (A, B), clothianidin (C, D), and dinotefuran (E, F). The concentrations of pesticides are 0, 2.5, 5, 10, 15, 25, 35, 50 and 70 μM. The concentration of AtGSTF8 is 5.0×10^{-7} mol/L.

3.5. Molecular docking investigation of AtGSTF8 towards fipronil and neonicotinoids

As multifunctional enzymes, glutathione S-transferases contribute to the cellular protection and detoxification through combining with environmental contaminants. Despite the glutathione *S*-transferase classes in plant have only limited sequence identity, they share a significant structural homology [46,47]. GSTs were reported to exhibit two spatially distinct domains: the N-terminal domain with a typical thioredoxin-like fold including α-helices and β-strands contains a glutathione binding site (G-site) and the C-terminal domain with all α-helical contains a hydrophobic substrate binding site (H-site) [48–50]. To predict the preferential binding sites of AtGSTF8 with the three pesticides, the molecular docking experiments were performed and the docked complexes with the lowest binding energy were selected for further analyzing. As displayed in Fig. 8, fipronil was predicted to be

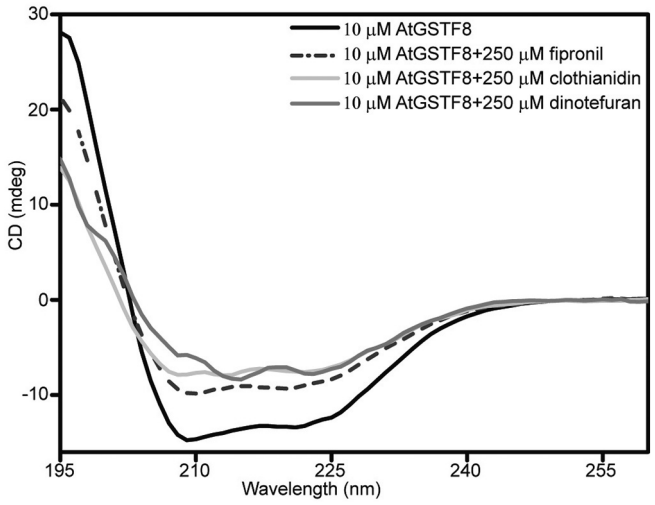

Fig. 6. Circular dichroism spectra of AtGSTF8 with and without the three pesticides. The concentration of AtGSTF8 is 1.0×10^{-5} mol/L.

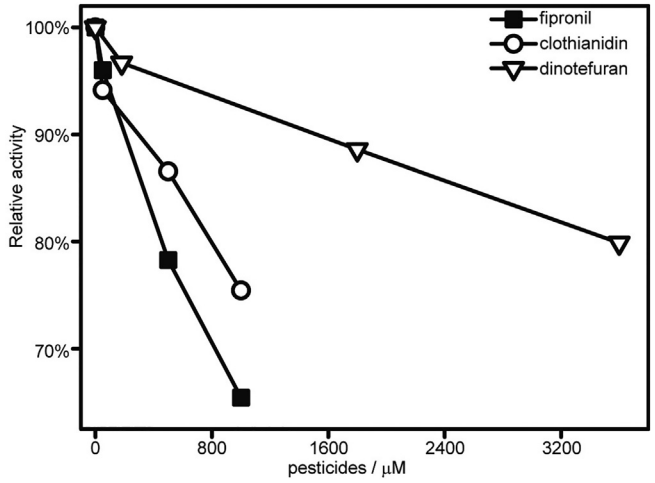

Fig. 7. Enzyme activity changes of AtGSTF8 under the exposure to different concentrations of pesticides.

inserted into the cavity of AtGSTF8 which was adjacent to the G-site in correlation with activity of the glutathione S-transferase, while clothianidin and dinotefuran were presumed to locate on the relative small pocket on the molecular surface of AtGSTF8. Therefore, the close binding site of fipronil from the active G-site may strongly affect the enzymatic activity, even though the complex formation of AtGSTF8 with fipronil not imposed significant influence on the structure in contrast with other two neonicotinic insecticides.

4. Conclusions

In this study, the toxic mechanisms of fipronil and neonicotinoids including clothianidin and dinotefuran were explored at molecular level for the first time. The intrinsic fluorescence of AtGSTF8 was quenched by all pesticides. Then AtGSTF8 was proven to bind with the two kinds of pesticides through bio-layer interferometry, among of which dinotefuran displayed weaker binding affinity to AtGSTF8 in contrast with another two pesticides. Besides, the conformational and structural alterations were also observed in the presence of these pesticides resulted in the increment of hydrophilicity around the vicinity of Tyr and Try residues and unfolding of the structural skeleton, especially for dinotefuran with the quite visible effects on the structure of AtGSTF8. With the addition of the three pesticides the activities of the glutathione S-transferase were decreased accompanied by the structural changes, but the latest generation of neonicotinic insecticide dinotefuran did not impose significant effects on the enzyme activity. At last, fipronil and neonicotinoids were presumed to locate on the different binding site of AtGSTF8. In brief, both fipronil and neonicotinoids posed adverse effects on AtGSTF8 at structure and enzyme activity aspects, while the especial latest generation neonicotinoid dinotefuran exhibited the weakest affinity with AtGSTF8 but with major effects on the structure. This work may provide comprehensive insights into the interaction mechanism of AtGSTF8 with three pesticides at molecular level and assessment of their toxicity to plants.

CRediT authorship contribution statement

Yanhua Xie: Investigation, Writing - original draft.

Xiaomin Hou: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

This research was supported by National Natural Science Foundation of China (No. 31200554), Project of Shandong Province Higher Educational Science and Technology Program (No. J16LE11), Key Research and Development Plan of Shandong Province (2019GSF107074) and Qingdao Science and Technology Program (No. 13-1-4-211-jch, No. 16-5-1-69-jch).

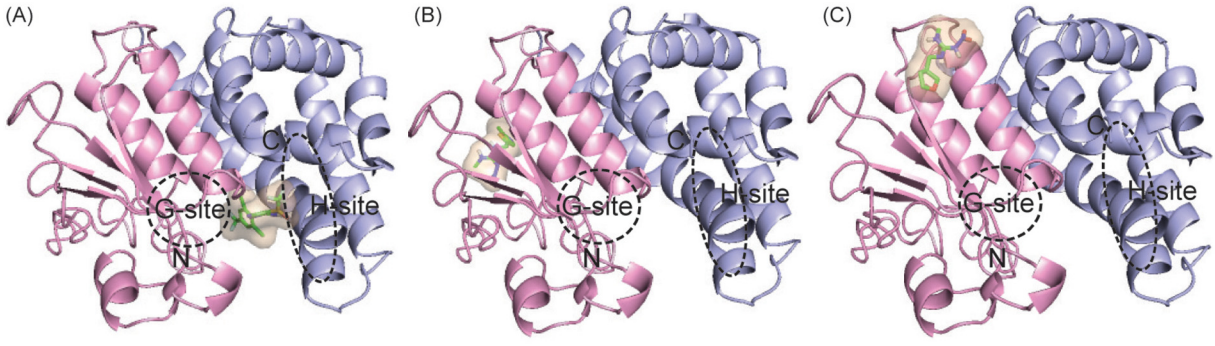

Fig. 8. Molecular docking results of AtGSTF8 with fipronil and neonicotinoids. AtGSTF8 was shown in cartoon. The pesticides were shown both in surface and stick representation with 50% transparency and colored as green. The N-terminal domain of AtGSTF8 was colored in pink and the C-terminal domain was in light blue. The G-site and H-site of AtGSTF8 were marked by black dashed circles.

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