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Short communication

Two–Dimensional Gel Electrophoresis of Ceolomic Fluid of *Eisenia foetida* Earthworm

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Earthworms possess antioxidant, antibacterial, antitumor, and hemolytic properties. To recognize the molecules responsible for various biological activities of earthworm's coelomic fluid, a detailed knowledge about its protein contents is required. The aim of this study was to characterize the proteins present within the coelomic fluid of *Eisenia foetida* earthworm. Polyacrylamide-gel-electrophoresis (SDS-PAGE), and two-dimensional gel electrophoresis (2-DE) were carried out. Several proteins with molecular masses varying from 10 to 150 kDa were further separated from the coelomic fluid of *Eisenia Foetida*. The biological activities of the coelomic fluid could be mediated by these proteins.

Key words: *Eisenia foetida*, coelomic fluid, electrophoresis

Earthworms are essential organisms in soil. They cause soil fertility through their burrowing, ingestion and excretion (1). Recently, it has been proven that the glycolipoprotein extract (G-90) from earthworm *Eisenia foetida* has effect on the wound healing process in alloxan-induced diabetic rats (2). Working on earth worms comes with some challenges; they do not contain a fully sequenced genome, and are too small to allow the internal organs or tissue dissection (3). *Eisenia Foetida* (phylum Annelida, family Lumbricidae) is a line of invertebrate life dating back to 540 million years (4-6). The segmented earthworm`s body cavity is filled up with coelomic fluid. It has also been reported that earthworm coelomic fluid contains molecules that exhibit hemolytic and agglutinative (7), antitumor (8), anti-oxidant (9), and antibacterial (10) properties. The function of coelomocytes, cells that are presented within the coelomic fluid, remains unclear. A detailed knowledge about their protein contents is required in order to recognize the molecules responsible for various biological activities of the coelomic fluid (11). In this regard, the key technology to proteome analysis is protein separation by two dimensional electrophoresis (2-DE) (12-14). Therefore, we focused on *Eisenia foetida* protein spots determination through 2-DE.

Materials and methods

Earthworms

Eisenia Foetida (phylum Annelida, family Lumbricidae) was provided from an earthworm

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farm in Babol, IRAN. The samples were 3–6 cm long, and weighed between 300 and 400 mg. All procedures involving the present study was were approved by the Ethics Committee of The Babol University of Medical Sciences, Babol, IRAN (no. 1365).

The protein extraction procedure

Since the coelomic fluid separation is time consuming, and procedure has a low quantitative effect, therefore we used earthworm tissue extract. Extracts were obtained from tissue homogenate of earthworm *Eisenia foetida*, according to the method described by Hrzenjak et al. (15). Earthworms were also held in 0.65 % NaCl (Sigma-Aldrich, Germany) solution until their digestive systems were clear. Then, their bodies were cut into small pieces. The earthworm tissues were homogenized within equal part chloroform-methanol (Sigma-Aldrich, Germany) solution (v/v), and incubated overnight at 4°C. The Next day, 16 ml of distilled water was added to the homogenate, and centrifuged at 2460 g for 10 min. The upper layer, water/methanol was removed, and evaporated so there was no more methanol. The remaining water solution was kept at 4°C.

Analytical polyacrylamide-gel-electrophoresis (PAGE and SDS-PAGE)

A polyacrylamide-gel, and two-dimensional gel electrophoresis of coelomic fluid protein of earthworm were performed using 12.5% gel, topped by a 7% stacking gel prepared based on the method proposed by Laemmli (16). The two electrode chambers were filled with the same buffer. Buffer migration ran for 3 h at constant current of 20 mA in cold room until the bromophenol blue marker moved 5 mm from the bottom of the gel. SDS-gel was calibrated using the low molecular weight (LMW) calibration kit. Then, the bands were detected by staining the gel with silver nitrate.

Two-dimensional (2-D) gel electrophoresis

The first dimension (isoelectric focusing, IEF) was performed using GE Healthcare Biosciences Immobiline Dry strip (24 cm, PH 3-10, nonlinear). A final volume of 500 µL was loaded. IEF gel solution contained 8 M urea (Sigma-Aldrich, Germany), 2 M thiourea, 4% m/v CHAPS, 65 mM DTT, 0.001% m/v bromophenol blue (Sigma-Aldrich, Germany), and 0.2% w/v Bio-lyte buffer. IEF was conducted at 20 °C with an EttanIP Gphor3 system applying the following program: 50 v for 30 min, 100 v for 20 min, 150 v for 20 min, 250 v for 20 min, 1000 v for 1 h, 2500 v for 1 h, 8000 v for 3 h, 8000 v for 7 h, and 500 v for 2 h. After the first dimension, strips were placed in equilibration buffer (0.05 M Tris–HCl, pH 8.8, 6 M urea; 30% glycerol; 2% w/v SDS; containing 1% w/v DTT) and were slowly shaken for 15 min. The strips were then incubated for another 15 min in the equilibration buffer with 2.5% (w/v) iodoacetamide without DTT (17). The second dimension was conducted on 12.5% SDS-PAGE. After electrophoresis, the gels were stained with silver (17-18). Images were captured by Image Scanner III.

All procedures involving the present study were approved by the Ethics Committee of Babol University of Medical Sciences, Babol, IRAN (no. 1365).

Results

To achieve a great understanding on the biology of *Eisenia foetida* coelomic fluid, glycol-lipoprotein extraction analysis of earthworm *Eisenia foetida* was carried out using SDS-PAGE and 2-DE.

SDS-PAGE analysis

The results of the present study revealed that the ceolomic fluid of earthworm *Eisenia foetida* contains several proteins. SDS-PAGE analysis after silver nitrate staining revealed at least 17 bands with an apparent molecular weight of 10 – > 150 kDa (Figure 1).

Two-dimensional electrophoresis analysis

The coelomic fluid proteins have been investigated by 2-DE. Staining of 2-DE with silver nitrate revealed the presence of proteins with pH ranging from 3-6 as shown in figure 2.

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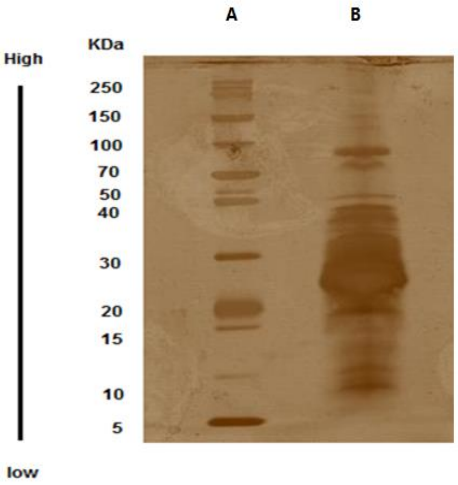

Figure 1. SDS-PAGE analysis of proteins from earth worms *Eisenia foetida*. Proteins were stained with silver nitrate. A: molecular weight standard; B: total protein extract.

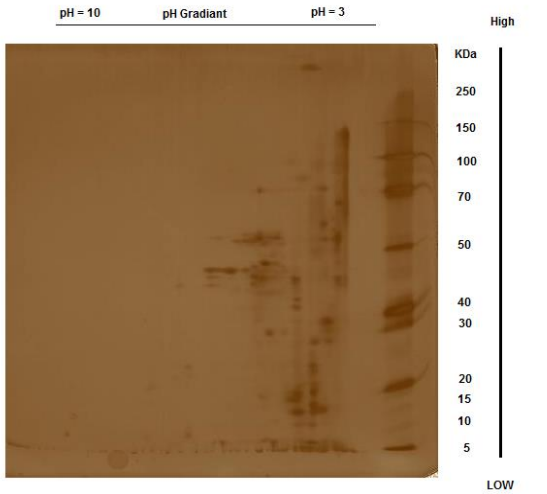

Figure 2. A representative 2-DE image of *E. foetida* extract. Proteins were submitted to isoelectric focusing on 3–10 IPG strips (24 cm) followed by electrophoresis on 12.5% SDS-PAGE.

Discussion

The coelomic fluid of the earthworm *Eisenia foetida* exhibits several biological activities including antibacterial, antitumor, anti-coagulative, and wound healing. The biological activities of coelomic fluid of earthworms must be related to components contained within the coelomic fluid. In this regard, researchers reported a wide variety proteins possessing several biological activities in coelomic fluid. The biological activities of the coelomic fluid could be mediated by its proteins. Correspondingly, a protein with 60 kDa molecular weight was found in *Eisenia foetida*. This protein might be involved in the immune reaction of earthworms. Researchers reported that the biological activities of the coelomic fluid of the earthworm *Eisenia foetida* could be related to some molecules with 33, 40, 42, 45 and 60 kDa molecular masses (15). One small peptide was isolated from the earthworm coelomic fluid with a molecular weight of 510.3 Da. Also, by immunochemical analyzes, these proteins were shown to belong to the immunoglobulin group.

The major finding of the present study was that the coelomic fluid of *Eisenia foetida* earth worm contains more proteins than previously thought. In this regard, 2DE is a powerful method for protein mixture analysis. We used this technique to determine the protein contents of coelomic fluid obtained from *Eisenia foetida* earthworm. The results of our study demonstrated a number of proteins with pH ranging from 3-6, and molecular weight of 10-150 kDa (mostly around 30-70 kDa) which were detected by SDS-PAGE, and 2DE in coelomic fluid of the earthworm *Eisenia foetida*. Previously, researchers have reported proteins with 30 to 60 kDa molecular weight. For the first time, in this study, we identified further proteins with molecular masses of 10 to 150 kDa. It might be possible that many of these proteins are responsible for earthworm's biological activities. Investigation of these proteins might be helpful to know the mechanisms of coelomic fluid action. These findings might introduce a new method for treating diseases caused by bacteria, inflammations, and enhancing the healing process. Our results corroborate with previous reports (15). Many biological functions such as anticoagulative and fibrinolytic, hemolytic and agglutinative (19), bacteriolytic (20), anti-oxidant (9), and growth promoting (10) activities of the extracts obtained from *Eisenia foetida* the homogenate were referred to two tyrosine-like serine peptidases (PI), and (PII) with molecular masses of 34 and 23 kDa, respectively (21-22). These findings support the traditional use of coelomic fluid of *Eisenia foetida*

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earthworm as a potential therapeutic agent that has been reported in literature (9). These proteins may contribute to accelerate the wound healing process but their structure, function, and roles are still unclear (23). The physiological relevance of our finding, as well as the factors contributing to wound healing in human`s, need t o be further evaluated in future experiments. The determination of the main proteins leading to biological activities of coelomic fluid are still under investigation by our group. It should also be taken into account that there was no study comparable to the present study. Also, the mechanism of action of glycolipoprotein extracts of *Eisenia foetida* (G-90) on wound healing cannot be explained by this report.

In conclusion, the coelomic fluid of earthworm *Eisenia Foetida* which includes a wide variety of biological activities could be mediated by its various proteins.

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Conflict of interest

The authors declare that they have no competing interest.

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