Molecular Cloning and Characterization of cDNA Encoding Fibrinolytic Enzyme-3 from Earthworm *Eisenia foetida*

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Abstract

The earthworm fibrinolytic enzyme-3 (EFE-3, GenBank accession No: AY438622), from the earthworm *Eisenia foetida*, is a component of earthworm fibrinolytic enzymes. In this study, cDNA encoding the EFE-3 was cloned by RT-PCR. The cDNA contained an open reading frame of 741 nucleotides, which encoded a deduced protein of 247 amino acid residues, including signal sequences. EFE-3 showed a high degree of homology to earthworm (*Lumbricus reballus*) proteases F-III-1, F-III-2, and bovine trypsin. The recombinant EFE-3 was expressed in *E. coli* as inclusion bodies, and the gene encoding the native form of EFE-3 was expressed in COS-7 cells in the medium. Both the refolding product of inclusion bodies and the secreted protease could dissolve the artificial fibrin plate.

Key words: earthworm; *Eisenia foetida*; earthworm fibrinolytic enzyme; cloning; expression; refolding

Earthworm fibrinolytic enzyme (EFE), a multi-component protease purified from some earthworm breeds, belongs to serine protease family with fibrinolytic activity [1]. It has been used in prevention and treatment of cardiac and cerebrovascular diseases in China for many years [2]. Previous studies focused mainly on the purification, physical/chemical properties, and clinical application of earthworm fibrinolytic enzyme isolated from *Lumbricus rubellus*, *Eisenia foetida* or *Lumbricus bimastus* [3–7]. Most EFEs showed characteristics of high stability and strong tolerance to organic solvents and high temperature. Chemical modification of these enzymes has been performed for parenteral administration [6,8]. Comprehensive clinical trials have also been done. The intestinal absorption mechanism of EFE has been clarified by Fan et al. [2]. The study of expression of EFE has also been performed and the fibrinolytic activity of the expression protein was demonstrated [9].

In this paper, we presented the molecular cloning and sequencing of EFE3, one of EFEs from *Eisenia foetida*, and its prokaryotic expression in *E. coli* BL21 (DE3) and eukaryotic expressions in COS-7 cells. This work may lay a foundation for bioengineering of EFEs.

Materials and Methods

Materials

Earthworm, *Eisenia foetida*, was raised as described by Nakajima et al. [5].

RNA isolation and cDNA synthesis

Total RNA from the mature earthworm tissue was prepared with TRIZol reagent (Gibco-BRL). Aliquots (2 μg) of total RNA were reverse transcribed to cDNA by using oligo (dT)16 primers and MMLV (moloney murine leukemia virus) reverse transcriptase (Promega, USA). The generated cDNA was used as the template for PCR.

Polymerase chain reaction (PCR) amplification

PCR was performed with 2 μl cDNA template, 0.5 μl Taq DNA polymerase, and 20 pM of each primer in a 50 μl reaction mixture for 30 cycles in a minicycler. Each cycle was held at 97 °C for 30 s, 48 °C for 60 s, and 72 °C for 60 s. The oligo-nucleotide primers were designed based on the sequences of U25643 and U25648 (GenBank accession number); sense primer: 5’-CGGAATTCAATTGATTGTCGGAATTGAAG-3’; antisense primer: 5’-CC-ATGCTTCTAGTTGGTGGAATTGAAG-3’ (synthe-
Gene cloning

PCR products were subcloned into a pUCm-T vector (Sangon). The recombinants were determined by restriction enzyme analysis and sequencing (Sangon) [10].

Sequence analysis

The ORF prediction and translation of nucleotide sequence into amino acid sequence were performed with Finder program (NCBI) and Primer 5 program respectively. Homology search and alignment of DNA with protein sequences were done by using the BLAST program [11,12].

Construction of the prokaryotic expression plasmid
cDNA encoding the mature form of EFE-3 was amplified by using pUC-EFE-3 as the template by PCR. The sense primer, 5'-CGGAATTCCTAGTTGGTGGTAA-TATGTCT-3' contained an EcoRI site at 5' end; and the antisense primer, 5'-CCAAGCTTAGTTATCTCGCcCTGCTGC-3' (Sangon) contained a HindIII site and a stop codon. The PCR product and pET22b(+) vector (Novagen) were digested by restriction enzyme analysis and sequencing. Sense primer: 5'-CGAAGCTTACTTCTCGCCTGCG-3' (Sangon) contained a HindIII site and a stop codon. Both the PCR product and pcDNA3.1 (+) vector (Invitrogen) were digested by NotI and EcoRI. After purification and ligation, it subsequently transformed into the JM109 cells for the purpose of selecting the correct insertion. The recombinant of pcDNA-EFE-3 was confirmed by restriction enzyme analysis and sequencing.

Expression of pcDNA-EFE-3 in COS-7 cells

2×10⁶ exponentially growing COS-7 cells were inoculated in a 35-mm Nunc plates and cultured for 24 h. Transfection mixture, containing 100 µl sterile, serum-free and antibiotic-free DMEM, 12 µl GeneJammer transfection reagent and 2 µg pcDNA-EFE-3, was prepared. The old medium was removed and 900 µl fresh serum-containing medium was added. The transfection mixture was added dropwisely to the Nunc plate and distributed evenly by gently rocking. Then incubated cells at 37 °C with 5% CO₂ in a humidified incubator for 3 h. Subsequently, added 1 ml RPMI-1640 (Hyclone) medium containing 10% fetal bovine serum to incubate for a further 72 h for transient express EFE-3. Collected the culture medium at 24 h, 36 h, and 48 h after transfection, respectively [15,16].

Protein and fibrinolytic activity assays

The protein was analyzed by SDS-PAGE, and the fibrinolytic activity was measured with standard fibrin plates by the method described previously [17]. 20 µl of protein solution was spotted onto the fibrin plates and incubated at 37 °C for 3 h. The protein concentration was measured by using Bradford method [18] with crystalline bovine serum albumin as standards.

Results

Cloning of the cDNA encoding EFE-3
After isolation from earthworm *Eisenia foetida*, the quality of total RNA checked by agarose gel electrophoresis containing formaldehyde and quantitated the ratio of $A_{260}/A_{280}$, was found to be good. The target gene was amplified from the cDNA by PCR. After subcloning the fragments and analyzing the nucleotide sequence of the clones, one cDNA fragment was obtained. The nucleotide sequence and the deduced amino acid sequence of the target gene were presented in Fig. 1.

**Bioinformatics analysis**

The cDNA of *Eisenia foetida* EFE-3 we got contained 859 nucleotides, with an open reading frame starting from position 112 to 853, encoding a polypeptide of 247 amino acid residues.

DNA sequence similarity searching against GenBank by using BLAST program revealed that the EFE-3 cDNA has significant identity to earthworm fibrinolytic enzyme F-III-1 (AB045720, 93%; AF433650, 98%; AF304199, 98%; and U25643, 99%) [9]. The identity between the deduced amino acid sequence of EFE-3 with F-III-1 is 89% (shown in Fig. 2). Same to F-III-1, EFE-3 also has the catalytic triad, His$^{43}$, Asp$^{91}$, and Ser$^{188}$; and its primary substrate specificity determinant site is Asp$^{182}$; while those different residues are located out of important function sites (shadow indicated the difference in Fig. 2). According to Sugimoto et al. [9] and our analysis, the EFE-3 cDNA encodes a preproenzyme that included an N-terminal signal peptide, MELPPGTK, with a cluster of hydrophobic residues, followed by an active form. The native
mature EFE-3 comprised of 239 amino acid residues.

Homology searching of the deduced amino acid sequence of EFE-3 against the protein entries available at the GenBank; and SWISS-PROT also revealed that the EFE-3 had considerable similarities with mammalian serine proteases such as bovine trypsin, bovine chymotrypsin, etc. (data not shown), indicating the active sites of the trypsins are conserved in the earthworm fibrinolytic proteases. Our results are consistent with that of Sugimoto [9].

Prokaryotic expression of EFE-3

The native form of EFE-3 amplified by PCR was fused with the pel signal peptide sequence, which is beneficial to the expression of EFE-3 cDNA. The fusion EFE-3 protein was expressed in BL21 (DE3). Fig. 3 showed that the expression of EFE-3 protein was time-dependent after induction by IPTG. A maximal yield of fusion EFE-3 was attained at 5 h after IPTG induction. A relatively pure protein was readily obtained by simple washing with 2% Triton X-100. The mobility of EFE-3 fusion protein was determined by SDS-polyacrylamide gel electrophoresis. The result showed the molecular weight of EFE-3 fusion protein was about 30 kD.

Eukaryotic expression of EFE-3

The complete form of EFE-3 cDNA containing the signal peptide sequence was cloned into the eukaryotic expression vector pcDNA3.1(+), then, the recombinant vector was transfected into COS7 cells. SDS-polyacrylamide gel electrophoresis of the harvested medium showed the molecular weight of EFE-3 protein was less than 30 kD (Fig. 4).

Activity assay of EFE-3

The lytic activities of the refolded fusion EFE-3 protein and the culture medium of COS-7 cells were assayed by fibrin plates, which were incubated at 37 °C for 3 h. The lytic area generated by the refolded fusion EFE-3 protein [Fig. 5(A)] were 148.41 mm²/200 µg protein, 82.47 mm²/20 µg protein, and 35.77 mm²/10 µg protein, respectively and the lytic area generated by the EFE-3 protease in the medium of transfected COS-7 cells was 190.30 mm²/10 µg protein [Fig. 5(B)]. According to the standard curve of urokinase, the lytic activity on the fibrin plate of the refolded fusion EFE-3 protein was about 2000 urokinase unit (UU)/mg protein, and the lytic activity of the medium from transfected medium were about 2600 UU/mg protein. Both of controls have no fibrinolytic activities.

Discussion

Although we designed primers based on the sequence of Lumbricus rubellus’s F-III-1, there were difference between the sequence of PCR product and F-III-1, indicating genetic diversity existed between different earthworm breeds (Eisenia fetida and Lumbricus rubellus). EFE-3, fused with a pel signal sequence at N-terminal,
was expressed in prokaryotic system. The presence of the pel didn’t direct the EFE-3 to the periplasm. The fusion protein was expressed as inclusion bodies. Fusion protein EFE-3 gained activity after refolding, suggesting pel didn’t affect the refolding and function of the EFE-3.

Lytic activity of COS-7 medium increased from 24 h to 48 h, but disappeared at 72 h. The increasing concentration of protease in the transient expression system before 48 h contributed to the detected increasing lytic activity. After 48 h, COS-7 cells started dying, which resulted in the disappearance of lytic activity at 72 h after transfection.

Earthworm fibrinolytic enzymes were considered as one of the important dissolution tools against both cardiac and cerebrovascular clotting disease [2]. To date, there were more than ten fibrinolytic enzymes genes purified from earthworm, but it was only one report about expressing recombinant EFEs [9].

The most remarkable feature of fibrinolytic enzymes was its way of absorption, the EFE-3 could be transported into blood through intestinal epithelium and exerted its biological function in circulation [2]. Our study also evidenced this viewpoint (data not shown). Other fibrinolytic enzymes such as urokinase and tissue plasminogen activator could only be used by intraperitoneal injection rather than oral administration. The differences provide interesting clues for clarifying the relationship between the protein action mode and its structure.

Success in expressing active EFE-3 protein in prokaryotic cells suggested the EFE could be produced by bioengineering method. The eukaryotic expression of EFE-3 provided a forceful evidence for the application of EFE bioengineering products. However, it is still necessary to characterize the differences between the prokaryotic product and the eukaryotic one.

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References


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