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Identification of Immunity Inducing Biological Compounds from Coelomic Fluid of Eudrilus Eugeniae

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Abstract

The coelomic fluid of Annelids exerts numerous biological activities that are involved in effective defense mechanisms against invaders. The coelomic fluid of the earthworm contains more than 40 proteins and exhibits several biological activities. About 20µl of *Pseudomonas aeroginosa* culture inoculated broth were injected into earthworm and disturbed at 24 hours to obtain the coelomic fluid. The uninoculated broth alone served as the control. *P. aeroginosa* injected coelomic fluid exerted higher antibacterial, proteolytic and erythrolytic activity along with a newly expressed protein in HPLC. This concludes that when the appearance of newly expressed protein in the body cavity of earthworm automatically kills various foreign intruders.

Key words: *Earthworm; Coelomic fluid; Pseudomonas aeroginosa; HPLC; Erythrolytic activity.*

1. Introduction

Invertebrates evince different immune mechanisms against environmental pathogens. Earthworms are relatively long living invertebrate organisms which persist of rich micro-organisms, fungi, and other potential pathogens. The success of the earthworms' survival depends on the conditions supported by efficient innate immune mechanisms based on both the components of the earthworm coelomic fluid such as cellular activity and humoral activity of coelomocytes. The coelomic fluid is very important to carry immune properties to affected parts in vertebrates. The coelomocytes present in the coelomic fluid plays a significant role in the active functioning of the humoral and cell-mediated immune response. They are also responsible for eliminating foreign materials by proteolytic, hemolytic and cytolytic properties. Lysozyme is the factor responsible for the destruction of the antibacterial activity of the coelomic fluid checked against Gram positive and Gram negative bacteria (1). This function is mediated by two proteins namely fetidin and lysine of presumed molecular mass 40 kDa and 45 kDa respectively that has a similarity of 35% when compared with the immunoglobulins (2). The major component of the coelomic fluid peptides was found to be present in both the above-mentioned proteins (20% of total CF proteins) and are released from the chloragogue cells(3). Similar cytolytic activities are also elucidated in bacteria (4), (5) and in some eukaryotes such as Molluscs, Echinodermata, Polychaeta, Oligochaeta, Arthropoda (6), (7) and vertebrates(8), (9), (10), (11). Lysenin a consequence of its specific binding to sphingomyelin (SM), lysenin induced the smooth muscle contraction(12), red blood cells were lysed (13) and destruction of vertebrate spermatozoa(14), (15). The coelomic fluid had strong hemolytic activity against various mammalian erythrocytes. The hemolytic proteins of molecular weight 38 kDa inducted eiseniapore that was also isolated from the coelomic fluid of the same species(16). The coelomocytes were immunocompetent cells that are amoebocyte, chloragocytes, and eleocytes. The inducted coelomic cytolytic factor 1 (CCF-1) 42-kDa protein triggered the prophenoloxidase cascade (17). A novel protein of 297 amino acids with a molecular mass of 33 kDa from the CF, This protein induced the contraction of smooth muscle and was named lysenin(12), (18), (19).

2. Material Methods

2.1 Extraction coelomic fluid

The coelomic fluid was extracted by using the cold shock method. In this method, gut cleared worms were washed with distilled water and injected with 50µl of *Pseudomonas aeruginosa*. After 24 hours of injecting microorganisms, the worms were placed in a sterile Petri plate to which 1ml of 1X PBS was added. Then it was kept between the ice packs for 5 minutes. The fluid was collected in a sterile tube and stored at 4°C.

2.2 Preparations of Tissue Extract (G90 Protein)

The earthworms were washed with running tap water. It was separated into two groups. One group of earthworms was injected with the microorganism *Pseudomonas aeruginosa*, another one group was used normally. Both the normal and injected worms were kept in 0.65% NaCl at room temperature for 1 hour. Later the worms were cut into small pieces. Three gram of the earthworm tissue was homogenized in 40ml of chloroform: methanol 1:1 (v/v) and kept at 4°C for overnight. Next day, about 16 ml of distilled water was added to the homogenate and centrifuged at 3000 rpm for 10 minutes. Three clearly visible layers were obtained. Bottom and middle layer was chloroform and tissue layer. Upper layer contained water and methanol. The methanol was evaporated in a laminar airflow chamber for one day. Remaining water extract of both the normal and injected worms tissue extract (G90) was collected separately and stored at 4°C.

2.3 TLC

The slurry was prepared by mixing silica gel with water in the ratio3:2 the slurry was coated onto the glass plate at a thickness of about 0.25mm and the plates were allowed to dry at room temperature for 15-30 minutes. Then the plates were kept in a hot air oven at 100-200°C for 2 hours. The samples were resolved using the solvent system Butanol: Acidic acid: Water (60:15:25) and then visualized in gel documentation.

2.4 Antibacterial Activity

Antibacterial activity of compound 1 and compound 2 from TLC were tested against selected Gram-positive (*B.subtilis*, *S.faecalis*, *M.luteus*) and Gram-negative bacteria (*S.abony*, *P.aeruginosa*, *E.coli*) using well diffusion method. The bacteria were grown on nutrient broth. Mueller-Hinton agar medium was poured into a sterile Petri plate and allowed to solidify. The 24 hours bacterial culture was swabbed on the medium and wells were bored. About 50µl of the samples were added into the well and the plates were incubated at 37°C for 24 hours. After incubation, the inhibition zone was observed around the well and recorded.

2.5 Proteolytic Activity

The proteolytic activity of the compound 1 and compound 2 from TLC were determined by using Gelatin lysis activity. Gelatin is the denatured product of collagen and is an abundant protein. About 20 ml of gelatin-agarose gel was prepared and poured into the Petri plate and allowed to solidify. About 20µl of each sample was 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 a solution containing 15g of HgCl2 and 20ml of 12N HCl in 80 ml distilled water. The diameter of the clear circle around each well was measured.

2.6 Erythrocytic Activity

About 1.5 % of agarose was prepared. 20 ml of sheep blood was collected and subjected to centrifuge action at 10000 rpm for 10 minutes. RBC was collected. 9ml of 0.9% freshly prepared NaCl was added to 1ml of collected RBC. 20 ml of prepared RBC was added to sterilized 1.5 % agarose and poured on plates, and cooled. Samples of compound 1 and compound 2 from TLC were loaded on the wells cut on the plate. It was incubated for 24 hours at 37°C. After incubation, the clear zone was recorded.

2.7 HPLC for active compounds

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. Two mg of the sample (active compound 1) was prepared by dissolving in 2ml methanol in a volumetric flask. The solution was then filtered. Stock solution 1mg/ml was kept in the fridge at 4°C. HPLC for various layers were performed.

3. RESULT

3.1 Thin layer chromatography

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Coelomic fluids and skin extracts of *Eudrilus eugeniae* were prepared for thin layer chromatography. Two metabolites (blue (compound 1) and yellow (compound 2)) were observed (figure 4) in coelomic fluids and *P.aeruginosa* injected coelomic fluid and skin extract and there is no spot was observed in normal skin extract. Two metabolites were synthesized in coelomic fluids and *P.aeruginosa* injected skin extract but there is no metabolites were noticed in skin extract.

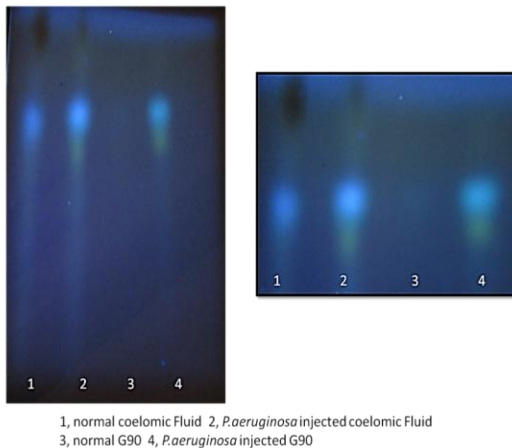

Fig.4.Thin layer chromatography of earthworm samples

3.2 Antimicrobial activity

TLC compound 1 and compound 2 extract from *Eudrilus eugeniae* were used to examine the antibacterial activity against gram-positive (*B.subtilis*, *S.faecalis*, *M.luteus*) and gram-negative (*S.abony*, *P.aeruginosa*, *E.coli*) bacterial pathogens. compound 1(50µl), (10µl) and compound 2 (10µl) showed high antibacterial activity against the pathogens. Compound 1(50µl) rendered the maximum zone of inhibition against *P.aeruginosa* whereas Compound 2 (50µl) (figure 1). Compound 2 showed less antibacterial activity against all pathogens when compared to Compound 1.

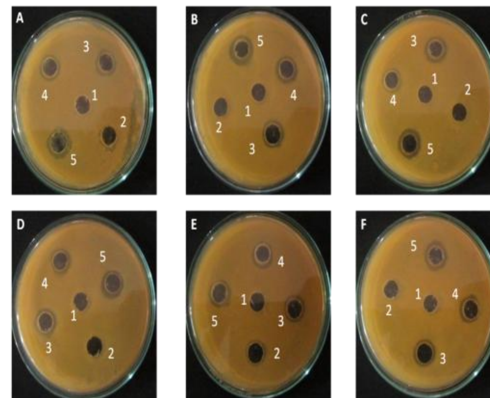

Fig.1. Antimicrobial activity of earthworm samples

3.3 Proteolytic activity

A simple agar diffusion assay incorporating gelatin as substrate was used to demonstrate the characteristics of TLC compounds extracts from *Eudrilus eugeniae*. After 4-6 hours of incubation of 30 µl different sample with agarose medium, the clear zone was viewed around the well which was due to the degradation of gelatin. The clear zone was observed in the Compound 1(50,10µl) and Compound 2 (50µl), and there is no zone observed in Compound 2 (10µl). High proteolytic activity was observed in the Compound 1(figure 2)

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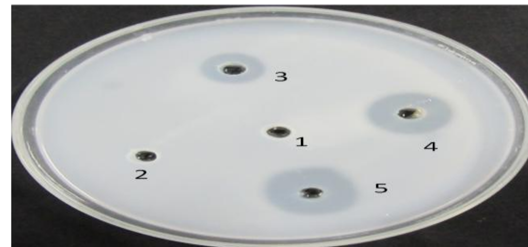

1, PBS 2,compound 2(10µl) 3, compound 2(50µl)
4, compound 1 (10µl) 5, compound 1 (50µl)

Fig. 2: Proteolytic Activity of earthworm samples

3.4 Erythrolytic activity

Compound 1 and Compound 2 were examined against Red Blood Cells (RBC) for erythrolytic activity.

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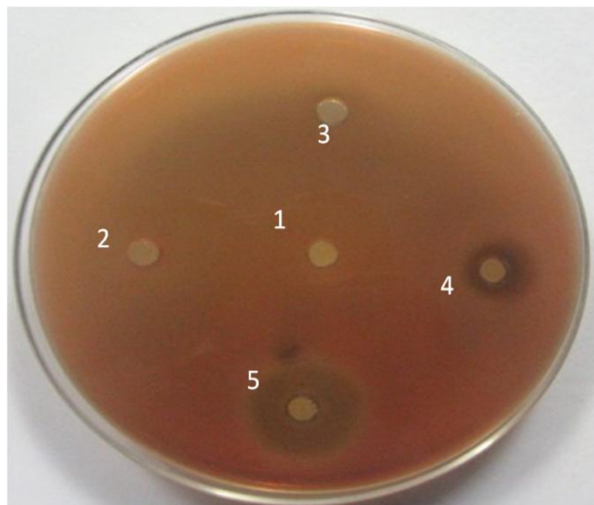

1, PBS 2,compound 2(10µl) 3, compound 2(50µl)
4, compound 1 (10µl) 5, compound 1 (50µl)

Fig.3.Erythrolytic activity of earthworm samples

After incubation of 30 µl different sample with blood agar media, the clear zone was viewed around the well. Degradation of red blood cells was observed in the Compound 1, whereas there is no lytic activity was observed in Compound 2 (figure 3).

3.5 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 compound 1. Purified fractions of the compound 1 were analyzed for HPLC. The HPLC profile compound 1 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). Herein, the intense peaks were attained at 4.3 and 6.0 min with the area 38688882 and 85811534, respectively.

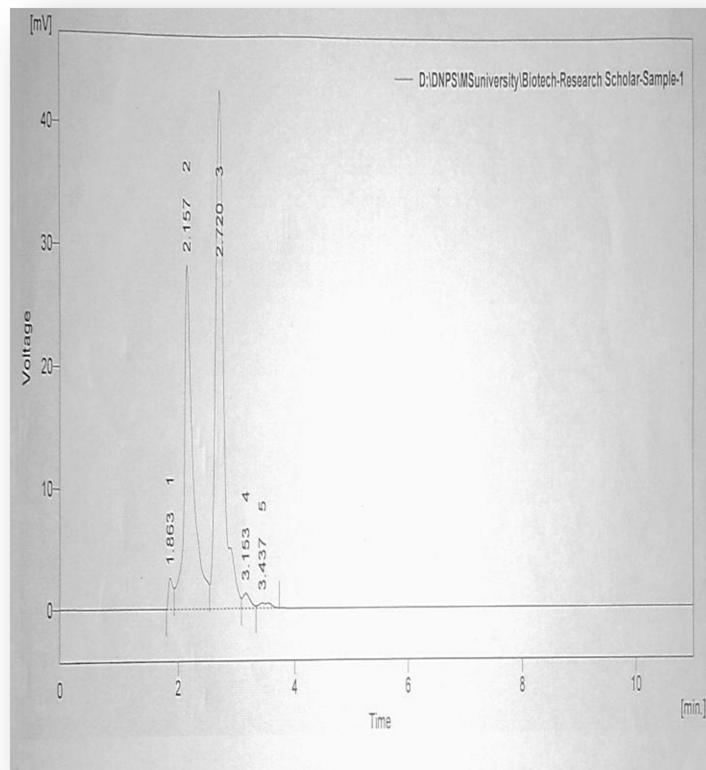

Fig.5.HPLC Analysis of specific spots (yellow and brown color spots)

Discussion

The coelomic fluid of *Dichogaster bolaui* showed antibacterial activity against pathogenic bacteria like *E.coli*, *S.aureus*, *Pseudomonas*, *Proteus* and *Bacillus sp*. The best inhibitory effect of coelomic fluid of *Dichogaster bolaui* on the growth of *Proteus* is 16 mm and that on *Pseudomonas* 8mm, whereas the zone of inhibition is only 7mm against *Escherichia coli*, *Staphylococcus aureus,* and *Bacillus* (28). In that *Eudrilus eugeniae* injected coelomic fluid had high zone against in gram-positive and negative bacteria better than to normal coelomic fluid. Earthworm paste of *Eudrilus eugeniae* was tested for their antibacterial effect against five strains of bacteria viz., *E.coli*, *S.abony*, *S.aureus*, *K.pneumoniae,* and *B.subtilis*. The earthworm pastes highly inhibit the growth of *S.aureus* and inhibit the growth of *S.abony* in minimum level (20). (21) Reported that the ethanol extracts of dried earthworm powder obtained from *Lampito mauritti* were a potent antibacterial agent against *Aeromonas hydrophila* and antifungal agent against *Candida albicans*. In that result compound 1 have high zone better than to compound 2 in gram positive and gram negative pathogens.

In earthworms, proteolytic enzymes have been found in the coelomic fluid (22), (23), (24) reported that the wounding and injection of foreign material into the coelomic cavity increase coelomocyte numbers and the activity of easily measurable humoral immune factors like agglutinins, lysins and protease. An increase in the number of proteases after stimulation has been shown before, but it is still unclear whether this increased number as demonstrated after native PAGE separation is due to newly synthesized proteins or whether it results from limited proteolysis. Compound 1 and Compound 2 possessed proteolytic activity. Compound 2 (50µ) was able to lyse the protein but the Compound 2 (10µ) showed no activity on Gelatin Agarose medium. Among the two samples, Compound 1(50µ) exhibited higher proteolytic activity. Compound 1(10µ) possessed low activity Normal coelomic fluid had lower activity in protein degradation.

Roch et al. and Milochau both found proteins with molecular weights of 45 kDa and 40 kDa possessing several activities, such as antibacterial, hemolysis, and hemagglutination from E. fetida. Some studies about the hemolytic and antibacterial effect of coelomic fluids have exhibited that antigenic determinant on the surface of sheep erythrocytes and bacteria which are inhibited by coelomic fluid are similar (25), (24). In this experiment Compound 1 possess highly erythrolytic activity and no activity in Compound 2.

The peak at retention time 4.3 exemplifies the presence of lysenin protein in the coelomic fluid of *Perionyx escavatus*. 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 (26), (27). Similar the *Eudrilus eugeniae* also exhibits major peaks at 2.1 and 2.7 min with an area 299.074 and 420.248, respectively (fig). The peak at retention time 2.1 and 2.7 min indicates the presence of biological active compound in the coelomic fluid of *Eudrilus eugeniae.*

Conclusions

This work is concluded normal coelomic fluid possess some biological activity without skin extract. Any substance disturbed from outside of body cavity suddenly the immunological compound are secreted in 24 hours. The secreted the compounds are involved in the biological function and destroy the all entry the foreign particles. . So this protein compound is highly applicable and used in pharmaceuticals for inducing immunity in animals and humans.

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Conflict of Interest

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

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