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iTRAQ-based quantitative proteomic analysis of the earthworm *Eisenia fetida* response to *Escherichia coli* O157:H7

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ABSTRACT

Soil environment contaminated by *Escherichia coli* O157:H7 which come from the waste of infected animals. Earthworms can live in the pathogens-polluted soil by their innate immunity. How the proteins of earthworms *E. fetida* will response to *E. coli* O157:H7-contaminated-soil still unclear? To identify the defense proteins under *E. coli* O157:H7 stress, we performed a proteomic analysis of earthworm under *E. coli* O157:H7 exposure through an iTRAQ technology. In total, we found 283 non-redundant proteins, including fibrinolytic protease 1, lombricine kinase, lysozyme, gelsolin, coelomic cytolytic factor-1, antimicrobial peptide lumbricin-l, lysenin, and et al. The proteins participate in metabolic processes, transcription, defense response to bacterium, translation, response to stress, and transport. The study will contribute to understand why earthworm can live in the pathogens-polluted environment.

1. Introduction

The pathogenic *E. coli* strain O157:H7 is a foodborne bacterium that bring about human bloody diarrhea, and other sequelae (Mayer, 2012). *Escherichia coli* O157:H7 not only causes human diseases because of contaminated food (Stromberg, 2015; Williams et al., 2017), but also contaminates several environments, such as soil (Ma Jincai, 2011), and invertebrates (Williams et al., 2006; Castro et al., 2013). *E. coli* O157:H7 can survival in dairy and poultry composts (Chen et al., 2018), the soil in the farmland or garden with animal composts as the fertilizers will be easy to find *E. coli* O157:H7 contamination. *E. coli* O157:H7 also can be found in wildlife faecal samples (Jay, 2007). *E. coli* O157:H7 is foodborne pathogen, so the source of pollution is animal wastes (Guan and Holley, 2003).

Earthworms live in polluted-soil, which help in the expanding of *E.coli* O157:H7 (Artz et al., 2005; Williams et al., 2006). Earthworms can decrease the number of pathogenic bacteria using their antioxidant systems and antimicrobial immune functions (Liu et al., 2009). In our previously published paper, we characterized differential gene expression of earthworm response to *E.coli* O157:H7 by suppressive subtractive hybridization and protein expression by 2-DE (Wang et al., 2010a, 2010b, 2011a, 2011b). 2-DE has several limitations; for example, it provides poor separation of alkaline proteins, automation is limited (Ong, 2005), and the dissociate of membrane or hydrophobic proteins remains difficult using this system (Han et al., 2001).

iTRAQ technology is importment for finding biomarkers using tissues (Bouchal et al., 2009; Liu et al., 2017). To answer why earthworm can live in the *E.coli*- polluted soil, iTRAQ-based proteomic were used to find up- and down-regulated proteins. Differentially proteins response in whole *E. fetida* on days 3, 7, and 14 after treatment with *E. coli* O157:H7. The study will increase our understanding of earthworm innate immunity.

2. Materials and methods

2.1. Animals and artificial soil

The species of earthworm is *E. fetida* recommended by the OECD as a test organism (OECD, 1984). Earthworm culture, artificial substrates, 10^7 CFU g^{-1} soil *E. coli*:O157:H7 treatment was prepared as described in our published paper (Liu et al., 2009; Wang et al., 2010a, 2010b). The stress times of *E.coli* O157:H7 were 0, 3, 7, and 14 days.

After *E. coli* O157:H7 exposure at the days of 3, 7, and 14, the earthworms were collected for voiding the gut (Stein, 1981). Masks and medical latex gloves were worn while using *E. coli* O157:H7.

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2.2. Protein sample preparation

Protein sample were referred to our published paper (Wang et al., 2010a, 2010b).

2.3. Peptide labeling

Protein extract was subjected to disulfide reduction with dithiothreitol at 56 °C for 1 h and alkylation with 55 mM iodoacetamide at 25 °C for 1 h. Proteins were precipitated with acetone for 2 h and washed with ethanol. The protein was redissolved in tetraethylammonium bromideand and 0.1% sodium dodecyl sulfate. Each sample contained 100 μg of protein. Protein was digested by trypsin (Promega, Madison, WI, USA; 1:25, w/w) according to manual. After enzymatic cleavage, the sample was centrifuged and the supernatant was dried in a vacuum centrifuge. Each trypsinized peptide was then tagged with iTRAQ reagents (Applied Biosystems, Foster City, CA, USA). The earthworm sample was divided into four parts and treated as Fig. S1. The samples were labeled with iTRAQ reagent 114, 115, 116, and 117, respectively, at room temperature. The labeled samples were pooled together post 2 h and dried in a vacuum centrifuge.

2.4. SCX chromatography

The dried iTRAQ-labeled mixture was fractionated by SCX chromatography. iTRAQ-labeled samples were injected via an autosampler (Agilent 1100 series, Santa Clara, CA, USA) and loaded to a 2.1 mm × 200 mm SCX-column (Poly-SULPHOETHYL). Ten fractions were collected from the SCX separation and then dried in a vacuum centrifuge.

2.5. Reverse-phase LC

Peptides in the SCX fractions were separated on a C18 nano LC coalumn For the second dimension of LC separation, (Zorbax300SB, 5 μm; Agilent). Each SCX salt step elution was desalted. Fractions were mixed with matrix, and were spotted onto a MALDI target plate (Applied Biosystems).

2.6. MALDI TOF/TOF MS analysis

4800 Analyzer equipped with TOF/TOF ion optics were used for MS analysis (Applied Biosystems, Foster City, CA, USA).

2.7. iTRAQ data analysis

The following database search settings were used: (a) ProteinPilot (version 3.0, ABI); (b) The search engine is paragon algorithm and the NCBInr database 20111206 (2,796,749 sequences) were used; (c) The digesting reagent was set as trypsin and one missed cleavage was allowed; (d) fixed modification was carbamidomethyl, variable modifications were methionine oxidation and alkylation; (e) The mass tolerances for the precursor and product ions were 0.2 Da; (f) Common contaminants (e.g., trypsin and keratins) were deleted manually; and (g) In this study, protein confidence threshold was at least one peptide with 95% confidence. Relative quantification was performed with ProteinPilot2.0 software, and all iTRAQ ratios of $>$ 0.77 or $<$ 1.3 were not considered changed proteins. Lower of 0.77 were considered down-regulated, while those above the higher range 1.3 were considered up-regulated, as previously described (Gan et al., 2007; Ho et al., 2009).

2.8. Validation by real-time PCR analysis

Total RNA of earthworms *E.fetida* was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). SYBR Green reagents were used for qRT-PCR. Primes used for qRT-PCR in the Table S1. ΔΔCt method was used for calculating the genes expression.

2.9. Quantitative analysis

Gene Cluster 3.0 and Java Treeview software were used for Temporal protein expression pattern was performed using (de Hoon et al., 2004).

2.10. Bioinformatic analysis- network analysis

Ingenuity Pathway Analysis software (Ingenuity IPA 8.5; CA, USA) was used for analysis the protein network. Biological interpretation was based on pre-existing knowledge from mammalian interaction databases.

3. Results

3.1. Proteins identified in earthworm

In total, 283 proteins were identified from NCBInr database using ProteinPilot 3.0 software. Fig. S2 shows an example of 2-D high-performance LC (HPLC) separation followed by MALDI-TOF/TOF analysis spectrum matching the peptide EPYWIDC[MSH]GR (A) and the iTRAQ marked quantitative information (B) of the earthworm (*Eisenia andrei*) lysozyme (gi|85003097). Uniprot was used for searching these proteins function.

3.2. Proteins cluster analysis

To more effectively analyze the entire protein profiles, the proteins expression pattern in response to *E.coli* O157:H7 was analysis with a tree-clustering method (Fig. 1). Quantitative variations in protein abundance using ProteinPilot 3.0. Two protein response patterns according the clustering analysis (correlation coefficient $>$ 0.8) (Fig. 1). Group I showed proteins an increased accumulation, and group II comprised proteins decreased accumulation in response to *E. coli* O157:H7 stress.

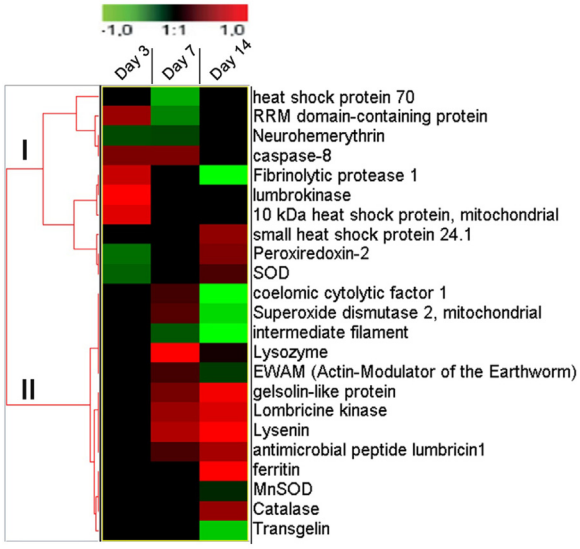

Fig. 1. Clustered analysis of the 23 proteins. The green color (−1) shows decrease while the red color (+1) means increase. Dark boxes (0) means no changes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)