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Cloning of fibrinolytic protease-0 (*Efp-0*) gene from diverse earthworm individuals

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Fibrinolytic enzyme has potential role as therapeutic agents for the treatment of some blood clotting diseases. It is a novel oral-administered fibrinolytic agent used for the prevention and treatment of cardiac and cerebrovascular diseases. As a drug, it has been widely studied and used for clinical treatment. In the present study, we have cloned and sequenced partial *Eisenia fetida* fibrinolytic protease (*Efp-0*) gene from three earthworm strains collected from diverse habitats. The gene was amplified by using polymerase chain reaction and cloned in pGEM-T easy vector. The size of *Efp-0* gene, cloned from the two strains of *E. fetida* and one strain of *Eudrilus eugeniae,* was 658 bp, 658 bp and 660 bp, respectively after sequencing,. Multiple sequence alignment of the cloned gene along with gene sequences available in public database showed 21 SNPs and 5 InDels in the coding region. However, diversity observed in *Efp-0* gene cloned from different earthworm strains will provide basis for allele mining of this gene. It is concluded that *Efp-0* is a novel gene and can be utilized for industrial applications. The gene sequences obtained in this study have been submitted in the GenBank (Accession no. GQ385193, GQ385194, GQ385195).

Keywords: Earthworm, *Eisenia fetida, Eudrilus eugeniae,* fibrinolytic protease, DNA sequencing, structural characterization, therapeutic agents

Introduction

Earthworm fibrinolytic enzymes also referred to as lumbrokinases are alkaline serine proteases having stronger degradable fibrinolytic activity with potential role as therapeutic agents for the treatment of some clotting diseases, prevention and treatment of cardiac and cerebrovascular diseases, and pulmonary embolism. The earthworm fibrinolytic enzyme (EFE) is a complex protein enzyme that is widely distributed in the earthworm's digestive cavity. Several investigators purified and further characterized the fibrinolytic enzymes in *Lumbericus rubellus* and found the hydrolysis of the plasmogen-rich fibrin and plasmogen-free fibrin[1-3]. The fibrinolytic enzymes dissolve blood fibrin clots, which are important for clinical application as chemotherapeutic agents[4-6]. The diagnostic values of the different genes have also been evaluated[7]. Culturing of earthworms is limited by the seasons and duration of growth period, and it is also difficult to assure the consistence of the EFE extracted from earthworms in terms of purity, specific activity, and therapeutic efficacy[8]. They obtained complete sequences for genes coded for 18S rDNA of three earthworm species and compared them based on sequence homology approach. Cloning of the genes that encoded the components in earthworm fibrinolytic protease will help to get more information about the molecular mechanisms involved to degrade fibrin. The objective of this study was to find variation in sequences of the earthworm fibrinolytic protease-0 (*Efp-0*) gene cloned from different species.

Materials and Methods

Isolates of earthworms including two of *Eisenia fetida* from Himachal Pradesh and one of *Eudrilus eugeniae* from Indian Agricultural Research Institute, New Delhi were selected on the basis of diversity analysis[9]. Samples were collected by digging and manual soil sorting and by chemical sorting using formalin. The earthworms were anaesthetized by soaking in 70% ethanol and then washed with tap water to remove the soil. All the samples were stored in 70% ethanol until DNA extraction.

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Genomic DNA Isolation and PCR Amplification

Extraction of genomic DNA was carried out the using a Geneaid Genomic DNA Mini Kit (Geneaid Biotech Ltd, Taiwan) following the manufacturer's protocol with minor modifications. DNA was quantified on 0.8% agarose gel using known concentrations of λ phage uncut DNA as standard and also by using spectrophotometer.

The nucleotide sequence (DQ836917) of *E. fetida* fibrinolytic protease-0 (*Efp-0*) gene was used to design gene specific forward primer (5′TGCGAGTGACT GGATCTCAC3') and reverse primer (5'ACTGCA CTCCAGAGGACCAC 3'). Gradient PCR was used for the amplification of *Efp-0* gene. Thermocycling conditions for the gene specific primer were: DNA denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 67°C for 1 min, primer extension at 72°C for 2 min. This was followed by a final extension step at 72°C for 10 min. The samples were stored at 4°C for further use. PCR products were subjected to agarose gel electrophoresis on 1% agarose gel in 1X TBE buffer at 60V for 2 h and photographs were taken by using gel documentation system (Alpha Innotech Corp., USA).

Cloning of PCR Products

The PCR products were gel purified to remove primers/nucleotides/polymerase and salts by using Gel/PCR DNA fragments extraction kit (Geneaid Biotech Ltd, Taiwan) following the manufacturer's protocol. Purified PCR products were cloned in pGEM-T easy vector (Promega Corporation, USA) in *E. coli* strain DH5α cells prepared by Z-Competent ™ *E. coli* Kit (Zymo Research Corporation, CA, USA). The transformed cells were selected by blue-white screening and plasmid DNA extraction was performed by High Speed Plasmid Mini kit (Geneaid Biotech Ltd., Taiwan). Plasmid DNA was isolated from several transformed colonies and analyzed for the presence of insert by *Not*1 restriction digestion. Finally, six recombinant clones were selected for DNA sequencing.

DNA Sequencing of Cloned Fragment

PCR amplicons cloned in pGEM-T easy vector were used for sequencing by Sanger's dideoxy method on automated DNA sequencer (MegaBACE 4000). For each sequencing reaction 100 ng (2 µL) template DNA was mixed with 4 µL DYEnamic ET mix (Amersham Biosciences), 3 µL sterile MQ water and 1 µL of universal sequencing primers. PCR was performed for 34 cycles with denaturation of template DNA at 94°C for 10 sec, primer annealing at 50°C for 10 sec and extension at 60°C for 2 min. After cleaning, the PCR products were sequenced using Sanger's dideoxy method on automated sequencer. Each fragment was sequenced atleast 4 times to generate high quality consensus sequences.

Sequence Annotation

The nucleotide sequences were assembled using Phred/Phrap/Consed suite of programs. Forward and reverse primer binding sequences were detected by pair wise alignment at NCBI (www.ncbi.nlm.nih.gov/) to ensure the completeness of the sequence. The nucleotide sequence data have been deposited in the GenBank Nucleotide Sequence Database under the accession numbers GQ385193, GQ385194 and GQ385195. The sequences of already cloned *Efp-0* gene were downloaded from the NCBI database and used for comparative analysis using ClustalW 2.0.9 (http://www.ebi.ac.uk/Tools/clustalw).

Results and Discussion

Cloning of *E. fetida Efp-o* Gene

PCR amplification of *Efp-0* genes was obtained from the genomic DNA of *E. fetida* and *E. eugeniae* using flanking primers yielded single DNA band from all the isolates (Fig. 1). The PCR products were cloned in pGEM-T easy vector and transformed *E. coli* cells were selected based on blue white screening. Restriction digestion of the plasmid extracted from the recombinant white colonies with *Not*1 restriction enzyme released the expected 500-700 bp insert size thus confirmed the cloning process (Fig. 2).

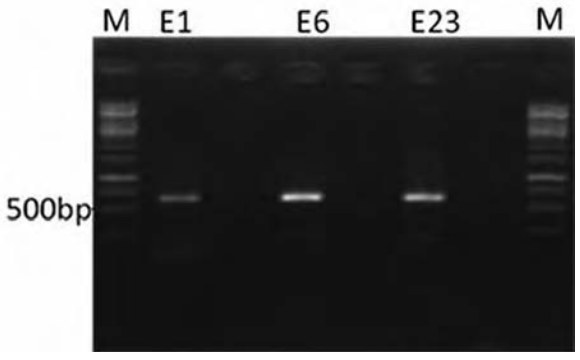

Fig. 1—PCR amplification of genomic DNA from three isolates of earthworm, E1 (*E. fetida)*, E6 (*E. fetida)* and E23 (*E. eugeniae*) with gene specific primers (*Efp-0* F and *Efp-0* R). M = 1 Kb DNA ladder. Lanes 1 to 3 are the PCR products separated on 0.8% agarose gel.

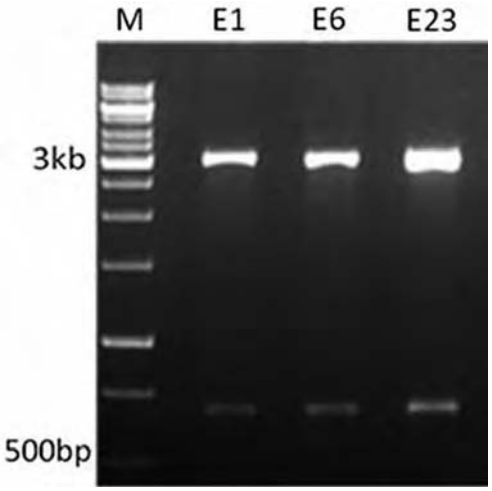

Fig. 2—Confirmation of PCR product cloned in pGEM –T Easy vector after restriction digestion with *Not*1. M = 1 Kb DNA ladder. Lane 1, Lane 2 and Lane 3 are the isolates of earthworm, E1 (*E. fetida)*, E6 (*E. fetida)* and E23 (*E. eugeniae*), respectively.

Cloned PCR fragments were sequenced from forward and reverse directions for at least four times and high quality consensus sequences were obtained by assembling these sequence reads. The actual nucleotide length of *Efp-0* gene of two strains of *E. fetida* and one strain of *E. eugeniae* obtained in the present study was 658, 658 and 660 bp, respectively. The partial nucleotide sequences of 3 clones were obtained and homology search was done using BLASTn program (http://www.ncbi.nlm.nih.gov). These sequences showed high homology with the *Efp-o* gene sequences of *E. fetida* (DQ836917), *Lumbricus rubellus lumbrokinase* (U36338), *Tetradon nigroviridis* (CR651223), *E. fetida* (AB 284053) and *L. rubellus* (AJ299434) in the genome database. In GenBank, 21 nucleic acid sequences of EFE have been published. Among them, most of the N-terminal amino sequences belong to the F-III type, and few to the F-II type. Few of the sequences were similar to the F-I type, but only one sequence was similar to F-I-0[10]. The gene sequences from three earthworm strains studied in the present investigation belonged to F-I-0. It has been reported that *Efp*-0 has 678 bp size and encodes a protein of 225 amino acids. Cloning of earthworm fibrinolytic protease in this study is a serine protease belonging to the trypsin family. It has amino acid composition similar to F-I-0[9]. Multiple sequence alignment for three nucleotide sequences from this study and known sequences in the database was performed by using ClustalW software Multiple sequence alignment of all the three individuals showed 21 SNPs and 5 indels. A phylogenetic tree of

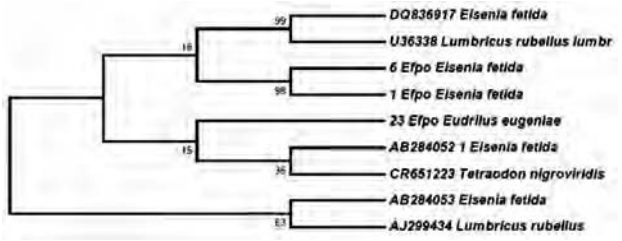

Fig. 3—Phylogenetic tree of *Efp-0* nucleotide sequences of three earthworm individuals and known nucleotide sequences obtained from multiple sequence alignments with their accession numbers. Values on the nodes are bootstrap values of 500 replicates. The accession numbers are given on the termini of branches.

these sequences along with the sequences with maximum similarity was constructed with 500 bootstrap replicates. Three clusters were formed in this phylogenetic tree (Fig. 3). Cluster-I includes individuals E1 and E6, both belonging to *E. fetida* species, showing 98% bootstrap value. Similarly, cluster II shared by *Efp-0* gene cloned from earthworm *E. eugeniae* (E23), *E. fetida* and *T. nigroviridis*. Cluster III consisted of *Efp-0* gene cloned from *E. fetida* and *L. rubellus* with 63% bootstrap value. This analysis of *Efp-0* gene showed that individuals belonging to *E. eugeniae* can be distinguished from other species based on the sequences of single gene.

Earthworm fibrinolytic enzymes are a group of serine proteases, which have strong fibrinolytic and thrombolytic activities. The proteases have the abilities not only to hydrolyze fibrin and other proteins, but also activate proenzymes such as plasminogen and prothrombin[11]. Compared to the present thrombolytic drugs, earthworm fibrinolytic enzyme is cheap, can be easily stored, and can be administered orally[3]. As a new drug for thrombosis, it has been widely studied and used for clinical treatment. In an earlier study, the diagnostic values of the different genes have been evaluated[9]. Evaluation of sequence difference in *Efp-0* gene cloned from diverse earthworm species will provide an opportunity to mine genes responsible for the production of lumbrokinase protein with more efficient activity. Lumbrokinase also referred to as earthworm powder fibrinolytic enzyme (EPE), which is a new product and shows promise in dissolving blood clots without the risk of hemorrhage. Clinical studies have shown complete recovery of stroke patients by using lumbrokinase (www.digitalnaturopath.com). The commercially available lumbrokinase capsules are taken orally,

which indicates that the lumbrokinase can be expressed into physiological secretions such as milk, egg-yolk or egg-albumin. It can be concluded that *Efp-0* gene cloned and characterized from three diverse earthworms in this study may facilitate future research on gene expression analysis for its commercial production.

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