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Original article

Transformation of soil humic acids by *Aporrectodea caliginosa* earthworm: Effect of gut fluid and gut associated bacteria

Vladimir Tikhonov [a,*], Julia Zavgorodnyaya [a], Vladimir Demin [b], Boris Byzov [a]

[a] *Faculty of Soil Science, Lomonosov Moscow State University, Russian Federation*
[b] *Institute of Ecological Soil Science, Lomonosov Moscow State University, Russian Federation*

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ABSTRACT

The decomposition of recalcitrant organic compounds by soil organisms plays an important role in the carbon and nitrogen cycles. Earthworms are assumed as “hot spots”, where humic acids (HAs) are being transformed. In present work the transformation of HAs, extracted from Dystric Albic Planosol soil, was studied in presence of i) sterilized gut fluid (GF) of the earthworm *Aporrectodea caliginosa*, ii) one of the five bacterial strains (*Aeromonas encheleia*, *Bacillus thuringiensis*, *Acinetobacter* sp., *Aeromonas* sp. and *Streptomyces* sp.) isolated from the native GF and iii) simultaneous present of GF and one of the five bacterial strains.

The average molecular mass of the HAs decreased from 31 to 12 kDa upon incubation with the GF and to 22–23 kDa upon incubation with the bacteria only. Incubation of HAs with GF + bacteria decreased the molecular mass to 14–18 kDa, depending on the bacterial strain. The less intensive transformation of the HAs in the presence of the GF and bacteria was due to high concentration of easily accessible organic compounds in the GF. These substances are preferentially decomposed by bacteria, whereas HAs are less preferential substrate for bacteria consumption in these conditions. Thus, in the earthworm intestine, which is normally rich in organic matter and populated by gut-associated bacteria, the transformation of the HAs is poor. In conclusion, earthworms can intensively utilize HAs, and the rate of transformation depends on the presence of gut enzymes and gut-associated bacteria.

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1. Introduction

Earthworms are among the main participants of the various recycling and decomposition processes occurring in soil. The feeding strategy of earthworms involves assimilation of the most easily degradable fractions of soil organic matter [3–7,13,26], soil microorganisms (mainly fungi [7]) and also assimilation of soil humic substances [9,10]. At the same time, balance calculations have shown that earthworms are incapable of depolymerizing plant polymers [1,2]. Thus, the principal source of food for earthworms is still unknown. Feeding behavior of earthworms is different and depends on ecological strategies [1]. Soil can remain in the gut tract of earthworms for several hours and during this period the organic matter can drastically change [8]. Several studies have shown that earthworms can assimilate soil humic substances, in particular humic acids (HAs) [10,20]. Microorganisms have a number of enzymes that can breakdown the components of humic substances, e.g., laccases [11,12] and peroxidases [13–15]. Recently, we have demonstrated a decrease in molecular mass of HAs from peat as it passes through the digestive tract of *Aporrectodea caliginosa*, and have also reported a decrease in the molecular mass of humic substances under vermicomposting of cow manure by the earthworm *Eisenia fetida*. [20]. The growth of the bacterial isolates of *Aporrectodea caliginosa* intestine on HAs as sole carbon source has also shown [16,17]. The decrease in the molecular mass of HAs occurs via two processes—present of enzymes in the gut of earthworms and degradation by gut associated bacteria. We hypothesized that bacteria and the digestive tract enzymes may interact and promote decrease in HAs molecular mass. However, the effects of the combined action of the GF and gut-inhabiting bacteria in processing of humic substances are unclear; and seem to be important for understanding the role of gut-associated processes in transformation of soil recalcitrant substances like HAs.

The aim of this study was to determine the degree of transformation of HAs extracted from podzolic soil by the earthworm

* Corresponding author. Faculty of Soil Science, Moscow Lomonosov State University, Leninskie gory 1-12, 119991, Moscow, Russian Federation.
E-mail address: vvt1985@gmail.com (V. Tikhonov).

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Aporrectodea caliginosa. The role worm's gut fluid and gut-associated bacteria in the process was also assessed. We hypothesized that bacteria and the digestive tract enzymes may interact and promote decrease in HAs molecular mass.

2. Materials and methods

2.1. Humic acids

Humic acids were extracted from Dystric Albic Planosol (Ruptic) soil, collected in the Central-Forest National Reserve, Tver' region, Russian Federation. The annual temperature is 3.6 °C and annual precipitation is 770 mm.

The basic characteristics of HAs are as follows (%): ash, 6.5; C, 40; H, 37.8; N, 4.2; O, 18; oxidation state (w), 0.05. Dry HAs were dissolved in 0.1 M sodium phosphate buffer (pH 7) and filter sterilized (0.22 μm, Millipore).

2.2. Obtaining the gut fluid

Adult earthworms, *Aporrectodea caliginosa* (Sav.), were collected from the cultivated soddy-podzolic soil. To clean their digestive tracts from soil, the worms were kept for 4 days at room temperature in sterile sand (preheated to 700 °C), and then on moist filter paper for 2–4 days at 8 °C. For the isolation of intestines, the earthworms were autopsied on a freezing table (Peltier element, −16 °C) during thawing. A section of the digestive tract was taken out from the area of clitellum. The clean digestive tracts were homogenized and centrifuged at 12000 rpm for 10 min to obtain the gut fluid. The supernatant fluid was centrifuged and filter-sterilized. Sterility of the sample was confirmed by plating the filtered fluid on R2A-agar (Difco). The obtained gut fluid (GF) was lyophilized and stored at −18 °C [18].

2.3. Bacteria

For isolation of bacteria, the supernatant of the centrifuged GF was plated on R2A-agar. The bacteria were identified according to their morphological, physiological and 16S rRNA gene sequence by employing PCR amplification of the 16S rRNA genes with the subsequent sequencing of the amplicons and data analysis. For amplification of the nucleotide sequences, PCR of the 16S rRNA genes was carried out using the primers 27F (AGA GTT TGA TCCTGG CTC AG) and 1492R (ACG GCT ACC TTG TTA CGA CTT) on an ABI Prism 3130xl automatic sequencer (Applied Biosystems). The bacteria were stored in liquid R2A medium with glycerol (25%) at −18 °C. The obtained nucleotide sequences were compared with those in the GenBank database. For each sequence, the closest homolog with a known taxonomy was used for bacterial genotypic identification. All of the obtained sequences had at least 99% similarity with the prototype strain sequence available in the GenBank database. Five bacterial strains were identified as: *Aeromonas encheleia*, *Bacillus thuringiensis*, *Acinetobacter* sp., *Aeromonas* sp. and *Streptomyces* sp. For the preparation of the inocula, the bacteria grown for 48 h on R2A liquid medium were washed off twice with sterile water.

2.4. Determination of the molecular mass of the humic acids

The molecular mass of HA was determined by size-exclusion chromatography (Agilent Technologies) with a diode array detector and a ChemStation data processing system (LCChem). The employed parameters of the chromatographic process were as follows: column TSK-2000SW 7.5 × 60 (Tosoh Bioscience), 100 μL sample volume, eluent 0.1 M sodium phosphate buffered saline (pH 7), 1 g L^{-1} sodium dodecyl sulfate, the eluent flow rate 0.75 mL/min, scan wavelength 280 and 465 nm. The column was calibrated by using a mixture of globular proteins 13.7–65 kDa (Pharmacia). The average molecular masses of HAs fractions were calculated using a standard calibration curve. High molecular mass products eluted earlier. The peak, V_0, on the chromatogram represented HAs high-molecular mass fractions (>100 kDa), which were not considered for calculations. To compare molecular masses of the individual HAs fraction, peak offset was determined (i.e., an increase or decrease in the weight of the individual fractions). To determine the peak offset and the excess of the new fractions, analysis of the first and second derivatives in the chromatogram was carried out. A typical chromatogram of HAs for first derivative is presented in Fig. 1, $V_0 - V_3$ represent major chromatogram peaks. Every sample were repeated tree times, and confidence interval was calculated ($p = 0.05$).

2.5. Measurement of bacterial growth

The growth of bacteria was measured every hour on a 96-well microplate reader (Tecan, Sunrise) at a wavelength 620 nm and 25 °C; the microplates were automatically shaken before measuring. Five replicates were used.

2.6. The effects of bacteria on humic acids

Each cell of the microplates was supplemented with 25 μL of the mineral base Czapek-Dox medium, 25 μL of the HAs dissolved in 0.4 M sodium phosphate buffer (4 mg mL^{-1}) as a sole carbon source, 50 μL of distilled water, and 2 μL of the bacterial suspension. The controls were HAs with no bacterial cells and the bacterial cells without HAs; the volume was adjusted with 25 μL of 0.4 M sodium phosphate buffer.

2.7. The effects of the gut fluid on bacterial growth

To each cell of the microplates, 25 μL of the mineral base Czapek-Dox medium, 25 μL of 0.4 M sodium phosphate buffers, 50 μL of the GF and 2 μL of the bacterial suspension were added. The controls were GF with no bacterial cells and the bacterial cells without GF.

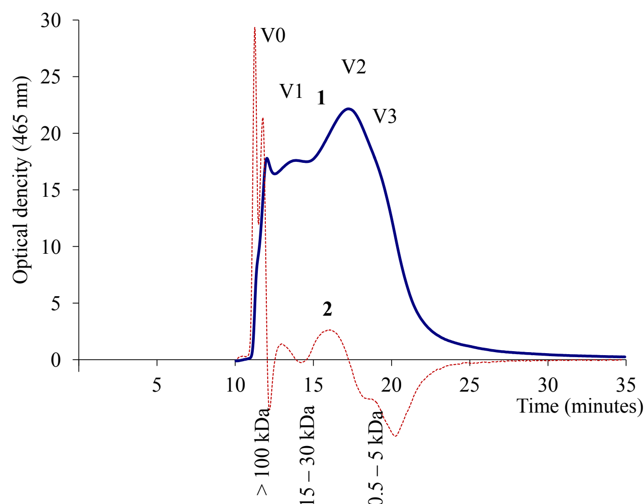

Fig. 1. Chromatogram of the humic acids (1) and of their derivate (2). V_0 – the peak with molecular mass of more than 100 kDa. V_1, V_2, V_3, – the peaks of the HAs fractions characterizing changes in HAs molecular mass.

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