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ORIGINAL PAPER

Earthworms (*Eisenia foetida*, Savigny) mucus as complexing ligand for imidacloprid

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Abstract Earthworms can excrete copious amounts of mucus that may affect the fraction, transport fate, and bioavailability of contaminants in soil. However, interaction of mucus with organic contaminants is still not well-known. In the present study, complexation properties of surface mucus (from the earthworm species *Eisenia foetida*, Savigny) with imidacloprid were investigated using fluorescence excitation emission matrix (EEM) spectroscopy. It was found that carbohydrates and proteins are major components in mucus of this species. Two fluorescent peaks belonging to protein-like substances were identified in the EEM spectrum of mucus. The protein-like fluorescence was clearly quenched by imidacloprid, indicating that the protein-like substances reacted strongly with imidacloprid. The fluorescence quenching processes was governed by a static process. The values of effective quenching constant ($\log K_a$) for these two peaks were 11.46 and 7.96, respectively, indicating that there is a strong interaction between mucus and imidacloprid and mucus–imidacloprid complexes are formed. Higher binding constants ($\log K_b$= 25.6 and 14.0) than those for heavy metals binding to dissolved organic matter or organic pollutants binding to proteins confirm the strong complexation between mucus and imidacloprid. Our study implies that earthworm surface mucus may significantly affect the fraction, toxicity, and bioavailability of organic contaminants in the soil due to its high affinity for organic contaminants.

Keywords Earthworm · Mucus · Complexation · Fluorescence titration

Introduction

Imidacloprid (1-[(6-chloropyridin-3-yl) methyl]-*N*-nitro-4, 5-dihydroimidazol-2-amine) is a systemic chloronicotinyl insecticide that specifically blocks the nicotinergic neuronal pathway. It is widely used in agriculture against sucking pests, such as aphids, thrips and white flies, soil insects, and termites (Moriya et al. 1993; Poland et al. 2006). Imidacloprid can be persistent in environment being not readily biodegradable (Tišler et al. 2009) and can be toxic to algae, isopod animals, and fishes (Drobne et al. 2008; Tišler et al. 2009). Due to its increasing use in crop protection and due to its persistence in soil up to 2 years (Kalpana et al. 2002), the use of imidacloprid is of growing environmental concern (Drobne et al. 2008).

Earthworms are ubiquitous in most soils. Earthworms improve soil structure, increase soil fertility, and plant growth (Lee 1985; Stephens et al. 1994; Pawlett et al. 2009). Earthworms can also significantly affect the distribution of heavy metals in soil and thus affecting the bioavailability of these metals (Cheng and Wong 2002; Wen et al. 2004; Udovic et al. 2007). Earthworms have also been shown to retard the binding of organic pollutants to soils, desorb previously soil-bound pollutants for subsequent degradation, and thus promoting biodegradation of organic pollutants (Hickman and Reid 2008). Therefore, earthworms have been used to assist bioremediation of soils

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contaminated with organic compounds (Eijsackers et al. 2001; Verma et al. 2006; Schaefer and Filser 2007).

Earthworms excrete mucus from body surface. The mucus is a mixture of water, electrolytes, and a number of macromolecules including glycoproteins, mucopolysaccharides, lectins, and hemocyanin (Chen et al. 1990; Heredia et al. 2008).

The mucus yield is high (Dayrup-Olsen and Luchtel 1998), and it was reported that 1 g of earthworms can, on average, produce 5.6 mg of mucus (dry weight) in 24 h (Chen et al. 1990). The large amount of mucus excreted by earthworm can exert an influence on the transport and fate of organic pollutants in the soil and plays a role in bioremediation of polluted soil. Recently, earthworm mucus has been shown to enhance plant growth and increase Cd uptake and transport in plants (Zhang et al. 2009a). However, effects of mucus excreted by earthworms on transport and fate of organic pollutants have been poorly known.

Fluorescence excitation emission matrix (EEM) spectroscopy is a rapid and sensitive method for characterizing fluorescent organic compounds and their interaction with pollutants (Lu and Jaffe 2001; Zhang et al. 2010). Since proteins are the key components that show fluorescence properties in earthworm mucus and mucus was demonstrated to have autofluorescence properties (Heredia et al. 2008), EEM spectroscopy can be employed to study the complexation of mucus with imidacloprid.

The aim of the present study was to investigate the complexation properties of mucus of earthworm with imidacloprid using EEM spectroscopy, with calculation of important parameters, including effective quenching and binding constants, which help understanding the role of earthworm surface mucus in influencing the fate and bioavailability of organic pollutants in soil.

Materials and methods

Earthworm mucus collection

Earthworms (*Eisenia foetida*, Savigny) were collected from the grassland of Institute of Geochemistry, Chinese Academy of Sciences, Guiyang, China. The 0–10-cm-depth soil was dug with a stainless steel shovel, and the earthworms were picked up by hands and then were immediately transferred to the laboratory. Two hundred mature clitellate earthworms with an individual biomass of 350–500 mg were selected for collection of mucus. The earthworms were rinsed with deionized water at least five times and then laid on moist filter paper for 2 days to completely empty their gut contents in order to minimize the effect of cast. Earthworms were then thoroughly rinsed with deionized water, dried with filter paper, weighed, and placed in ten 150×20-mm Petri dishes at 20°C in the dark. Twenty earthworms were placed in each Petri dish. After 12 h, the earthworms were removed from the Petri dishes. A part of Petri dishes was weighted before and after vacuum dry at 35°C to calculate the water content of mucus. The rest of Petri dishes with mucus were washed with deionized water, and the washing water was considered as the mucus sample; it was filtered through 0.45-μm membrane and freeze-dried. The mucus yield was calculated from the weight of earthworms and mucus. The freeze-dried mucus sample was stored at 4°C in the dark until use. The mucus solution (15 mg L^{-1}) was prepared by dissolving the freeze-dried mucus sample in deionized water for chemical analysis and fluorescence measurement.

Chemical analysis of mucus

Carbohydrate content in mucus was measured by the phenol sulfuric acid method with glucose as the standard (Dubois et al. 1956). Protein content in mucus was determined by Bradford's (1976) method using bovine serum albumin as the standard.

Preparation of imidacloprid solution

Imidacloprid with purity of 96.4% was obtained from Shandong LianHe Pesticide Inc., China. The stock imidacloprid solution (0.002 M) for fluorescence titration was prepared by dissolving imidacloprid in deionized water. The imidacloprid was stored at 4°C in the dark until use.

EEM fluorescence spectroscopy and fluorescence titration

The EEM spectra of the mucus solutions were recorded with a fluorescence spectrophotometer (F-7000, HITACHI, Japan). The EEM spectra were collected at 5 nm increments over an excitation range of 230–400 nm, with an emission range of 250–500 nm every 2 nm. The excitation and emission slits were set to 5 and 10 nm of band-pass, respectively. The scan speed was 1,200 nm min^{-1}. The Milli-Q water blank was subtracted from the sample's EEM spectra, and EEM data were processed using the software SigmaPlot 10.0 (Systat, USA). All experiments were conducted in triplicate, and the mean values were used.

Mucus solution (15 mg L^{-1}) in a 1×1-cm quartz cuvette was titrated with incremental additions of 5 μL 0.002 M imidacloprid at 298 K. After each addition of imidacloprid, the solution was mixed using a magnetic stirrer for 15 min. Fluorescence quenching titrations of mucus by imidacloprid were conducted.

Results and discussion

Mucus yield and its composition

It was found that, on average, 1 g of earthworm excretes 3.6 mg of mucus in dry weight in 12 h. The pH value of the mucus solution was 6.7. The water content of the mucus was 98.4% whereas the carbohydrate and protein contents were 174.2 and 147.8 mg g^{-1} mucus DW, respectively.

EEM spectra of mucus

Two fluorescence peaks were found in the EEM spectrum of *E. foetida* mucus. Peak A was detected at Ex/Em=280/342 nm and peak B at Ex/Em=230/344 nm. Because protein-like fluorophores have two excitation wavelengths at 220–230 and 270–280 nm in EEM spectra (Yamashita and Tanoue 2003) and proteins are the main components in mucus, peaks A and B could be assigned to protein-like fluorescence.

Fluorescence titration of mucus with imidacloprid

It can be seen from Fig. 1 that fluorescence of peaks A and B was clearly quenched by imidacloprid, indicating that the fluorophores of mucus reacted with imidacloprid.

Stern–Volmer equation was used to analysis the fluorescence quenching data (Gauthier et al. 1986).

$$F_0/F = 1 + k_q\tau_0[\text{imidacloprid}] = 1 + K_{SV}[\text{imidacloprid}] \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher, respectively. Parameter k_q is an energy transfer rate (liters per mole per second), while τ_0

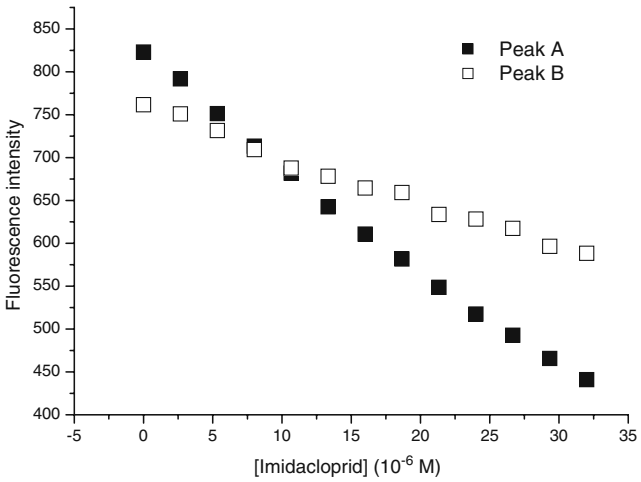

Fig. 1 Variation of fluorescence intensities of peaks A and B in EEM fluorescence spectroscopy of earthworm mucus with increasing imidacloprid concentration up to 32 μM at 293 K

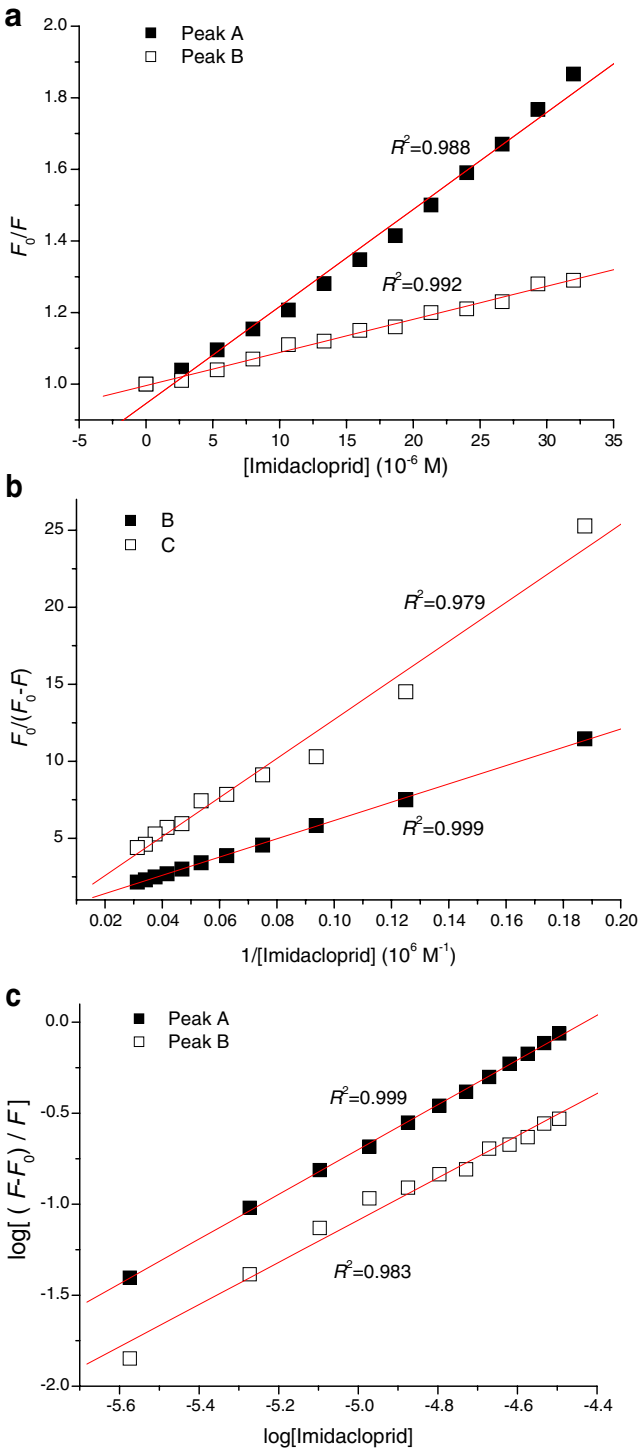

Fig. 2 **a** Stern–Volmer plots for the fluorescence quenching data of earthworm mucus by imidacloprid at 293 K, **b** modified Stern–Volmer plots of fluorescence emission quenching of earthworm mucus titrated with imidacloprid in the range of 2.67–32 μM at 293 K, and **c** plots of $\log[(F_0-F)/F_0]$ versus log[Imidacloprid] for binding of imidacloprid to earthworm mucus at 293 K

refers as to lifetime of fluorescence (seconds), which taken as 10^{-8} s (Lakowicz 1999), and [imidacloprid] is the concentration of the imidacloprid. If the fluorescence titration data are represented by the Stern–Volmer equation, a single quenching mechanism can involve either static or dynamic quenching. In the present study, good linearity was obtained for the plot of F_0/F against [imidacloprid] for both peak A and peak B (R^2>0.987; Fig. 2a). The K_{SV} and K_q were 2.71×10^4 and 2.71×10^{12} M^{-1} for peak A and 2.71×10^4 and 2.71×10^{12} M^{-1} for peak B, respectively. The values of logK_{SV} for mucus–imidacloprid system were close to that for human serum album (HAS)–imidacloprid system (Wang et al. 2009). The values of K_q for both peak A and peak B were 2 orders of magnitude greater than the maximum diffusion collision quenching rate constant (2.0×10^{10} M^{-1} s^{-1}) for a variety of quenchers with biopolymer (Hu et al. 2005). Therefore, the fluorescence quenching process of mucus by imidacloprid may mainly be static rather than a dynamic quenching process. In other words, interaction of mucus and imidacloprid was initiated by complex formation, and the dynamic collision could be negligible. This confirms the results of interaction between dissolved organic matter (DOM) and other organic pollutants (Traina et al. 1989; Chen et al. 1994; Doll et al. 1999; Lee et al. 2003). Lee et al. (2003) showed that interaction of leonardite humic acid with phenanthrene or pyrene is not dynamic and the values of logK_{SV} were 4.92 for phenanthrene and 4.56 for pyrene, respectively. In comparison with this study, the values of logK_{SV} (9.84 for peak A and 36.9 for peak B) were much greater. The values of logK_{SV} were close to those for pure protein and organic pollutant system. Silva et al. (2004) showed that the values of logK_{SV} for interaction of methyl parathion with human and bovine serum albumin were 7.9 and 12.2, respectively.

The fluorescence quenching data were further analyzed by the modified Stern–Volmer equation:

$$F_0/(F_0 - F) = 1/(f\,K_a[\text{imidacloprid}]) + 1/f \quad (2)$$

where F_0 and F are the fluorescence intensities in absence and presence of dicamba, respectively, f is the fraction of the initial fluorescence which corresponds to the binding fluorophore, K_a is the effective quenching constant, and [imidacloprid] is the imidacloprid concentration. Good linear relationship (R^2>0.978) was obtained for the plot of $F_0/(F_0-F)$ versus 1/[imidacloprid] (Fig. 2b). The values of f were 4.41 for peak A and 19.8 for peak B, respectively, indicating that 22.7% of the total fluorescence of peak A and 5% of the total fluorescence of peak B were accessible to imidacloprid, respectively. The values of logK_a for peak A and peak B were 3.58 and 2.02, respectively, which were lower than those for heavy metals binding to DOM but close to those for organic pollutants binding to protein (Table 1). This indicates that there is a strong interaction between mucus and imidacloprid, and they may form mucus–imidacloprid complexes.

Binding constants and binding sites

When small molecules are bound independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the following equation (Hill 1985):

$$\log[(F_0 - F)/F] = \log K_b + n\log[\text{Imidacloprid}] \quad (3)$$

where F_0 and F are the fluorescence intensities of fluorophore in the absence and presence of imidacloprid, respectively, K_b is the binding constant, and n is the binding site number.

Figure 2c showed that the plots of log $[(F_0-F)/F]$ versus [Imidacloprid] gave rather good linearity (R^2> 0.98). The values of logK_b for peak A and peak B were 25.6 and 14.0, respectively. This confirms that there is a strong interaction between mucus and imidacloprid and a complex formation of mucus–imidacloprid. The values of n for peaks A (1.16) and B (1.23) were a little higher than 1, suggesting that one class of sites was present in the fluorophores of mucus for imidacloprid. The values of logK_b and n were bigger than those for HAS–imidacloprid system (Wang et al. 2009).

Table 1 Effective quenching constant (logK_a) and fraction of initial fluorescence (f) for fluorophore–pollutant system in literature and in this study

Fluorophores	Pollutants	logK_a	f	Reference
River DOM	Hg(II)	5.01–5.62	28.2–44.2	Fu et al. 2007
DOM	Hg(II)	4.12–4.8	9.1–58.8	Lu and Jaffe 2001
DOM leached from biomass	Hg(II)	4.23–5.26	37.8–75.1	Lu and Jaffe 2001
Bovine serum albumin	Malachite green	2.16–3.73	–	Zhang et al. 2009b
Bovine serum albumin	Sudan I	3.49–4.46	–	Zhang et al. 2008
Exopolymers from biofilm	Hg(II)	3.28–4.48	76–93	Zhang et al. 2010
Mucus from earthworm	Imidacloprid	7.96 (peak A), 11.46 (peak B)	4.41 (peak A), 19.8 (peak B)	This study

Conclusion

Our study demonstrates that EEM fluorescence spectroscopy is a sensitive and useful tool for investigating interaction of mucus and organic pollutants. However, this method is only limited to the fluorescent components and cannot provide information on the components that do not emit fluorescence such as carbohydrates. Future studies can focus on complementary use of other methods including electrochemical titration and infrared spectroscopy in order to gain a comprehensive understanding of the interaction between mucus and pollutants.

Mucus contains strong complexing organic ligands for organic pollutants. In addition, mucus from earthworms may significantly affect the adsorption/desorption process and bioavailability of organic pollutants in the following ways: (1) the abundant water in mucus acts as solvent even in relative dry soils as earthworms move through the soil and (2) the presence of mucus with a strong binding capacity from earthworm may interact with organic pollutants in soil solution, mobilize organic pollutants from soil, alter the partition of organic pollutant between soil and soil solution, and finally enhance bioavailability or bioremediation. Recently, mucus from earthworm body surface has been demonstrated to enhance Cd accumulation in tomato seedlings (Zhang et al. 2009a).

There is dispute over whether dermal or intestinal uptake is the dominant mechanism for contaminant uptake in earthworms (Lanno et al. 2004). The high affinity of mucus for organic pollutants shows that body surface mucus can play an important role in the uptake of pollutants by earthworms. However, whether the mucus serves as a protective barrier against the toxic pollutants or promotes pollutants to enter the earthworm needs further study.

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