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Proteomic analysis of the earthworm *Eisenia fetida* exposed to oxytetracycline in soil

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Increasing attention has been paid to the toxicity and hazards of antibiotics on non-target organisms in soil ecosystems because redundant antibiotics in the excretion of treated animals are being brought into the soil by way of manure and sewage irrigation. In order to understand the toxic mechanisms of antibiotics in soil ecosystems, the earthworm *Eisenia fetida* was exposed to 500 mg kg^{-1} of oxytetracycline (OTC) as a typical antibiotic for 7, 14 and 21 days. The total proteins of *E. fetida* in each treatment were separated by two-dimensional gel electrophoresis and differential expressed proteins were identified by MALDI-TOF/TOF-MS. A total of 30 proteins were successfully identified and divided into four categories based on the function. It was surprisingly found that more than 50% of identified proteins belong to the actin family, and all of them were down-regulated more than 2.0-fold. In the meantime, the fibrinolytic enzymes, an important protease with plasminogen activator activity, were suppressed in the last two weeks. The validations in the mRNA level were performed using RT-PCR. However, due to the incomplete genome sequence of *E. fetida*, we failed to identify more proteins response to OTC stress. This study may provide a new insight into the discovery of novel biomarkers for continuous-poured and low-toxicity pollutants.

1 Introduction

As food additives or veterinary and human medicines, most of antibiotics are poorly retained in the stomach and intestine and cannot totally be absorbed by animals and human beings. According to some literature articles,[1–3] about 40–90% of the antibiotics in their bodies could be excreted in faeces and urine in the forms of parent or metabolites. According to an investigation made by Lin,[4] the concentration of tetracyclines in some agricultural soils from suburbs of Beijing and Tianjin was up to 119–307 mg kg^{-1} due to irrigation with domestic wastewater and application with pig feces and sewage sludge.[5] Excessive antibiotics in the environment may potentially and adversely affect non-target organisms.[6,7] Thus, more and more concerns have been paid to the toxicity and hazards of antibiotics in soil ecosystems with their wide application in agriculture, including disease therapy, health maintain, growth rate enhancement and production improvement of livestocks. Particularly, continually accumulated antimicrobials and their byproducts probably bring about the resistant bacteria which are discharged, fertilized or irrigated into waters, soils or other environmental media and allowed to enter the food chain.[8] Although we can partially know the adverse effects of these pharmacologically active substances in soil ecosystems, their toxicity and hazards at the level of proteomics are still vague.

In soil ecosystems, earthworms are identified as the key species of invertebrates and can indicate soil health and feedback contamination of terrestrial ecosystems.[9] Increasingly, the earthworm species *Eisenia fetida* is suggested as the model organism for sensitive diagnosis of soil contamination, because they keep constantly exposed to the contaminated soil,[10] which has been regarded as the standardization of toxicological experiments for industrial chemicals by the International Standard Organization (ISO).

Oxytetracycline (OTC) is a kind of widely used tetracycline antibiotics. Bao[11] found that the concentration of OTC in soil and aquaculture industry sediments was up to 200 mg kg^{-1} and 285 mg kg^{-1}, respectively. A few studies have disclosed toxic effects of OTC on the structure and function of soil microbial communities, the activity of enzymes and the growth of plants.[12,13] Hu *et al.*[14] investigated the distribution of antibiotic OTC resistance gene in the environment. However, few research reports are available on toxic effects of OTC on terrestrial organisms, especially on earthworm species at the level of proteomics.

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In this study, the earthworm species *Eisenia fetida* was adopted as the model organism for examining the toxicity and hazards of OTC as a typical antibiotic at the level of proteomics. In particular, changes in the total protein expression profiles of the earthworm species under the stress of OTC would be analyzed comparatively. The results from this present study can undoubtedly help in understanding about toxicological mechanisms of antibiotics in soil ecosystems.

2 Materials and methods

2.1 Test animals and chemicals

The earthworms *E. fetida*, weighing between 300 and 600 mg, were purchased from the Earthworm Breeder Company in Tianjin, China. After transported to a laboratory without any physical stress, the worms were bred at ambient temperature of 20 ± 2 °C, using the peat moss as the bedding material. Worms were fed with commercial cattle manure once a week and the humidity of the soil was maintained around 70% by spraying purified water every 3 days. Adult worms with the weight of 400 ± 50 mg and fully developed clitellum were used for all the experiments. Oxytetracycline hydrochloride with more than 95% purity was purchased from Sigma-Aldrich and all other reagents were of analytical or molecular biology grade with the purity of 95–99%.

2.2 Tested soils

Clean soils were collected from the surface layer (0–20 cm in depth) of a natural forest park in Tianjin, China, then air-dried and sieved with 2 mm mesh. The physical chemical properties and baseline concentration of OTC in the tested soil were listed in Table 1. Use the sterilized soil.

Sub-acute toxicity test of *E. fetida*. According to the reported concentration of OTC in the actual environment and the results of relevant toxicological experiments, 500 mg kg^{-1} of OTC were added to the soil after introducing the worms for about half an hour, and using deionized water was as a control. The soil was placed in a 1000 mL beaker. The beaker is sealed with a film with the small hole for ventilation and then placed in a biochemical incubator for testing. Exposure times were 7, 14 and 21 days. Six earthworms were introduced in each treatment group. After earthworms had been exposed to OTC for 7, 14 and 21 days, the earthworms were weighed again and then placed onto clean moist filter paper for 24 h at 20 ± 2 °C to force them to empty their gut, and then frozen with liquid nitrogen and stored at −80 °C.

2.3 Protein extraction

Total proteins were extracted based on the trichloroacetic acid-acetone (TCA-A) method previously described by Wang[15] with a little modification. Briefly, fresh whole *E. fetida* samples (2 g) collected from three replications were pulverized to a fine powder with liquid nitrogen and a mortar and pestle. The powdered homogenate was placed in a 50 mL centrifuge tube and the protein was precipitated overnight with 8–10 times volume of ice-cold TCA-A solution containing 20% TCA (w/v) and 0.1% DTT (w/v). After centrifugation at 4 °C and 12 000 × g for 40 min, the precipitate above was washed twice with 30 mL pre-chilled acetone solution containing 0.1% DTT (w/v) and once with 80% washing solution above. The resulting pellets were vacuum freeze dried until the remaining acetone was all removed, and then redissolved in lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS (w/v), 65 mM DTT, and 0.5% (w/v) Bio-lyte (pH 4–6 and pH 5–7, Bio-rad, USA)]. The undissolved protein particulars in the lysis buffer were sonicated with the ultrasonic cell disruptor (JY92-II Ultrasonic Crasher, Ningbo Scientz Biotechnology Co., Ltd, China) at 4 °C and 300 W for 40 apm (actions per minute) and then centrifuged at 4 °C and 13 500 × g for 40 min. The supernatant was collected and the protein concentration was measured according to the Bradford's method using Coomassie Brilliant Blue G-250 (Bio-Rad, USA) and BSA as a standard.[16] And then the protein samples were stored in 1.5 mL EP tubes in 100 μL aliquots at −80 °C.

2.4 Two-dimensional electrophoresis (2-DE)

Isoelectric focusing (IEF) was carried out using immobilized pH gradient (IPG) gel strips (17 cm, pH 4–7; Bio-Rad, USA). And the dry gel strips were passively rehydrated for 8 h with 300 μL rehydration buffer containing about 2 mg of protein and focused on a PROTEAN IEF Cell (Bio-Rad, USA) at 20 °C. The condition performed was set at 50 V for 8 h to active rehydration and 200 V for 1 h slow (the build up mode of voltage), 1000 V for 1 h slow, 8000 V for 2 h linear then 8000 V for 75 000 V h (voltage × hours). Prior to the second dimension separation, the focused strips were equilibrated for 15 min in 6 mL equilibration solution (6 M urea, 2% SDS (w/v), 0.375 M pH 8.8 Tris–HCl and 20% glycerol (v/v)) with 2% (w/v) DTT. This is followed by a further 15 min equilibration using the same solution containing 2.5% (w/v) iodoacetamide (IAA) instead of DTT.

The separation in the second dimension was performed with a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 30% acrylamide (w/v), 1.5 M pH 8.8 Tris–HCl, 10% SDS (w/v), 10% ammonium persulphate (w/v) on a PROTEAN II xi Cell system (Bio-Rad, USA). Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 and then destained.

Table 1 Physical and chemical properties and the baseline of oxytetracycline in the tested soil

Soil property	pH	OC[a]	Sand	Silt	Clay	CEC[b]	OTC[c]
Tested soil	8.18	18.85 g kg^{-1}	36.99%	28.91%	34.10%	16.33 cmol kg^{-1}	<0.001 mg kg^{-1}

[a] OC: organic matters content. [b] CEC: cation exchange content. [c] OTC: oxytetracycline.

Table 2 Specific primer pairs for the two *E. fetida* genes used in this study

Gene	Spots no.	Sense primer (5′–3′)	Antisense primer (5′–3′)
Actin[a]	5555	CCGCCCTGGTCGTCGATAATG	TACCTCTCTTGCTCTGGGCCTCAT
β-Actin	—	TCTCCACCTTCCAGCAGATG	CGAAAAATGTCCTCCGCAAG

[a] The sequence of *E. fetida* actin gene was sequenced by the Beijing Genomics Institute.

2.5 Image scan and analysis

The gels incompletely destained by destaining solution containing 50% methanol (w/w) and 10% glacial acetic acid (w/w) were then washed with boiled deionized water until the background was clear. Stained gels were scanned by ScanMaker i800 (Microtek, Shanghai, China). Automated protein spots detection and matching were carried out using PDQuest 8.0.1 software (Bio-Rad, USA) with subsequent manual editing and modifying. Three well-resolved gels of each sample were chosen as the replicate groups. By the software, the 'Analysis sets' of each treatment groups were quantitatively and qualitatively processed. The spots included in the Boolean's intersection of exhibiting 1.50-fold increase or 0.67-fold decrease between the controlled groups and treated groups and statistically different at a level of $p < 0.05$ based on one-way ANOVA analysis were considered to be differentially expressed proteins. And the statistical significance was determined by one-way analysis of variance (ANOVA) using SPSS 20.0.

2.6 In-gel digestion

Protein spots were excised with a diameter of 1–2 mm from the 2-D gels and transferred to a 500 μL Safe-lock tube (Eppendorf, Germany). The cut gels granules were washed twice for 10 min with 200 μL of Milli-Q water and then destained for 20 min in a mixture of 25 mM ammonium bicarbonate (NH_4HCO_3) and 50% acetonitrile (CH_3CN) (w/w) until the gels were clear. The destaining solution should be blotted up before the dehydration of the gels by 100% acetonitrile (w/w). The protein spots were digested overnight with 5 ng μL^{-1} trypsin (Promega, USA) dissolved in 25 mM NH_4HCO_3 at 37 °C and then centrifuged. The supernatant was collected for the next step.

2.7 Identification by mass spectrometry

Firstly, 1 μL of peptide mixtures from the 2-DE gel spots were spotted on the AnchorChip target (Bruker Daltonics, Bremen, Germany) and then the air-dried sample was covered with 1 μL of α-cyano-4-hydroxycinnamic acid (HCCA) in 70% CH_3CN (v/v) and 0.1% trifluoroacetic acid (TFA) (v/v) as a matrix. Analysis of mass spectra was carried out on the instrument Autoflex MALDI-TOF/TOF purchased from Bruker Daltonics (Bremen, Germany). Data acquisition was performed by Flex Control software 3.0 using PeptideCalibStandard II (Bruker Daltonics) as an external calibration the mass spectra were evaluated using Flex Analysis software. The protein matching using the peptide mass data were performed in the NCBInr database with the MASCOT search engine with the mass accuracy of 100 ppm and at most two peptide cleavage sites. The reliable identification results were selected considering high MASCOT score, maximum sequences coverage, and consistency of molecular weight (MW) and isoelectric point (pI) given by the database and the electropherogram. Function of the identified proteins was determined using the UniProt Knowledgebase (UniProtKB), which is a comprehensive resource for protein sequence and annotation data.

2.8 Quantitative real-time PCR

Total RNA was extracted from 50 mg of six whole earthworm tissues using TRIzol® Reagent (Ambion®, Invitrogen, USA) and the extraction was repeated three times for each group (three biological replicates). RNA purity and integrity were checked by electrophoresis on 2% agarose gels (w/v) stained with ethidium bromide and by ensuring the absorbance ratios (OD_{260}/OD_{280}) were between 1.8 and 2.0. First strand cDNA was synthesized using GeneAmp® PCR System 9700 (Applied Biosystems, USA) with the TransScript® First-Strand cDNA Synthesis SuperMix (TransGen, China). Primer pairs of the *E. fetida* actin gene were designed using Gene Tool 1.0 Lite software according to the sequence of *Lumbricus terrestris* actin gene which has a high sequence similarity (95%) to that of *E. fetida*, and the β-actin gene, which was submitted by Brulle *et al.*,[17] was selected as a reference gene (Table 2). Quantitative PCR reactions were performed in a StepOne Plus Real-Time PCR System (Applied Biosystems, USA) in triplicate wells following the instructions (1 cycle at 95 °C for 30 s and 40 amplification cycles at 95 °C for 5 s, 53 °C for β-actin/57 °C for actin 30 s, and 72 °C for 20 s). The mRNA expression level of actin gene and the *E. fetida* β-actin mRNA, selected as an internal reference gene, was determined in parallel for each group. The differential expression changes results were analyzed using the $2^{-\Delta\Delta C_T}$ method of Livak and Schmittgen.[18]

3 Results and discussion

3.1 Identification of OTC respective proteins

There were different protein profiles of the earthworm *E. fetida* between the control group and OTC-treated group at 7, 14 and 21 days (Fig. 1). On average, about 1200 protein spots could be detected on each 2-DE gel of the three replications. All of the protein spots from the earthworm *E. fetida* present a wide distribution on the gel between the isoelectric point range from 4 to 7 and an obvious molecular mass between 14 kDa and 100 kDa. The experimental MW and pI of the identified proteins were probably inconsistent with the theoretical values

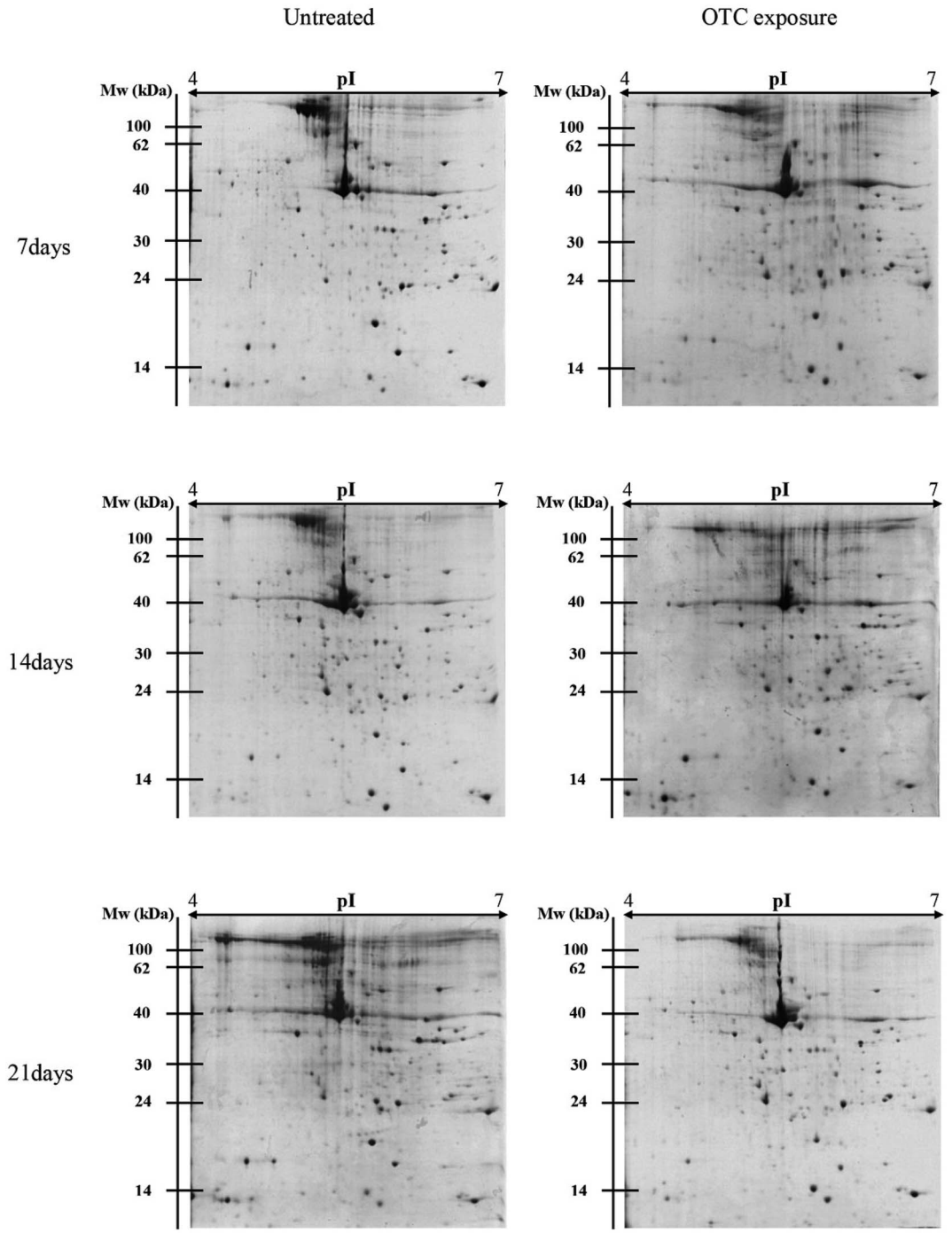

Fig. 1 Patterns of earthworm *Eisenia fetida* total proteins at 7, 14 and 21 days of OTC exposure stained by Coomassie Brilliant Blue R-250. 1 milligram of protein was used for IEF with linear IPG strips (pH 4–7) and for the second dimension vertical separation with 12.5% polyacrylamide gels. Blue Plus™ II Protein Marker purchased from TransGene (Beijing, China) was used as a molecular weight standard. The maps of control groups and OTC exposure groups were selected from three independent experiments.

in the consequence of the post-translational modifications (PTMs) of the proteins. Analysis of the gels with PDQuest 8.0.1 software expounded the significant ($p < 0.05$) changes of protein spots between OTC-treated groups and control groups at least during one time point. Total 107 spots were significantly ($p < 0.05$ and more than 1.50-fold variation) regulated and subsequently 31 proteins were determined with MALDI-TOF/TOF-MS and MASCOT Database (Table 3). The identification results of spot 5555 are depicted in Fig. 2 as a representative. The function information of some identified proteins was evaluated by UniProtKB resource and their NCBI database accession numbers.

Table 3 The list of identified proteins using MALDI-TOF/TOF-MS and related information from the NCBI database

Spot no.[a]	NCBI GI. no.	Protein name (organism)	Theor. MW/pI[b]	Exp. MW/pI[c]	MASCOT score[d]	SC[e] (%)
Actin family						
6453	gi|3319951	Actin (*Helobdella triserialis*)	41.703/5.38	34.62/6.0	95	29
6306	gi|3319951	Actin (*Helobdella triserialis*)	41.703/5.38	30.24/5.8	98	36
3344	gi|397881222	Actin (*Lineidae* sp. TWL-2008)	42.132/5.30	25.14/5.0	121	39
4113	gi|1707573	Actin (*Lumbricus terrestris*)	42.161/5.30	19.65/5.3	155	38
6003	gi|3046400	Actin 1 (*Schmidtea polychroa*)	8.257/5.48	12.95/5.8	81	79
5363	gi|358332531	Actin beta/gamma 1 (*Clonorchis sinensis*)	35.711/5.04	28.33/5.4	144	57
3642	gi|405964580	Actin, cytoplasmic (*Crassostrea gigas*)	42.010/5.30	48.12/4.9	163	55
4014	gi|3452277	Beta actin (*Pseudopleuronectes americanus*)	14.537/5.28	13.26/5.4	84	60
9002	gi|3452277	Beta actin (*Pseudopleuronectes americanus*)	14.537/5.28	13.81/6.6	79	43
5260	gi|197320840	Beta-actin (*Gallus gallus*)	10.298/6.17	24.00/5.5	107	79
5555	gi|2829750	RecName: Full = actin (*Lumbricus rubellus*)	41.582/5.46	45.50/5.6	159	40
5749	gi|2829750	RecName: Full = actin (*Lumbricus rubellus*)	41.582/5.46	60.68/5.7	189	54
4117	gi|2492669	RecName: Full = actin, cytoskeletal 3; AltName: Full = LPC3 (*Lytechinus pictus*)	19.652/5.78	16.88/5.3	107	34
6313	gi|1703136	RecName: Full = actin, cytoskeletal; AltName: Full = M; flags: precursor (*Heliocidaris erythrogramma*)	42.063/5.30	32.36/5.9	134	42
4403	gi|1703137	RecName: Full = actin, cytoskeletal; AltName: Full = M; flags: precursor (*Heliocidaris erythrogramma*)	42.077/5.30	37.40/5.2	97	37
5024	gi|27883553	Alpha actin (*Ictalurus punctatus*)	16.770/5.28	14.10/5.7	92	60
Metabolism						
8401	gi|16660643	Fibrinolytic enzyme (*Eisenia fetida*)	20.221/4.59	34.10/6.2	80	31
6203	gi|110341195	Fibrinolytic protease 0 (*Eisenia fetida*)	23.588/5.31	24.79/5.8	88	40
9323	gi|220924493	GTP-binding protein EngA (*Methylobacterium nodulans* ORS 2060)	48.460/8.66	30.29/6.8	106	18
1107	gi|61657939	Myosin heavy chain, skeletal muscle, adult (*Gallus gallus*)	224.010/5.63	17.66/4.7	101	12
Hypothetical and predicted proteins						
4301	gi|268531882	Hypothetical protein CBG02838 (*Caenorhabditis briggsae*)	17.058/4.54	30.37/5.2	81	34
5558	gi|436835559	Hypothetical protein FAES_2173 (*Fibrella aestuarina* BUZ 2)	7.091/6.07	38.70/5.7	93	50
5750	gi|332017101	Hypothetical protein G5I_14087 (*Acromyrmex echinatior*)	150.374/8.67	64.18/5.5	92	22
3434	gi|395827452	Predicted: ATP-dependent zinc metalloprotease YME1L1-like (*Otolemur garnettii*)	80.347/8.98	36.21/5.0	96	27
5364	gi|530606293	Predicted: dnaJ homolog subfamily C member 25-like (*Chrysemys picta* bellii)	42.272/9.10	25.98/5.6	84	33
6112	gi|507644426	Predicted: dolichyl-phosphate beta-glucosyltransferase isoform X2 (*Octodon degus*)	33.609/9.34	20.55/5.8	84	33
3346	gi|514704523	Predicted: interleukin-17B isoform X3 (*Anas platyrhynchos*)	21.123/9.35	29.33/4.8	90	50
2666	gi|488526628	Predicted: low quality protein: vinculin (*Dasypus novemcinctus*)	104.217/5.38	54.93/4.7	123	22
6550	gi|488510571	Predicted: protein phosphatase 1B isoform 2 (*Dasypus novemcinctus*)	26.106/5.11	38.88/5.9	83	32
7006	gi|498969699	Predicted: restin homolog isoform X7 (*Ceratitis capitata*)	202.861/4.99	14.16/6.1	94	19
5191	gi|512836262	Predicted: torsin-1A-interacting protein 2 isoform X9 (*Heterocephalus glaber*)	15.666/6.88	16.75/5.4	80	35

[a] The spot numbers are acquired from the PDQuest 8.0.1 software during analysis of 2-D gels. [b] The theoretical MW and pI are offered by the MASCOT search engine. [c] The experimental molecular mass (kDa) and pI are estimated from 2-DE gels according to their relative position. [d] Proteins identified are listed with the MASCOT score as a criteria for MS or MS/MS identification. [e] SC is short for sequence coverage.

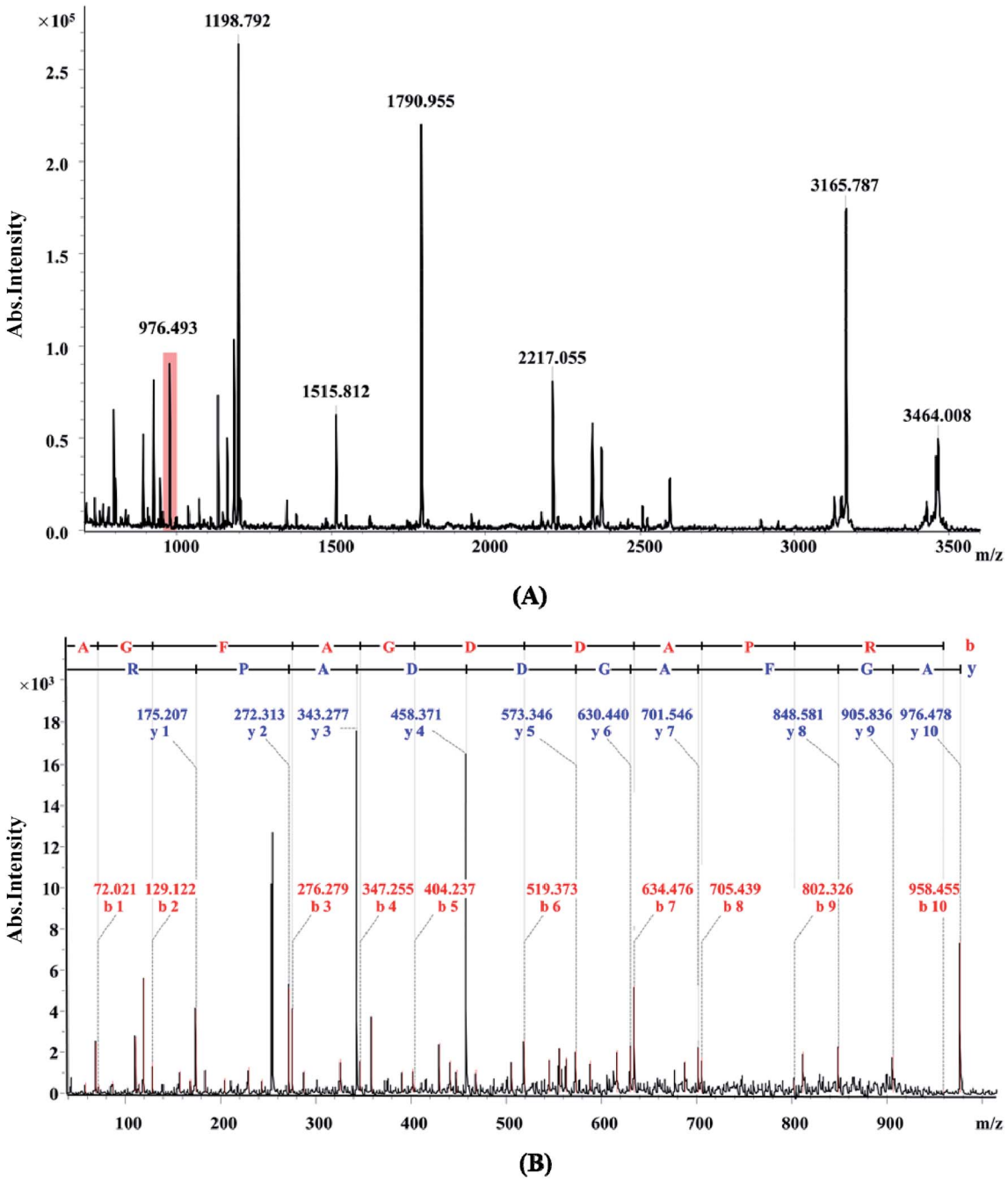

Fig. 2 Identification of spot 5555 *via* MALDI-TOF/TOF-MS. The protein excised from the gels was digested with trypsin and the peptides were analyzed by a MALDI-TOF/TOF ultraflex mass spectrometer (Bruker Daltonics). (A) MS spectra. The marked ion 976.493 was analyzed by MS/MS. (B) MS/MS spectra of ion 976.493.

3.2 Changes of protein expression under OTC stress

To avoid the errors caused by the experiment operation and biological variances, the identified proteins would be considered if there were more than 1.50 times regulation (up-regulation or down-regulation) of protein expression at one or more time point between the control groups and the OTC-stressed groups at a significant level ($p < 0.05$). Totally, 107 spots satisfied the condition and were cut down from all the gels to be analyzed by MALDI-TOF/TOF-MS. By the use of Biotools 3.0 (Bruker, Daltonics) and MASCOT search engine with NCBI database, 5 spots were identified by matching the sequence of amino acids with 31–54% sequence coverage from the earthworm species. Due to the genome of earthworms was not fully sequenced, so most of proteins were identified from other organisms. The identified proteins could be divided into three categories according to their function, among them, surprisingly, more than 50% of identified proteins belong to the actin

Table 4 The expression changes of the actin proteins up-/down-regulated

Spot no.	Exposure time (day)		
	7	14	21
5555	1.14	1.12	−2.22[b]
5363	1.57[a]	−1.74[a]	−2.80[b]
5260	1.65[a]	−2.03[b]	−1.84[a]
4113	1.37	1.03	−3.53[c]
4117	3.46[c]	−1.71[a]	−2.54[b]
5024	1.45	−1.09	−2.13[b]
4014	2.11[b]	−1.50[a]	−2.54[b]
6003	1.85[a]	−1.40	−1.69[a]
6453	2.17[b]	−2.41[b]	−2.54[b]
6306	1.49	−1.74[a]	−1.25
3642	2.81[b]	1.57[a]	−2.06[b]
4403	2.06[b]	−1.09	−2.07[b]
5749	1.57[a]	0.00	−2.09[b]
9002	1.40	1.05	−1.95[a]
6313	1.70[a]	−1.69[a]	−1.55[a]
3344	2.07[b]	−1.38	−1.70[a]

[a] 1.5 fold < the protein expression changes < 2.0 fold. [b] 2.0 fold < the protein expression changes < 3.0 fold. [c] The protein expression changes > 3.0 fold.

family which suggested the OTC exposure increased the cytoskeleton degradation *in vivo*.

After a 7 day exposure to 500 mg kg^{-1} of OTC, 23 proteins were up-regulated while 7 proteins were down-regulated. However, the situation reversed when there were 22 and 20 proteins down-regulated after a 14 and 21 day OTC exposure, respectively, which might suggest a stress-adaptation-compensation mode if we investigate as long as enough. There were both 7 proteins up-regulated at the last two time points. The cytoskeletal protein actin (spot 4117) was the most up-regulated (3.46-fold) after a 7 day exposure, while the myosin heavy chain protein was the most down-regulated (−11.99-fold) after a 14 day exposure.

We also found that the regulation of some spots from different position on the gel (with different MW and pI) showed the same changing trends. The combined results of 2-DE and MALDI-TOF-MS showed that 16 spots were identified as the actin protein (or actin-like fragments) in Table 4, although 3 of them were annotated as earthworm actin proteins, both gene and protein sequences of the other actin proteins had a quite high similarity of more than 95%. Most of these actin proteins were nearly intact proteins, but a few of them seemed to be the fragments of actin. All of the actin proteins were up-regulated and 11 of them increased more than 1.50-fold compared with the normal group after 7 days exposure to OTC, and among them about 50% increased more than 2.00-fold of normal group. However, on the 14th day, the expressions of the 16 actin proteins consistently decreased. Moreover, 11 out of 16 actin proteins showed the obviously down-regulation. At the last exposure time point of 21 days, expression of almost all the actin proteins were down-regulated relative to the control group shown in Fig. 3, and the most down-regulation was 3.53-fold (Spot 4113).

3.3 mRNA level expression changes of actin genes

To investigate the changes in mRNA level during the OTC stress and validate the novel interesting changes that so many actin proteins all down-regulated, quantitative PCR of three biological replicates was performed. The sequence of forward and reverse primers are listed in Table 2. The result indicated that

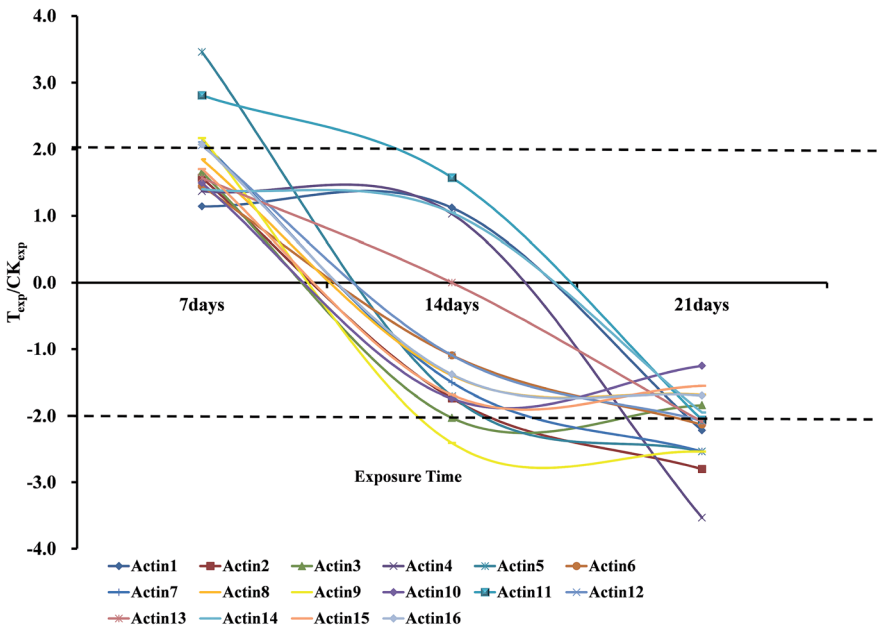

Fig. 3 The change trends of the Actin proteins expression ratio of treated group to control group at 7 days, 14 days and 21 days. The areas outside the dashed line indicate the ratio is more than 1.5; T_{exp} means protein expression of treated group; CK_{exp} means protein expression of control group.

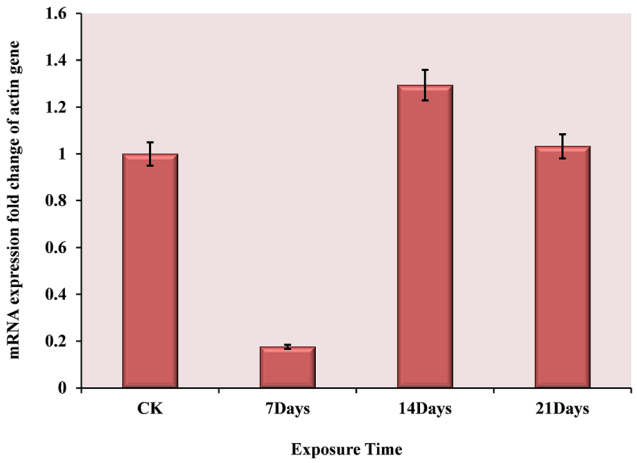

Fig. 4 The mRNA expression changes of actin gene during the exposure time of 7 days, 14 days and 21 days under the oxytetracycline stress. The comparation between treatment groups and control group was performed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

the mRNA–protein expression correlation was relatively inconsistent at the same time point. Comparing with the normal group, the actin gene was very significantly down-regulated at 7 days, but it turned to be up-regulated after exposure to OTC for another week. Finally, at 21 days, its expression returned to the normal level shown in Fig. 4. There may be spatial–temporal differences in transcription and translation of the actin genes caused by complicated biological processes, such as transcriptional splicing, post-transcriptional splicing, translational modifications and translational regulation.

4 Discussion

Many studies have focused on physiological mechanisms including cellular structure, DNA damage and enzyme activity variation of earthworms under the stress of pollutants.[4,19–22] In recent years, Wang *et al.*[10] investigated effects of cadmium (Cd) on earthworms at the level of proteomics. However, the effect of antibiotics on earthworms has not been reported at the level of proteomics. Thus, protein expression profiles of the earthworm *E. fetida* exposed to OTC was analyzed as a demonstration by the two-dimensional gel electrophoresis. Totally, 107 protein spots with a significant ($p < 0.05$) variation (1.50-fold) between the control and the OTC-stressed groups during at least one time point were identified using the MALDI-TOF/TOF-MS. It indicated that almost of the proteins were up-regulated after a 7 day exposure and then down-regulated after a longer exposure.

Actin is a natural and abundant protein. It is responsible for a wide range of cellular activities including cytoplasmic streaming, cell transport, and cell division. It comprises more than 12–15% of the total proteins in motile cells or cells which exhibit cytoplasmic streaming, and accounts for about 30% of muscle cell proteins. Even in nonmotile cells such as red blood cells, actin comprises about 1–2% of the cytoskeletal proteins.[23] Actin is present in all eukaryotic cells, and most of the organisms have several genes encoding this protein.[24]

Oxidative stress routinely caused by cellular processes, xenobiotic intoxication or exposure to metals was an imbalance between reactive oxygen species (ROS) production and the antioxidant defence systems.[25] The cytoskeletal protein composed of actin protein was reported as a target for oxidative stress. Proteomic study of four model contaminants (Aroclor 1254, Cu^{2+}, tributyltin and As^{3+}) stress on the clam *Chamaelea gallina* found that two actin proteins of different species with high homology were down-regulated, and one of them was identified as a actin-like fragment.[24] Coincidentally, Cu^{2+} exposure of the mussels caused a severe perturbation and rearrangement in *Mytilus galloprovincialis* haemocyte actin, and the phalloidin staining showed that cell morphology was altered including the disappearance of filamentous actin and the disorganization of actin cortical meshwork, due to the carbonylation and glutathionylation of the actin in order to response to the oxidization-altered state.[26,27] The similar results were also achieved when the *E. fetida* was exposed to 80 mg kg^{-1} Cd that the intermediate filament protein, which consist of actin monomer, significantly down-regulated at 21 days exposure.[10] As well, the genes expression results showed that the intermediate filament protein gene was down-regulated when exposed to B(a)P, Cd and PCP.[28]

During exposed to OTC, it could be observed that the outer layer of the earthworms came to be soft and festered after 14 and/or 21 days exposure, and the activity of worms in the stressed group were obviously weakened compared with the normal group. Maybe, the perturbation of water[29] and calcium ion[30] homeostasis in the worms caused by the oxidative damage, through the xenobiotics interacting with the membrane receptors trigger a signaling pathway, then the activated inositol-triphosphate (IP_3) released from the pathway subsequently triggers the release of the intracellular storaged calcium ions. As a result, the increase in cytoplasmic free Ca^{2+} concentration guides reorganisation of cytoskeletal proteins, mainly actin and tubulin. High free Ca^{2+} concentrations activate some specific protein-kinases phosphorylating cytoskeletal proteins, and calmodulin-dependent cytoskeletal phosphodiesterases and phosphatases, which could hydrolyze the actin filaments and then impair cell morphology and motility.[31]

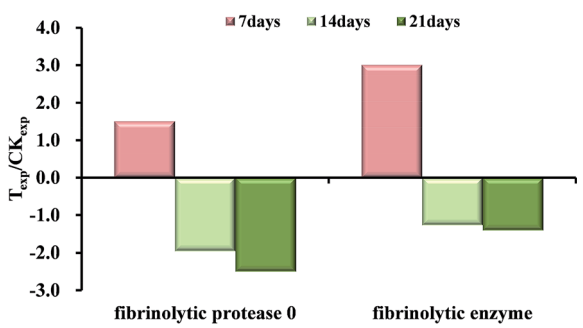

Fig. 5 The expression fold changes of the fibrinolytic enzymes at 7 days, 14 days and 21 days compared with respective control groups. T_{exp} means protein expression of treated group; CK_{exp} means protein expression of control group.

Fibrinolytic enzyme (Spot 8401) and fibrinolytic protease 0 (Spot 6203) were found to be significantly ($p < 0.05$ and fold change > 1.50) up-regulated at 7 days, but they both turned to be down-regulated during the following two-week exposure to OTC shown in Fig. 5. Earthworm fibrinolytic enzymes (EFEs) also referred to as lumbrokinases (LK), are a group of alkaline serine proteases with both strong plasminogen activator activity and direct fibrin-degrading activity.[32–34] The changes were possibly caused by the prevention mechanism against the stress activated by xenobiotics just like high-concentration OTC, or the oxidative injury caused by the toxicity of the antibiotics. In previous study, earthworm fibrinolytic enzymes could decrease the pulmonary fibrosis induced by bleomycin in rat.[35] Maybe, we speculated that the high-concentration OTC stress, to some extent, led to the formation of blood clot or the tissue fibrosis in the early days. As a result, the expressions of fibrinolytic proteases were induced for hydrolysis to repair and remodel the damaged tissue or cytoskeletal structure discussed above.

As a result of the validation test, at all the exposure time points, the contrary expression changes were shown in mRNA level (Fig. 4) comparing with protein level (Fig. 3). Presumably, the toxicity of OTC was not so strong as heavy metal, polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyl (PCBs) or other organic pollutants for earthworm, which was harmed only by contacting or eating the contaminated soil. In contact with the high concentration OTC, the outer layer of the earthworm was damaged on the mechanism discussed above. These may cause the oxidative damage in the outer layer cells during the first seven days, which suppressed the expression of actin genes. Subsequently, the body tissue 'sensed' the damage, such as degradation of cytoskeletal proteins, and enhanced expression of the actin genes for repair at the phase of 14th day. Finally, the long-term stress may cause an irreversible damage on gene, so 21 days later, the mRNA expression started to decrease with respect to 14th day.

Previous studies of our group[21,36] showed that tetracycline antibiotics could cause the breakage of earthworm *Eisenia fetida* DNA by a comet assay, and with an positive dose-response relationship, the changes in antioxidant enzymes (CAT and SOD) activities and DNA damage were observed in earthworms exposed to 300 mg kg^{-1} of chlortetracycline and tetracycline. But enzymes activity was confirmed as an unreliable, susceptible and poorly repeatable indicator. In this study, it's the first time to investigate the expression changes of earthworms on the antibiotics (oxytetracycline) stress in the proteome level using the 2-DE and MALDI-TOF/TOF-MS technology. The results suggested that unlike the heavy metal, PAHs and PCBs with a relatively high values of LC_{50}/EC_{50}, toxicity of OTC with a concentration in field and soil environment, as a widely used veterinary, couldn't make a significant adverse effect on the earthworms' important physiological and biochemical process in short or medium term exposure. However, we found that 500 mg kg^{-1} of OTC caused the significant changes of cytoskeletal proteins which were discovered due to the sensibility of the proteomics. The injury of cellular structure and the alteration of membrane permeability may directly expose the earthworms to the contaminated surroundings without sound membrane systems protection. In total, all the previous and current studies results indicated that the cytoskeletal and membrane proteins of some invertebrate, which were characterized by their soft bodies and living in physically contact with environment, like earthworm and molluscs, were refered to as the first mainly protective barrier to toxicants. So they should be the most sensitive as both targets and biomarkers. The future studies should focus on the mechanism and pathway of the cell structure damage caused by the pollutants with low dose and low toxicity. In addition, various contaminants may be existed simultaneously in realistic environments, the proteomic analysis of combined effects of pollutants mixture should be equally investigated.

5 Conclusions

This study investigated the toxicity and hazards of earthworms exposed to soil containing OTC, and changes in the total protein expression profiles of the earthworm species under the stress of OTC. A total of 30 proteins were successfully identified and divided into four categories based on the function. More than 50% of identified proteins response to OTC stress belong to the actin family. All of the actin proteins were significantly up-regulated at 7 days, but down-regulated during the following two-week and the fibrinolytic enzyme and fibrinolytic protease were induced as the same trend, which might suggest a stress-adaptation-compensation mode. Meanwhile, it could be observed that the outer layer of the earthworms came to be soft and festered after 14 and/or 21 days exposure. This study may provide an new insight into the prevention mechanism against the stress activated by xenobiotics just like high-concentration OTC, or the oxidative injury caused by the toxicity of the antibiotics.

Conflicts of interest

The authors declare that they have no conflict of interest.

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