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IDENTIFICATION OF LYSENIN PROTEIN FUNCTION IN COELOMIC FLUID OF *EUDRILUS EUGENIAE*

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Abstract

The coelomic fluid (CF) is the body fluid present in the space between the body wall and gut in the earthworms. The earthworm's coelomic fluid contains various molecules and exhibits important biological properties such as antimicrobial substances, hemagglutination and anticoagulant agents. The coelomic fluid of earthworm possesses primary function that has the ability to destroy the membranes of foreign cells. It is a mechanism that leads to cell death by the release of cytosol and attributes coelomocytes which secretes humoral effectors into the coelomic fluid. In this work, bacterial cultures namely *Pseudomonas aeruginosa* and *Bacillus subtilis* were injected into the earthworm body cavity. Then the coelomic fluid extract from the bacteria injected earthworms were collected out. The extracted coelomic fluids were partially purified and precipitated using ammonium sulphate and analyzed to biological activities such as antibacterial activity, proteases activity and heamolytic activity. Then the active compounds present in the coelomic fluids were analysed to FTIR, HPLC and LCMS. This concludes that the microbes introduced into the earthworm body containing coelomic fluids have lysenin protein that inhibits antibacterial, protease and haemolytic activity. Hence the induction of bacterial cultures Pseudomonas aeruginosa and Bacillus subtilis into the earthworm's body cavity leads to the secretion of lysenin protein in the coelomic fluid of earthworms. Thus the presence of proteins presence in the coelomic fluid can be used for pharmaceutical purposes.

Key words: Earthworm; coelomic fluid; FTIR; HPLC; LCMS; lysenin.

Introduction

Invertebrates are commonly called "worms". In day to day life, the term worm is also applied to various other living forms such as larvae, insects, centipedes, shipworms etc. To most people the most familiar worms are the earthworms, members of phylum Annelida. Earthworms have been recognized in oriental medicine as anti-inflammatory, analgesic and antipyretic agent (Yegnanarayan *et al*. 1987) and (Valembois et al. 1982b). Moreover, earthworms have been used to treat upper respiratory tract infections, typhoid and diarrheal pathogenic bacteria as a natural drug in Indonesia more than 50 years (Bakti et al. 2003). Earthworms have been proved as excellent inexpensive animals for studying regeneration, aspects of neuro-physiology, neuro-endocrinology and excretion. Now the attention of comparative immunologists are towards

Annelid worm's, to unveiling the immune protein potentially in it (Cooper 2003); (Cooper and Roch 2003). During evolution, earthworms have developed defense strategies against living pathogens such as bacteria, fungi and parasites. Earthworms lack true antibodies and hence an adaptive immune response and instead have efficient innate immunity system to defend themselves against invading foreign materials (Cooper 2003); (Cooper and Roch 2003). Coelomic fluid cells usually present in the fluid of coelomic cavity known as coelomocytes and skin of the earthworms play a major role in the defence mechanisms of earthworm.

Material and Methods

Collection of Earthworms

Earthworms *Eudrilus eugeniae (E.eugeniae,* annelid, Oligochaeta, Eudrilidae) were collected from Microbial Biotechnology Laboratory, Department of Biotechnology, Manonmaniam Sundarnar University, Tirunelveli, Tamil Nadu.

Bacterial Injection and Coelomic Fluid Harvesting

The gut cleared earthworms were washed with distilled water and injected with 50µl of *Pseudomonas aeruginosa* (*Pa*) and *Bacillus subtilis* (*Bs*) and incubated for 24 hours. After 24 hours post bacterial injection, the worms were placed in a sterile petriplate to which 1ml of 1X PBS was added. After harvesting the coelomic fluid (CF) was centrifuged at 12,000 g for 10 min at 4ºC in a Remi C24 plus (Remi, Vasai, India). The cell-free supernatant of *Pseudomonas aeruginosa* injected coelomic fluid (*Pa.*CF) and *Bacillus subtilis* injected coelomic fluid (*Bs.*CF) were collected and sterilized with 0.45 µm syringe filter (Hi- Media) for immediate use and stored at -20°C until needed.

Ammonium Sulphate Precipitation

The salt precipitation is an ideal method to recover total protein content of crude extract. The precipitation of proteins via ammonium sulphate does not hamper protein activity. Ammonium sulphate was used to precipitate total protein from crude filtrate. More than 90% of total protein was precipitated at 60% concentration of ammonium sulphate. The precipitate was separated from soup by high speed centrifugation at 20,000 rpm at 4^0C for 30 minutes. The precipitate was suspended in 20mM phosphate buffer pH 7.5 and further filtered with membrane filter of pore size 0.4µm.

Antimicrobial Activity

The protein fraction was assessed for antibacterial activity by well diffusion method. Different types of bacterial strains including thermophiles was used for examining the antimicrobial activity. Both Gram negative bacterial strains (*Escherichia coli, Salmonella abony, and Pseudomonas aeruginosa*) and Gram positive bacterial strains (*Streptococcus faecalis, Bacillus subtilis, Micrococcus luteus*) were used. The determination of antibacterial activity was carried out by using well diffusion method where nutrient broth was used for the growth of liquid bacterial cultures and nutrient agar for preparation of bacterial cultures in petriplates. The wells were punched into the nutrient agar plates and allowed for solidification. The CF was filled (50µl) in each well with PBS (100µg/µl) which was alone treated as negative control. The plates were incubated at 37ºC for 24 hrs and antibacterial activity was determined by measuring the diameter of zone of inhibition.

Protease Activity

The protease activity was determined by purified protein fraction. About 20 ml of gelatin-agarose gel was prepared and poured into the petriplate and allowed to solidify. About 50µl of each CF sample were added to the well created in the agar plate. It was incubated for 4-6 hours at 37°C. The gelatin-agarose gel was precipitated with 5ml of solution containing 15g of $HgCl_2$ and 20ml of 12N HCl in 80 ml distilled water. The diameter of the clear circle around each well was measured.

Haemolytic Activity

Haemolytic activity of the CF of the earthworm *Eudrilus eugeniae* was determined by using well diffusion method. About 20 ml of blood agar was prepared by adding 5 ml goat blood and poured into petriplate and allowed to solidify. About 50µl of each sample were added to the well punched in the agar plate. It was incubated for 24 hours at 37ºC. The diameter of the clear circle around each well was measured.

FTIR Analysis

The cell-free CF of precipitated sample from earthworm's viz., *Eudrilus eugeniae* was taken in the sample block of FTIR instrument. The block was washed with acetone and allowed to dry prior to loading the sample. The vibrational spectra were recorded and averaged over 65 scans on a Digilab FTS 6000 FT-IR spectrometer equipped with a cesium chloride detector. The spectral range of 400-4000 cm1 was used for analysis. Background spectra were obtained subsequently. The vibrational spectra of different treated groups were recorded with a resolution of 4 cm and compared it with the FT-IR spectrum of test chemicals. The spectra were processed and drawings were made using the software, 'Origin' from Perkin-Elmer. FT-IR spectra of the CF collected from the control sample were used for comparison.

HPLC Analysis

The HPLC (High Performance Liquid Chromatography) is an analytical chemistry technique that combines the physical separation capabilities of liquid Chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. Mixtures of these types require aqueous mobile phases such as methanol-water or acetonitrile-water and these liquids do not work well on normal silica or alumina stationary phases. Instead of these polar phases, very nonpolar ones called "reverse-phase" packing are used. These are manufactured by bonding lots of hydrocarbon Molecules to the surfaces of a silica gel particles so that the silica gel is like a very nonpolar "grease ball." In this situation, the order of elution will be exactly opposite the behavior on an alumina or silica column. On a reverse-phase column, the more nonpolar materials will adhere to the stationary phase (or like material) longer and the polar compounds will elute first. 2 mg of the purified compound sample from *Eudrilus eugeniae* was prepared by dissolving in 2ml methanol in volumetric flask. Solution was then filtered. Stock solution 1mg/ml was kept in refrigerator at 4ºC. HPLC for various layers were performed.

LC-MS Analysis

CF samples from the earthworm *Eudrilus eugeniae* was analyzed by LC-MS/MS using Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, and Germany) equipped with a DPV-550 Digital Pico View nanospray source and coupled with an UltiMate 3000RS LC nano System (Dionex). Details concerning LC MS/MS measurement method and data analysis are described in (Swiderska et al. 2016).

Results

Antimicrobial Activity

The influence of protein fractions of CF in *Eudrilus eugeniae* on the *in vitro* growth of bacterial cultures was evaluated against pathogenic bacteria such as gram positive bacteria (*Streptococcus faecalis, Bacillus subtilis, Micrococcus luteus*) and gram negative bacteria (*Escherichia coli, Salmonella abony, Pseudomonas aeruginosa*). The best inhibitory effects of *Pa.*CF and *Bs.*CF were identified on the growth of *Pseudomonas aeruginosa* (*Pa*) and *Bacillus subtilis* (*Bs*) .

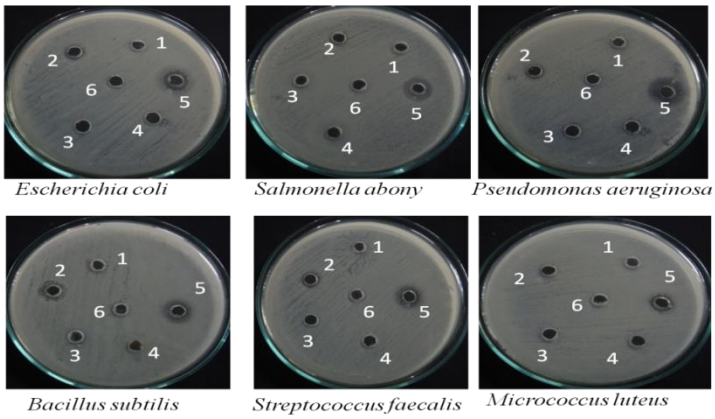

Figure 1 - Antibacterial activity of *Eudrilus eugeniae* coelomic fluids sample: 1: PBS 2: *Bs.*CF 3: Broth 4: Normal CF 5 *Pa.*CF: 6: Broth. CF

The inhibitory zones measured for *Pseudomonas aeruginosa* and *Bacillus subtilis* was 19 mm and 13 mm respectively.

Protease Activity

The protease activities of the CF were examined by simple agar diffusion method. After incubation, the clear zones were viewed around the well for all CF samples in the gelatine agarose medium. The degradation of the protein gelatine was observed in all the CF samples except in the PBS and broth. The best effect was observed in *Pa.*CF.

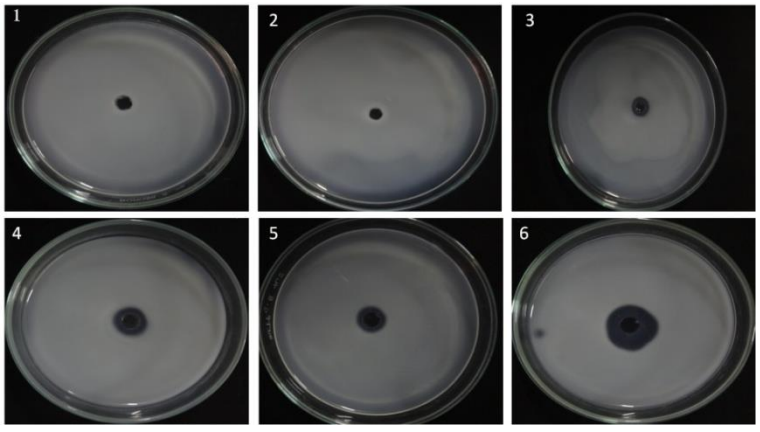

Figure 2 - Proteolysis activity of *Eudrilus eugeniae* coelomic fluids samples. 1: PBS 2: Broth 3: Normal CF 4: Broth. CF 5: *Bs.*CF 6: *Pa.*CF

Haemolytic Activity

A simple well diffusion method was used to demonstrate the characteristics of CF of earthworms *Eudrilus eugeniae* against the erythrocytes present in the red blood sample. Different CF with blood sample mixed with blood agar medium was visualized for the appearance of clear zones around the well after an incubation of 24 hours. The heamolytic activity was found to be present in all CF sample but the best inhibition was observed in *Pa.*CF.

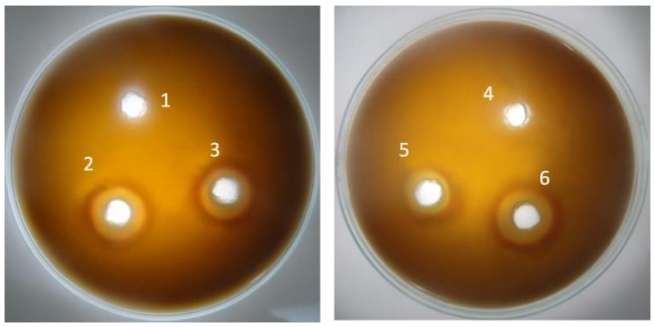

Figure 3 - Haemolysis activity of *Eudrilus eugeniae* coelomic fluids samples. 1: PBS 2: Broth. CF 3: *Bs.*CF 4: Broth 5: Normal CF 6: *Pa.*CF

FTIR Analysis

The FTIR analysis was performed to analyze the functional groups involved in the antibacterial, proteolytic and haemolytic activities. Herein, the antibacterial, proteolytic and haemolytic activities were examined for CF isolated from *Eudrilus eugeniae*. The peaks for *Pa.*CF were observed at 3748, 3405, 3389, 2355, 1505 and 1395 cm-1. The weak band was observed at 3748 cm-1 indicates the presence of phenols and alcohols with OH stretch. The peaks 3405 and 3389 cm-1 corresponds to the N-H stretching of primary amines. The peak observed at 2355 denotes C≡N stretching of nitriles. The peak at 1505 and 1395 cm^{-1} indicates the N=O stretching of nitro groups. The FTIR peaks for *Bs.*CF were observed at 3585, 2889, 2326, 1510 and 1446 cm^{-1}. The band at 3585 cm^{-1} corresponds to OH stretching of phenols and alcohols. The absorption band at 2889 cm^{-1} indicates the H-C-H stretching of alkenes. The band at 2326 cm^{-1} denotes the presence of C≡N stretching of nitriles. The peak at 1510 and 1446 cm^{-1} indicates the N=O bending of nitro groups. The peaks for normal CF appeared at 3489, 2954, 2910, 2355, 1716, 1508 and 1442cm^{-1}. The band at 3489 cm^{-1} corresponds to N-H stretching of secondary amines. The peak at 2954, 2910 and 1442 cm^{-1} indicates the presence of H-C-H stretching of alkenes. The peak at 2355 cm^{-1} corresponds to the nitriles with C≡N stretch. The peak at 1710 cm^{-1} indicates the C=O stretching of carboxylic acids. The peak at 1508 cm^{-1} corresponds to N=O stretching of nitro groups.

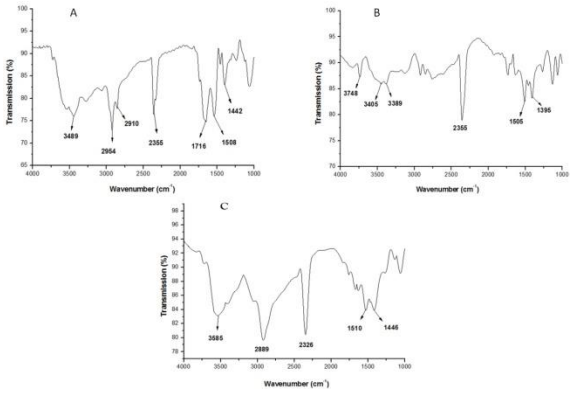

Figure 4 - FTIR analysis of *Eudrilus eugeniae* coelomic fluids samples. A : *Bs.*CF B: *Pa.*CF C: Normal CF

From the FTIR analysis, the amines, nitro groups and phenols in the lysenin protein might be involved in the Antibacterial, Haemolytic and Proteolytic activities.

HPLC Analysis

HPLC is an analytical chemistry technique that combines the physical separation capabilities of liquid Chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. The HPLC was performed to confirm the presence of active constituents in the CF. The CF sample was analyzed for HPLC. The HPLC profile of *Pa.*CF represents characteristic peaks at retention time 2.1, 4.3, 6.0, 14.8, 19.2, 23.2, 23.5 and 24.7 min at the wavelength 220 nm.

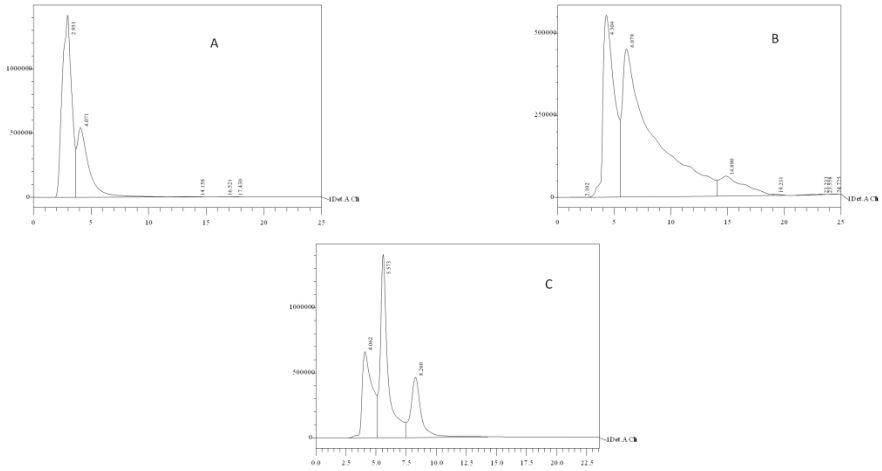

Figure 5 - HPLC analysis of *Eudrilus eugeniae* coelomic fluids samples. A: Normal CF B: *Bs.*CF C: *Pa.*CF

Herein, the intense peaks were attained at 4.3 and 6.0 min with the area 38688882 and 85811534, respectively. The peak at retention time 4.3 exemplifies the presence of lysenin protein in the *Pa.*CF. Coelomic fluid of *Bs.*CF also exhibits major peaks at 2.9 and 4.0 min with an area 80930829 and 39509424, respectively (figure 5). The peak at retention time 4.0 min indicates the presence of lysenin protein in the *Bs.*CF. The Normal CF also shows 3 intense

peaks at the retention time 4.0, 5.5 and 8.2 min (figure 5). In normal CF also, the peak confirms the presence of lysenin protein in the CF. From the results of HPLC analysis, the peaks at the retention time 4.0 min clearly shows the presence of lysenin proteins and it might be responsible for the Antibacterial, Haemolytic and Proteolytic activities.

LC-MS Analysis

The presence of lysenin protein in the CF identified by HPLC and FTIR analysis was further confirmed by LC-MS analysis. Herein, the mass analysis shows different molecular mass of the proteins ranging from 100 to 1450 m/z. The *Pa.*CF exhibits an intense peak at 255.20 m/z molecular mass reveals the presence of lysenin protein in the CF. Similarly, the *Bs.*CF also shows the peak at 255.27 m/z also indicates the presence of lysenin protein.

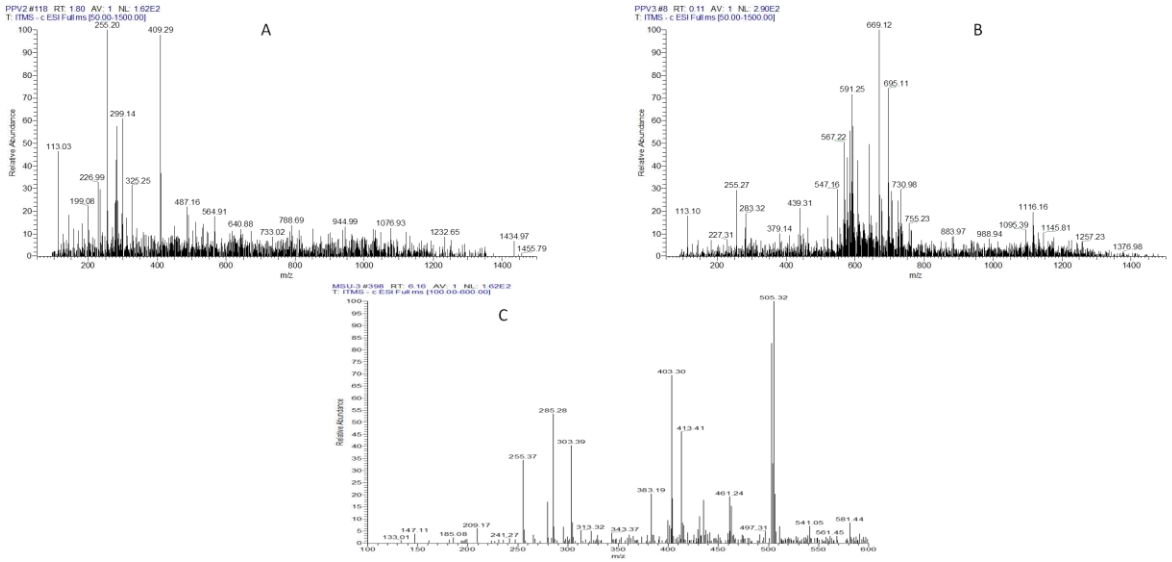

Figure 6 - LC-MS analysis of *Eudrilus eugeniae* coelomic fluids samples. A: *Pa.*CF B: *Bs.*CF C: Normal CF

Finally, Normal CF also shows the peak at 255.37 m/z also indicating the presence of lysenin protein. The mass spectroscopy indicates that lysenin is one the proteins in the CF of earthworm that might be involved in the antibacterial, haemolytic and proteolytic activities.

Discussion

The pathogenic bacteria *A. hydrophila* and *B. megaterium* enhance some antibacterial activity and infestation only in CF (Lassègues et al. 1989). (Valembois et al. 1994) and (Cooper et al. 1999) reported that the coelomocytes (cells of coelomic fluid) initiate the process of connecting with each other by their adhesive structures around the bacteria and form brown bodies. At the same time, the coelomocytes intensively synthesize and secrete proteins that attach to the bacteria, forming aggregations and may inhibit their further proliferation. One of these proteins is agglutinin of 56 kDa molecular mass which attaches to the lectin-like monosaccharides of the cellular membrane of the bacteria. The CF of chlorocytes secreted lysenin protein (33 kDa) which binds specifically to phospholipids of the bacterial cell membrane and causes cytolysis (Kauschke et al. 2007), (Ohta et al. 2000), (Opper et al. 2013) and Lysenin is one of the toxin

proteins present in the coelomic fluid of earthworm might be responsible for pore formation in the bacterial cell membranes with specifically bind to sphingomyelin and resulted in the death of bacterium cell (Yamaji et al. 1998); (Shogomori and Kobayashi 2008).Coelomic cytolytic factor 1 (42 kDa) from the CF which involved in the activation of prophenoloxidase cascade via recognition of Gram-negative bacteria cell wall molecules such as glucan and lipopolysaccharide (Beschin et al. 1998) but the agglutination (Valembois et al. 1984) and clotting (Laemmli 1970) were mediated by fetidin proteins (40 kDa and 45 kDa) from CF. The 40 kDa fetidin involved in 84% of haemolysis and 45 kDa fetidin involved in 77% of heamolysis (Milochau et al. 1997). The normal CF possesses heamolytic, agglutination, protease activity and clotting as it cannot be involved in antibacterial processes because normal CF carried normal micro biota (Dales and Kalac 1992). The CF involved in antibacterial activity was mediated by injecting the pathogenic germs within the CF (Valembois et al. 1982a). The pathogenic germs (Lassègues et al. 1989) specifically induced several protein mediated by culture that was injected after 24 hours. The specifically bacterial protein responsible for the gene destruction was translated. In the research activity carried out here shows that HPLC, after the stimulation of the above mentioned bacterial cultures indicated that lysenin peak level is high while lysenin level in normal CF was found to be low. The same result was initiated for LC-MS with normal CF having low lysenin level but the stimulated CF level was high. When the lysenin was present in CF at high level it was found that the CF possessed high antibacterial capacity and the level of heamolytic as well as proteolytic activity automatically increased. The result indicates that the stimulated CF that produced high antibacterial activity and no activity was observed in normal CF (Figure 1). The stimulation of CF was found to be better than the normal CF in proteolysis activity (Figure 2) because after the stimulation of CF that exhibited strong protease activity (Kauschke et al. 1997). Lysenin protein was highly secreted after the stimulation of bacteria (Figure 4, 5, 6).

Conclusion

This work is concluding normal CF doesn't possess antibacterial activity because of low lysenin level. When the CF was stimulated or disturbed by bacterial cultures, the lysenin protein was secreted. The secreted lysenin protein was involved into the antibacterial activity and destroyed the entry of all foreign particles. Hence the lysenin protein was confirmed to exhibit the antibacterial, haemolysis and proteolysis activity antibacterial protein in earthworm *Eudrilus eugeniae*. So this protein is highly applicable and be used in pharmaceuticals for inducing immunity in animals.

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Conflict of Interest

The authors confirm that the manuscript has no conflict of interest.

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