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Research Article

EARTHWORM- A POTENTIAL SOURCE FOR STABLE AND POTENT ANTIMICROBIAL COMPOUNDS- ISOLATION AND PURIFICATION STUDY

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ABSTRACT

In the current work, we have isolated and purified an antimicrobial peptide from celomic fluid of Indian earthworm *Pheretima posthumous*. The purified peptide was characterized as serine protease and possesses broad range of antimicrobial activity. Another important finding was stability of antimicrobial peptide towards higher temperature, different pH and various inhibitors. The celomic fluid of earthworm was subjected to series of purification step such as ammonium sulphate precipitation, dialysis followed by size exclusion chromatography and ion exchange chromatography. The average molecular weight of peptide was found 20KD by SDS PAGE analysis. The peptide was evaluated for antibacterial and antifungal activity in various temperatures, pH and in the presence of different inhibitors. We have evaluated antibacterial activity of purified peptides among physchrophiles, mesophiles and thermophiles.

Keyword: Celomic fluid, Antimicrobial activity, Purification, Earthworm, Electrophoresis and chromatography.

INTRODUCTION

Since ancient time, earthworm was known and used for its therapeutic potential[1]. Various therapeutic molecules have been isolated and characterized such as fibrinolytic, antiviral, antitumor, hepato-protective, cytotoxic and antioxidant in different species of earthworms around the world. In last few decades, the fibrinolytic property has been explored tremendously[2,3]. Earthworm has recognized as potential source of therapeutics not only because of availability of various molecules but also stability and potency of these isolates. The antimicrobial property (antibacterial and antifungal) of earthworm have been evaluated by many researchers in last decade[4,5,6]. The entire earthworms as paste has used for treatment of wounds and tropical ulcer[7]. Such potent molecules naturally reside predominantly in intestinal fluids and subsequently in tissue fluid of earthworm. Lumbricin I and II are peptides, which have been reported in *Eisenia. fetida* and *Lumbricus rubellu* [8,9]. These molecules characterized as glycoprotein, which offers strong antimicrobial activity[10]. The molecular mechanism and biophysical characterization of these molecules have not completely explored yet.

The question often arises that why earthworm possesses such potent molecules in their intestinal fluid and tissue fluid and their pharmacological significance in earthworm physiology. The most accepted theory concluded its habitat where earthworm encounters various microorganism including bacteria fungi and viruses [11]. Besides advanced animal's immune system such as humoral and cell mediated immunity, lower organism's immunity constituted with such type of molecules, which offer first line defense mechanism. Earthworm essentially requires such potent molecules as it feeds on organic matter with soil[12]. These glycoproteins are characterized as protease (especially serine protease) offer proteolytic degradation of microbial population and provide first line defense system[13,14].

In this study, we have isolated and purified protein offering antimicrobial property of celomic fluids from Indian Earthworm *Pheretima posthumous*. Further, we are looking for the complete molecular mechanism of these molecules, so these can be used for treatment of various bacterial and fungal borne diseases.

MATERIALS AND METHODS

The chemicals and consumables used in the following study purchased form Hi-Media, Sigma and GE healthcare. All the chemicals were molecular biology grade and prepared freshly at the time of use. For determination of antimicrobial activity, used bacterial and fungal strains were purchased from MTCC Chandigarh, India. Bacterial and fungal strains used were wild type and nonpathogenic for following study.

Preparation of Extract

For the current work, Indian Earthworm *Pheretima posthumous* was selected as source of antimicrobial molecules. The earthworms were collected from vermicomposting unit at Vijayawada, Andhra Pradesh, India. Fully-grown healthy Earthworms were subjected to autolysis and further homogenization in 20mM phosphate buffer pH 7.5 for one week at 50^0C with 0.02% sodium azide as bacteriostatic. Autolysed earthworms were further subjected to high-speed centrifugation at 20,000rpm for 30 minute and soup was collected in separate sterile tubes. The clear soup was filtered by membrane filters (0.4µm, Hi-Media) and stored at 4^0C for purification cascade[15,16].

Purification of Peptide

The crude celomic filtrate was further processed by series of chromatographic step for purification of desired peptide.

Ammonium Sulphate Precipitation

The salt precipitation is 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% of ammonium sulphate. The precipitate was separated from soup by high speed centrifugation 20,000 rpm at 4^0C for 30 minute. The precipitate was suspended in 20mM phosphate buffer pH 7.5 and further filtered with membrane filter of pore size 0.4µm.

Dialysis

To remove the salt proportion from protein approximately 12 hour Dialysis was performed to remove salt from filtrate. Dialysis membrane possessing Molecular Weight Cut Off (MWCO) of 12KD purchased from Hi Media was preferred for desalting. The 2ml of ammonium sulphate precipitate was loaded into dialysis bag and desalted against 20mM phosphate buffer pH 7.5. The buffer was replaced in each 4 hour and dialysis was run for 12 hour. After 12 hour, soup was collected in sterile tube and preserved at 4^0C.

Size exclusion chromatography

The soup collected after dialysis was subjected to size based separation of proteins and peptides from crude filtrate. In this study, we have used Sephadex G 50. The Sephadex G 50 (GE Healthcare) beads were allowed for complete swelling in 20mM phosphate buffer pH 7.5 for 48 hours at room temperature. The swelled beads were sonicated to remove traces of entrapped air. Further complete swelled beads were loaded in glass column (60cm*1.5cm) up to

paced bed length 30cm. 2 ml of dialyzed sample was loaded in the Sephadex G-50 column and eluted with excess of 20mM phosphate buffer pH 7.5 in automated fraction collector with 2ml per minute speed[17]. Total 30 fractions were collected (3ml each) and each of fractions was assessed for antimicrobial and protease activity. The fractions shown maximum activity were pooled together and subjected to further purification by charge-based separation. The total protein estimation of each fraction was carried out by Lowery method to calculate concentration.

Ion Exchange Chromatography

Pooled fractions possessing higher antimicrobial activity were further fractionated by ion exchange chromatography where DEAE Cellulose (GE Healthcare) was used for column preparation. The DEAE resin was charged by treatment with acid (HCl) pH (2-3), alkali (NaOH) (pH10-11), distilled water and finally in phosphate buffer 20mM, pH 7.5. The charged DEAE Cellulose resin was loaded on the glass column (60cm*1.5cm) with 20 cm packed bed length. 2ml of pooled fraction was loaded in column and eluted with excess of phosphate buffer and different concentration of Sodium Chloride (0.1% - 0.5%)[18]. A total 20 fractions were collected and each fraction was subjected to the antimicrobial and protease activity analysis.

SDSPAGE Analysis

The average molecular weight of purified peptide possessing antimicrobial activity was determined by SDS-PAGE. A 15% of acrylamide gel was prepared, 15µl of purified sample was loaded and electrophoresis was run for 3 hour at 50 milli-volts with 0.5 X tris glycine electrophoresis buffer. With purified sample protein marker was loaded to determine average molecular weight. After completion of electrophoresis gel was stained with Coomassie Brilliant Blue R-250 and destained subsequently with excess of methanol in destaining solution.

Determination of Caseinolytic Activity

The purified fractions were analyzed for the proteolytic activity by using casein as substrate. Casein agar plates were prepared with 2% casein (Hi-Media) with agar and phosphate buffer 20mM pH 7.5. The caseinolytic activity of purified fractions was assessed by well diffusion method where casein agar plates incubated overnight at 37^{0}C[19].

Assessment of antimicrobial activity

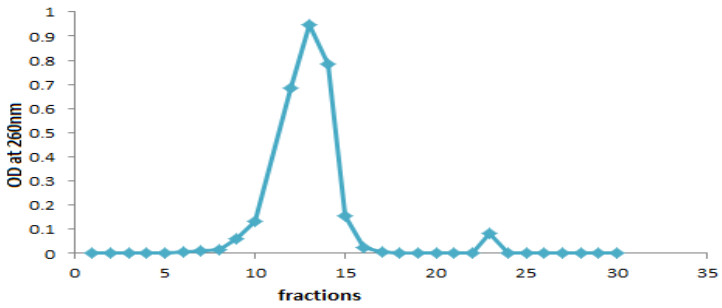
Purified fractions were evaluated for antibacterial and antifungal activity by well diffusion method. Different wild type bacterial and fungal strains including physchrophiles, mesophiles and thermophiles were used for assessing the antimicrobial activity. Both Gram positive and Gram negative bacterial strains were used in the study included Escherichia coli, Pseudomonas putida, Streptococcus aureus, Azotobactor, Bacillus streothermophilous and Pectobacterium carotovorum. For the determination of antibacterial activity, well diffusion method was used with slight modifications where nutrient Broth used for growth of bacterial cultures and nutrient agar for plate preparation. The 6 mm diameter wells were punched into nutrient agar plates and plates were left for solidification. The purified fraction of celomic fluid was filled (20µl) in each well with ampicillin (100µg/µl) positive control. The plates were incubated at 37oC for 14hours and antibacterial activity was determined by measuring the diameter of zone of inhibition. Further, we have incubated purified fractions in various temperature 200C-500C and various pH 4-12 further then analyzed for their antimicrobial activity.

For the determination of antifungal activity, we have used Sabouraud's dextrose/agar (SDA) for the growth of fungal culture. The protocol runs same as that for assaying, the antibacterial activity was evaluated and fungal cultures were kept for 48 h to determine the diameter of zone of inhibition. Here we have used ketoconazole (1mg/ml) as positive control. For the accessing antifungal activity Penicillium and candida, species have been used [20].

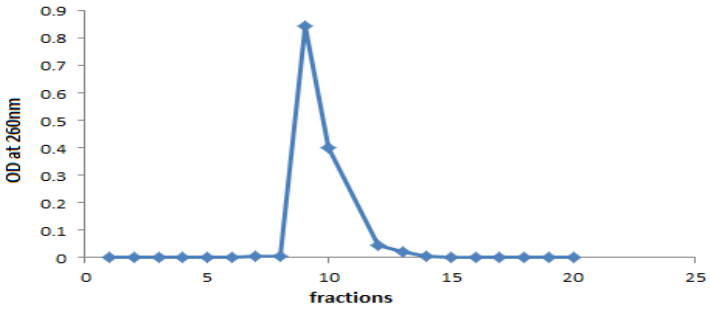
RESULT

Isolation and Purification

The crude celomic fluid was purified by successive chromatographic matrix. The total protein content of celomic fluid was precipitated by 60% ammonium sulphate and recovered more than 90% of total protein. Further precipitate was dialyzed against 20mM phosphate buffer pH 7.5 with dialysis membrane molecular weight cut off (MWCO) 12KD procured from HiMedia. In Gel filtration chromatography, we have collected 30 fractions and fraction number 12,13,14,15 shown maximum antimicrobial activities, which were further, pooled and loaded in run in ion exchange chromatography using DEAE Cellulose column. In 20 eluted fractions from Ion exchange chromatography 9 and 10 fraction were selected for antimicrobial activity as shown enormous activity for antibacterial and antifungal property and SDSPAGE study.



Graph 1: Sephadex G 50 eluted fractions and their absorbance at 280nm for dialyzed celomic fluid



Graph 2: DEAE Cellulose eluted fractions and their absorbance at 280nm for Sephadex G 50 fractions have shown maximum antimicrobial activity

Proteolytic Activity

The crude as well as purified fractions have shown proteolytic activity on casein agar plate as zone of clearance after incubation for 14 hour at 20^{0}C-50^{0}C. The activity was accessed in different temperature and various pH. The proteolytic activity was constrained for purified fraction, which was found maximum at 40^{0}C at pH 8.0 while least in 20^{0}C at pH 4.0.

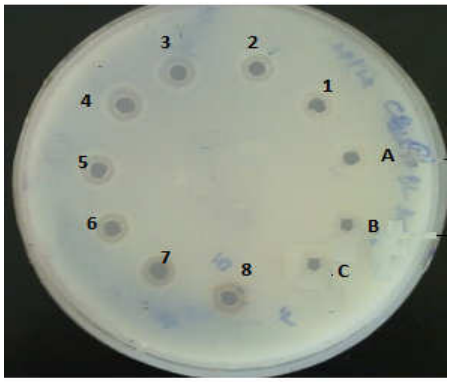


Fig. 1: Caseinolytic activity of celomic fluid while purification Dialyzed (1 & 2), Sephadex G 50 fraction (3, 4, 5 & 6) and

DEAE Cellulose fractions (7 & 8), while A, B and C are Phosphate Buffer, distilled water and native Protease

Molecular weight determination

The average molecular weight for purified fraction was determined by SDS-PAGE. The 15% acrylamide gel was prepared and purified fraction was resolved with standard protein marker. After staining with Coomassie Brilliant Blue R-250 in both two fractions after DEAE Cellulose column separation a thick band was observed corresponding to 20KD of standard protein marker.

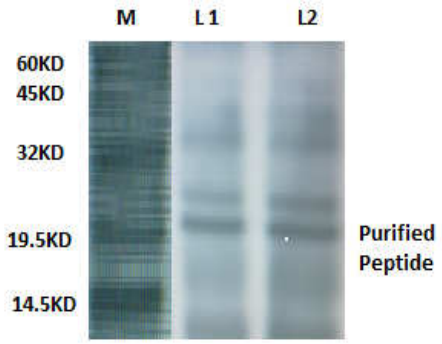


Fig. 2: SDSPAGE analysis of purified fraction on 15% acrylamide with standard protein ladder

Antimicrobial Activity

The antimicrobial activity of purified fractions was evaluated in the context with zone of inhibition on nutrient agar plate after overnight incubation. The following data is collected while accessing activity with different microbial population, which is shown in table-

Table 1: Assessment of antimicrobial activity of purified peptide by well diffusion method

Bacterial strains	Zone of Inhibition (mm)
Escherichia coli	19
Pactobacterium. carotovorum	14
Pseudomonas putida	16
Streptococcus. aureus	17
Bacillus streothermophilous	14
Azotobactor	16
Penicillium sp.	14
Candida sp.	13

Fig. 3: Zone of clearance after incubation of purified fraction on nutrient agar plate plated with *Escherichia coli* and *Pseudomonas putida*

CONCLUSION

In the current work, we have collected Indian earthworms and processed mature worms for collection of intestinal fluid. The intestinal fluid was further processed and purified by different chromatographic techniques including size based and charged based separation [21,22]. The purified fractions were analyzed for apparent molecular weight of purified protein was found 20KD. Further purified fractions were accessed proteolytic activity and found serine protease by casein agar plate as zone of clearance after overnight incubation with casein as substrate. Further purified fractions were evaluated for antimicrobial activity (antibacterial and antifungal activity) by well diffusion method and zone of inhibition was measured [31,24&25].

Many of facts about the molecules possessing antimicrobial property of earthworm's intestinal and tissue fluids need more attention in order to explore at molecular level[26,27] The biophysical characterization of such potent molecules led to understand precise molecular mechanisms and mode of action that further can be tuned to cure for various microbes borne diseases. Further, molecular characterization can lead to identification of DNA coding sequence of these molecules in earthworm genome[28]. That can provide a platform for production of antimicrobial peptides by r-DNA technology, which is economical and does not violate bioethics. Currently our team is working for finding exact molecular weight and N-terminal protein sequence[29]. That will be useful not only to understand molecular properties of antimicrobial molecules but also to predict evolution and biological inter-relation of biomolecules after generating phylogenetic tree in various species of earthworm .We are also looking for crystallography of purified peptide in order to generate pdb, essentially useful for insilico analysis and system biology for new generation antimicrobial compounds.

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REFERENCES

1. Cooper, E.L. 2009. A Closer Look at Clinical Analyses. *Evid based Complement Alternate Med. eCAM*. 6(3): 279–81.
2. Cooper, E.L., Hrzenjak, T.M., and Grdisa, T. 2004. Alternative sources of fibrinolytic, anticoagulative, antimicrobial and anticancer molecules. *Int. J. Immunopathol. Pharmacol*. 17: 237–244.
3. Costa-Neto, E.M. 2005. Animal-based medicines: Biological prospection and the sustainable use of zoo therapeutic resources. *An. Acad. Bras. Cienc.* 77 (1): 33–43.
4. Edwards, C.A., and Bohlen, P.J. 1996. *Biology and Ecology of Earthworms.* 3rd ed. Chapman and Hall, London.
5. Balamurugan, M. 2009. Studies on selective therapeutic properties of earthworm *Lampito mauritii* (kinberg). PhD diss., Annamalai University, Tamilnadu, India.
6. Balamurugan, M., Parthasarathi, K., Cooper E.L., and Ranganathan, L.S. 2007. Earthworm paste (*Lampito mauritii*, Kinberg) alters inflammatory, oxidative, haematological and

serum biochemical indices of inflamed rat. *Eur. Rev. Med. Pharmacol. Sci.* 11 (1): 77–90.

7. Balamurugan, M., Parthasarathi, K., Cooper, E.L., and Ranganathan, L.S. 2009. Antiinflammatory and antipyretic activities of earthworm extract—*Lampito mauritii* (Kinberg). *J. Ethnopharmacol.* 121: 330–332.
8. Balamurugan, M., Parthasarathi, K., Ranganathan, L.S., and Cooper, E.L. 2008. Hypothetical mode of action of earthworm extract with hepatoprotective and antioxidant properties. *J. Zhejiang Univ. Sci.* B 9 (2): 141–147.
9. Balamurugan, M., Parthasarathi, K., Ranganathan, L.S., and Cooper, E.L. 2008. Hypothetical mode of action of earthworm extract with hepatoprotective and antioxidant properties. *J. Zhejiang Univ. Sci.* B 9 (2): 141–147.
10. Tamamura H, Ikoma R, Niwa M, Funakoshi S, Murakami T, Fujii N. Antimicrobial activity and conformation of tachyplesin I and its analogs. Chem. Pharm. Bull. (Tokyo) 41: 978-980, 1993.
11. Arduino, R. C., Jacques-Palaz, K., Murray, B. E. & Rakita, R. M. (1994) Infect. Immun. **62,** 5587–5594.
12. Boggs, A. F. & Miller, G. H. (2004) Clin. Microbiol. Infect. **10,** Suppl. 4, 32–36.
13. Projan, S. J. & Shlaes, D. M. (2004) Clin. Microbiol. Infect. **10,** Suppl. 4, 18–22.
14. Tasiemski A, Schikorski D, Le Marrec-Croq F, Pontoire-Van Camp C, Boidin-Wichlacz C, Sautiere PE. Hedistin: a novel antimicrobial peptide containing bromotryptophan constitutively expressed in the NK cells-like of the marine annelid, Nereis diversicolor. Dev. Comp. Immunol. 31: 749-762, 2007.
15. Huycke, M. M., Spiegel, C. A. & Gilmore, M. S. (1991) Antimicrob. Agents Chemother. **35,** 1626–1634.
16. Lewis, K. & Lomovskaya, O. (2002) in Bacterial Resistance to Antimicrobials: Mechanisms, Genetics, Medical Practice and Public Health, eds. Lewis, K., Salyers, A., Taber, H. & Wax, R. (Dekker, New York), pp. 61–90.
17. Mihara, H. Sumi, H. and Akazawa, K. 1983. Fibrinolytic enzyme extracted from theearthworm. *Thromb. and Haemost.*, 50: 258.
18. Zhang X, Sun Z, Zhuo R, Hou Q, Lin G. Purification and characterization of two antibacterial peptides from *Eisenia fetida*. Prog. Biochem. Biophys. 29: 955-960, 2002.
19. N. Nakajima, M. Sugimoto, and K. Ishihara, "Stable earthworm serine proteases: application of the protease function and usefulness of the earthworm autolysate," *Journal of Bioscience and Bioengineering*, vol. 90, no. 2, pp. 174–179, 2000.
20. T. Hrˇzenjak, M. Hrˇzenjak, V. Kasuba, P. Efenberger-Marinculi´c, and S. Levanat, "A new source of biologically active compounds-earthworm tissue (*Eisenia foetida, Lumbricu rubelus*)," *Comparative Biochemistry and Physiology Part A*, vol. 102, no. 3, pp. 441–447, 1992.
21. Sahm, D. F., Kissinger, J., Gilmore, M. S., Murray, P. R., Mulder, R., Solliday, J. & Clarke, B. (1989) Antimicrob. Agents Chemother. **33,** 1588–1591.
22. Ciornei, C. D., Sigurdardottir, T., Schmidtchen, A. & Bodelsson, M. (2005) Antimicrob. Agents Chemother. **49,** 2845–2850.
23. Tamamura H, Ikoma R, Niwa M, Funakoshi S, Murakami T, Fujii N. Antimicrobial activity and conformation of tachyplesin I and its analogs. Chem. Pharm. Bull. (Tokyo) 41: 978-980, 1993.
24. Cho JH, Park CB, Yoon YG, Kim SC. Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization. Biochim. Biophys. Acta 1408: 67-76, 1998.
25. Tasiemski A, Schikorski D, Le Marrec-Croq F, Pontoire-Van Camp C, Boidin-Wichlacz C, Sautiere PE. Hedistin: a novel antimicrobial peptide containing bromotryptophan constitutively expressed in the NK cells-like of the marine annelid, Nereis diversicolor. Dev. Comp. Immunol. 31: 749-762, 2007.
26. Tasiemski A, Vandenbulcke F, Mitta G, Lemoine J, Lefebvre C, Sautiere PE, et al. Molecular characterization of two novel antibacterial peptides inducible upon bacterial challenge in an annelid, the leech Theromyzon tessulatum. J. Biol. Chem. 279: 30973-30982, 2004.
27. Wang X, Wang X, Zhang Y, Qu X, Yang S. An antimicrobial peptide of the earthworm Pheretima tschiliensis: cDNA cloning, expression and immunolocalization. Biotechnol. Lett. 25: 1317-1323, 2003.
28. Zhang X, Sun Z, Zhuo R, Hou Q, Lin G. Purification and characterization of two antibacterial peptides from *Eisenia fetida*. Prog. Biochem. Biophys. 29: 955-960, 2002.
29. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 415: 389-395, 2002.