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An enzyme from the earthworm *Eisenia fetida* is not only a protease but also a deoxyribonuclease

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ABSTRACT

The earthworm enzyme *Eisenia fetida* Protease-III-1 (*Ef*P-III-1) is known as a trypsin-like protease which is localized in the alimentary canal of the earthworm. Here, we show that *Ef*P-III-1 also acts as a novel deoxyribonuclease. Unlike most DNases, this earthworm enzyme recognizes 5′-phosphate dsDNA (5′P DNA) and degrades it without sequence specificity, but does not recognize 5′OH DNA. As is the case for most DNases, Mg^{2+} was observed to markedly enhance the DNase activity of *Ef*P-III-1. Whether the earthworm enzyme functioned as a DNase or as a protease depended on the pH values of the enzyme solution. The protein acted as a protease under alkaline conditions whereas it exhibited DNase activity under acid conditions. At pH 7.0, the enzyme could work as either a DNase or a protease. Given the complex living environment of the earthworm, this dual function of *Ef*P-III-1 may play an important role in the alimentary digestion of the earthworm.

1. Introduction

The living environment of the earthworm is complex. Earthworm food includes leaves, seeds and grass clippings, and contains protein and nucleic acid in addition to other nutrients. Enzymes for digesting proteins and nucleic acids are thus necessary during earthworm digestion.

Research on earthworm proteases began at the end of the 19th century with Frédéricq's discovery that an enzyme secreted from the alimentary tract of earthworm had proteolytic activity [1]. Several proteases which can digest casein, gelatin and albumin were later isolated from the earthworm in 1920 [2]. Large scale research on earthworm proteases began in 1980. Mihara et al. [3] isolated a group of proteases from the earthworm *Lumbricus rubellus* and studied their fibrinolytic activities. Subsequently, different purification methods, including gel filtration, affinity chromatography, ion exchange chromatography and high-pressure liquid chromatography (HPLC) have been used to isolate the enzymes. Proteases, such as earthworm fibrinolytic enzyme [4], earthworm-tissue plasminogen activator [5], and earthworm plasminogen activator [6–10], have been isolated from different species, and have been used as drugs to treat clotting diseases.

Eight glyco-proteases (*Ef*P-0-1, *Ef*P-0-2, *Ef*P-I-1, *Ef*P-I-2, *Ef*P-II-1, *Ef*P-II-2, *Ef*P-III-1 and *Ef*P-III-2) have been isolated from the earthworm *Eisenia fetida* using a stepwise-purification procedure [11]. Of these, a non-specific *Ef*P-I with different chromogenic substrate test in subsequent reactions of protease, both the "lock and key" and "induced fit" mechanisms are used depending on the degree of conformational change required [12]. *Ef*P-III-1, with a molecular mass of 30 kDa, is located in the epithelial cells of the alimentary canal around the clitellum [13] and has the highest trypsin activity [14].

The structural and functional characteristics of *Ef*P-III-1 have been widely investigated [13,15–19]. As an alimentary protease, *Ef*P-III-1 specifically recognizes the carboxylic sites of the arginine and lysine of its substrate. It functions in a similar manner to lumbrokinases involved in the plasmin–anti-thrombus pathway [20–23], and shows a relatively broad substrate specificity.

E. fetida proteases are thought to digest proteins in earthworm food. We expect that similarly, DNases should exist in the earthworm alimentary canal to digest DNA. So far, proteases which also function as DNases have not been found in animals. Here we report for the first time that the protease *Ef*P-III-1, an animal protease, acts as a DNase under acidic conditions.

2. Materials and methods

2.1. Purification of *Ef*P-III-1

Ten milligram of crude earthworm proteases, prepared as previously described [13], was obtained by ammonium sulfate

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precipitation of *E. fetida*. *Ef*P-III-1 was purified on a 4-aminobenzamidine dihydrochloride-coupled Sepharose CL-6B affinity column (Pharmacia, Pfizer, Canada) and eluted with a gradient of guanidine hydrochloride denaturant (from 0.1 to 1 M) using a Bio-Rad Gradient Maker [24]. Fractions were assayed with a chromogenic substrate from Roche (Switzerland) as previously described [25]. The active eluate was pooled and dialyzed against 0.05 M NH_4HCO_3 buffer (pH 8.0). The purified *Ef*P-III-1 was then lyophilized and stored at −20 °C before use. The isozyme retained its original activity after resuspension in 10 mM Tris–HCl buffer (pH 8.0) and presented as a single band on 12% SDS–PAGE with an apparent molecular mass of ~34 kDa (Panel A, Supplementary Fig. 1). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay with bovine serum albumin as the standard (Pierce, USA). To prove the purity of *Ef*P-III-1, we ran HPLC (LC20A, Shimadzu Corporation, Japan) with a Nanofilm SEC-250 column (Sepax Technologies, Int., USA). The column was first balanced with 2.5 mg/ml BSA (AMRESCO, USA) overnight (0.1 ml/min), and then washed by 0.05 M Tris–HCl buffer (pH 8.0) for 2 h (0.2 ml/min). 10 μl *Ef*P-III-1 was taken into the column and eluted with 0.05 M Tris–HCl buffer (pH 8.0) for 30 min (0.2 ml/min), followed by measurements of the absorbance at 214 nm. The eluate was pooled under the protein peak (Panel B, Supplementary Fig. 1), and the pooled fraction ran as a single band in 12% SDS–PAGE upon silver staining (Panel C, Supplementary Fig. 1). The band was transferred onto a poly-vinylidene difluoride membrane (Gelman, USA) and then cut out from the membrane for amino acid sequencing.

2.2. Synthesis of polynucleotides

A series of polynucleotides (DNA-1, DNA-2, DNA-3 and DNA-4) were designed and synthesized (Shanghai Sangon Co., China) with random sequences (Supplementary Table 1). The polynucleotides were denatured by heating to 95 °C in a water bath for 5 min, and then complementary strands were annealed by cooling slowly to room temperature. For effective phosphorylation, polynucleotides were first heated at 70 °C for 5 min, and chilled on ice for at least 1 min. T4 polynucleotide kinase (New England Biolabs, UK) was then incubated with the polynucleotides at 37 °C for 30 min. Phosphorylation was terminated at 65 °C for 20 min. The concentration of annealed double-stranded DNA was determined by measuring its absorbance at 260 nm on a Hitachi U-2010 UV Spectrophotometer (Japan).

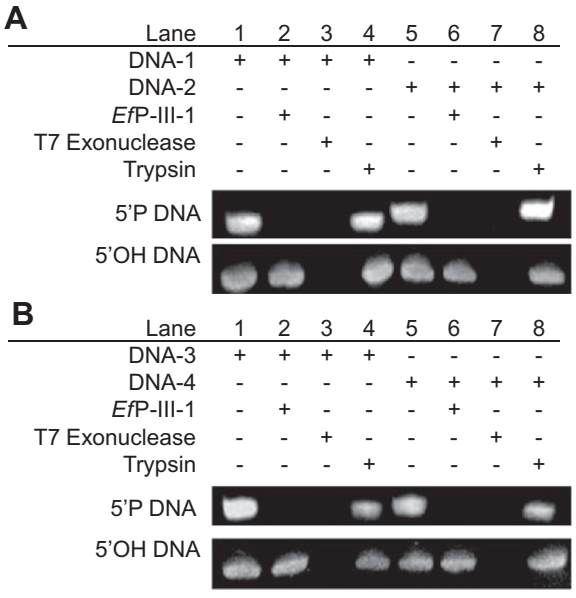

Fig. 1. DNase activities of *Ef*P-III-1. 5 μM of nucleic acids with different sequences (DNA-1, DNA-2, DNA-3, and DNA-4; Supplementary Table 1) were incubated separately with 7 μM of purified *Ef*P-III-1 in reaction buffer at 37 °C for 1 h. The DNA used contained either a phosphate or a hydroxyl group at the 5′ terminus. Trypsin was used as a negative control. T7 Exo, an exonuclease for double-stranded DNA, was included as a positive control. 5′P DNA, 5′-phosphate DNA; 5′OH DNA, 5′-hydroxyl DNA.

2.3. Assay DNase activity of *Ef*P-III-1

7 μM *Ef*P-III-1 was incubated with 5 μM 5′P DNA in reaction buffer (10 mM $MgCl_2$, 10 mM Na_2HPO_4–citrate, pH 6.0) at 37 °C for 1 h. Samples were then resolved by 45% polyacrylamide gel containing 7 M urea and electrophoresed at 100 V in 1× TBE running buffer at 4 °C for 2 h. The gel was then stained with ethidium bromide (0.5 μg/ml) for 10 min and visualized using a Herosbio Gel Imaging 39B (China). The relative activity of the enzyme was analyzed by grayscale scanning (The Discovery Series Quantity One 1-D Analysis Software Version 4.6.9, Mac) of the band. T7 exonuclease was used to react with the DNA as a positive control and trypsin as a negative control.

2.4. Assay of the DNase activity of *Ef*P-III-1 in the presence of magnesium or sodium

5 μM 5′P DNA-4 was incubated with 7 μM *Ef*P-III-1 in 10 mM Na_2HPO_4–citrate, pH 6.0, with increasing concentrations of $MgCl_2$ (1–10 mM) or NaCl (40–320 mM) [26] at 37 °C for 1 h and also analyzed by grayscale scanning.

2.5. Assay DNase activity of *Ef*P-III-1 at different temperatures

The optimal temperature of *Ef*P-III-1 was measured by performing a nuclease activity assay at different temperatures (16, 25, 37, 45, 55, 65 and 75 °C) for 1 h in the presence of 5′P DNA-4 as the substrate.

2.6. Assay DNase activity of *Ef*P-III-1 at different pH values

The effect of pH on DNase activity was evaluated at the optimal temperature in the presence of the 5′P DNA-4 for 1 h at different pH values: 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, using three buffers, Na_2HPO_4–citrate (pH 3.0–7.0), Tris–HCl (pH 8.0–8.9) and Gly–NaOH (pH 9.0–12.0), each at 10 mM [27].

2.7. Assay DNase and protease activity in the presence of an inhibitor

To inhibit DNase activity, 7 μM *Ef*P-III-1 was incubated with its inhibitor 4-aminobenzamiline dihydrochloride (Sigma, USA) at different concentrations (0.01, 0.02 and 0.04 mM) for 30 min, and then the DNase activity was measured in the presence of 5′P DNA-4. The inhibition of protease activity was measured under the same conditions, except that Chromozym TH was used instead of 5′P DNA-4 as the substrate. The absorbance was measured at 405 nm. The activity was calculated using the coefficient $\varepsilon_{405} = 9.75\ mM^{-1} \times cm^{-1}$ [28]. I_{50} is defined as the concentration of an inhibitor that causes 50% inhibition of an enzyme reaction [29].

3. Results

3.1. Purification of *Ef*P-III-1

*Ef*P-III-1 appeared as a single band on 12% SDS–PAGE with an apparent molecular mass of ~34 kDa (Panel A, Supplementary

Fig. 1). The pooled fraction under the peak (Panel B, Supplementary Fig. 1) ran as a single band in SDS–PAGE upon silver staining (Panel C, Supplementary Fig. 1). Sequencing of the N-terminal region (IVGGIEAR) revealed this protein had an identical amino acid sequence to *Ef*P-III-1 [19], confirming the purity of our *Ef*P-III-1 sample. The specific protease activity of the purified enzyme was 51×10^4 U, measured using a light scattering assay.

3.2. *Ef*P-III-1 exhibits DNase activity

To investigate the DNase activity of *Ef*P-III-1, four polydeoxyribonucleic acids with random sequences (DNA-1, DNA-2, DNA-3 and DNA-4) were synthesized (Supplementary Table 1). Four dsDNAs containing 5′-phosphate ends (5′P DNA; phosphorylated by T4 polynucleotide kinase), were incubated with *Ef*P-III-1 for 1 h. As shown in Fig. 1, all of the 5′P DNA bands disappeared after the incubation. However, the four untreated 5′OH DNA bands did not change under the experimental conditions. T7 exonuclease was used as a positive control and digested both 5′P DNA and 5′OH DNA, while trypsin was used as a negative control and did not digest either form of DNA. These results demonstrate that protease *Ef*P-III-1 functions as a DNase and can digest 5′P DNA.

3.3. Effects of magnesium and sodium on DNase activity

As reported by many authors [30–33], divalent metal ions such as magnesium act as important factors in the catalytic reaction of many DNases. Here, we used different concentrations of $MgCl_2$ in the protease-catalyzed DNase reaction to determine the requirement of *Ef*P-III-1 for Mg^{2+}. DNase activity increased markedly as $MgCl_2$ concentration increased and was 6-fold higher in the presence of 5 mM $MgCl_2$ than in the absence of $MgCl_2$ (Panel A, Fig. 2). NaCl showed little enhancement of *Ef*P-III-1 DNase activity under the same conditions, though an increase in the activity was observed at a high concentration of NaCl (Panel B, Fig. 2). This suggests that magnesium plays an important role in the digestion of DNA, similar to that in most DNases [34–36].

3.4. Effects of temperature and pH on the enzymatic activity

The optimal temperature for *Ef*P-III-1 DNase activity was found to be around 45 °C (Panel A, Fig. 3). The DNase activity increased as pH decreased (Panel B, Fig. 3).

3.5. Inhibition of the DNase in the presence of a protease inhibitor

To exclude the possibility that the above results were caused by a contaminating DNase, 4-aminobenzamidine dihydrochloride (a protease inhibitor) was used to inhibit the reaction of *Ef*P-III-1 with 5′P DNA-4. Significant inhibition of the DNase activity of *Ef*P-III-1 was observed in the presence of this inhibitor (Fig. 4). The I_{50} of the DNase was about 16 nM, at which concentration the protease activity was also inhibited.

4. Discussion

In this study, we have shown that the earthworm enzyme *Ef*P-III-1 acts as both a protease and a DNase, and recognizes 5′-phosphate dsDNA without sequence specificity under acidic conditions. Potyviral NIa protease, an enzyme from the pepper vein banding virus, has been reported to function as both a protease and a DNase. However, in this case, the viral enzyme functions as a DNase under alkaline conditions [37]. The authors suggest that the dual functions of this viral protease could play an important role in the life cycle of the virus.

It should be emphasized that the DNase activity of the earthworm protease detected here was not due to contaminating DNases for the following reasons. (i) The purified enzyme exhibited a single peak on HPLC and ran as a single band on SDS–PAGE upon silver staining (Panels B and C, Supplementary Fig. 1). (ii) The specific protease activity of the purified enzyme was 51×10^4 U, indicating the high purity of the protein [13]. (iii) With the HPLC results, identical N-terminal sequencing (IVGGIEAR) showed the high purity of *Ef*P-III-1. (iv) The protease inhibitor used inhibited not only the proteolytic activity but also the DNase activity, and

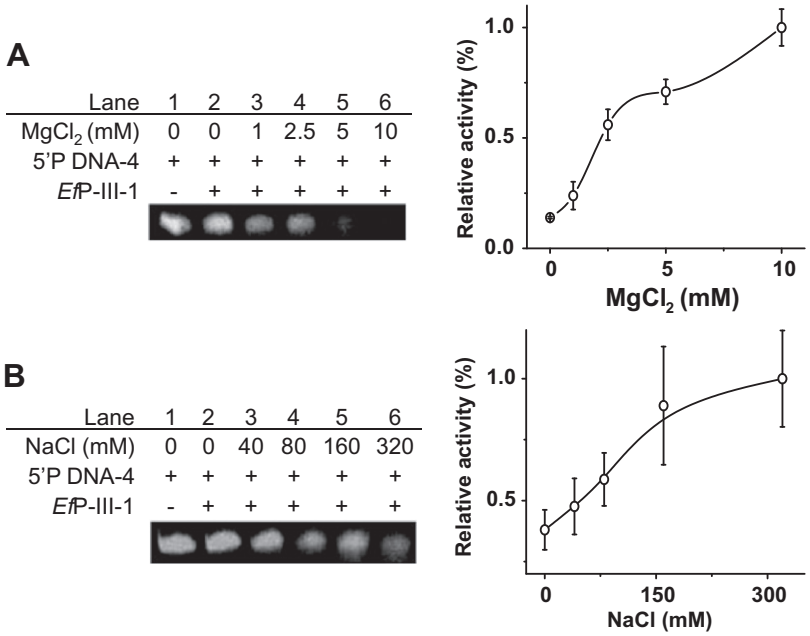

Fig. 2. Effects of magnesium and sodium on the DNase activity of *Ef*P-III-1. (A) 5 μM 5′P DNA-4 was incubated with 7 μM *Ef*P-III-1 in 10 mM Na_2HPO_4–citrate, pH 6.0, with increasing concentrations of $MgCl_2$ (1–10 mM) at 37 °C for 1 h. (B) 5 μM 5′P DNA-4 was incubated with 7 μM *Ef*P-III-1 in 10 mM Na_2HPO_4–citrate, pH 6.0, with increasing concentrations of NaCl (40–320 mM) at 37 °C for 1 h.

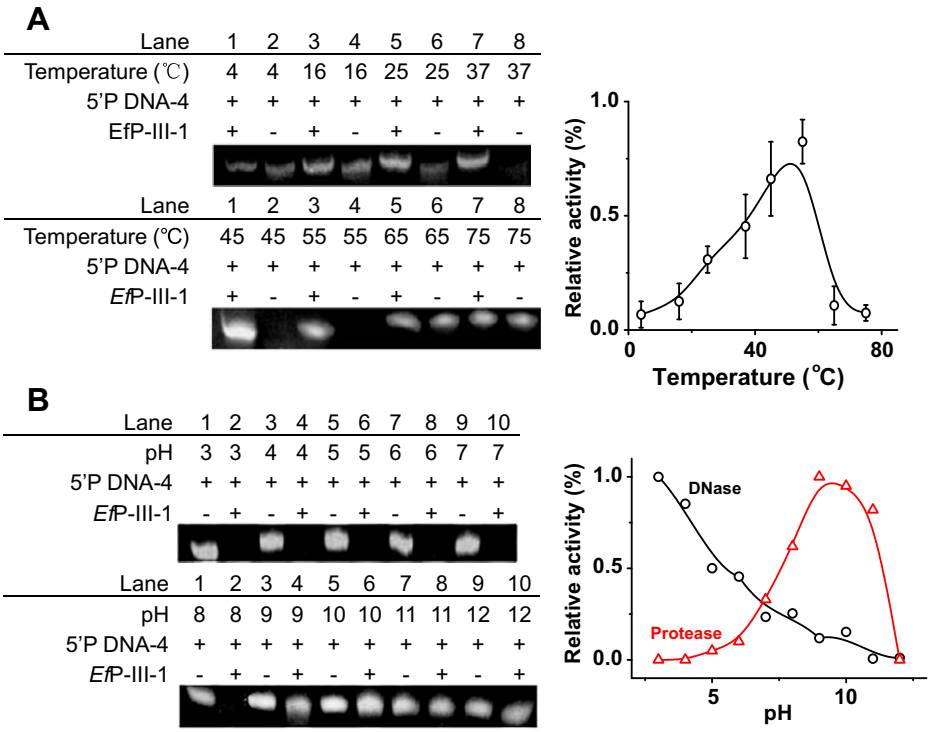

Fig. 3. Effects of temperature and pH on DNase activities of *Ef*P-III-1. (A) The optimal temperature of *Ef*P-III-1 was measured by performing the nuclease activity assay at temperatures ranging from 16 to 75 °C (16, 25, 37, 45, 55, 65 and 75 °C) for 1 h in the presence of DNA-4 as the substrate. (B) The effect of pH on nuclease activity was evaluated at 37 °C for 1 h over a pH range of 3.0–12.0 in the presence of the substrate 5′P DNA-4. The DNase or protease activity of *Ef*P-III-1 depends on pH.

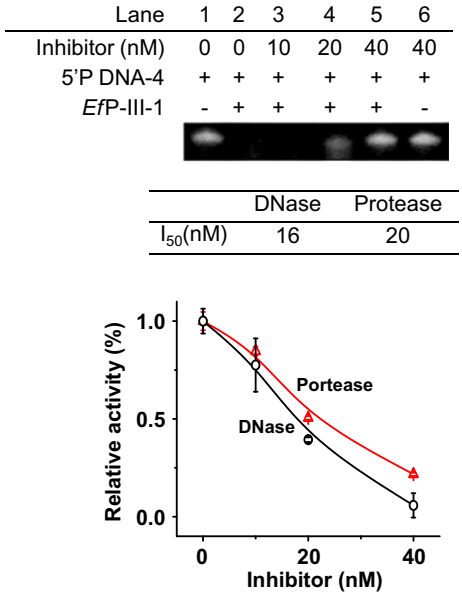

Fig. 4. Effect of a protease inhibitor on the DNase activity of *Ef*P-III-1. 7 μM *Ef*P-III-1 was incubated with its inhibitor (4-aminobenzamiline dihydrochloride) at different concentrations (0.1, 0.2 and 0.4 mM) for 30 min, followed by addition of 5′P DNA-4.

their I_{50} values were not significantly different. (v) Most DNases digest both 5′P DNA and 5′OH DNA [38,39], however, this earthworm enzyme was able to discriminate between 5′P DNA and 5′OH DNA, something that is rare in DNase contamination. All these observations indicate therefore that this earthworm enzyme functions as both a protease and a DNase.

To our knowledge, this earthworm enzyme is the first protease found in animals that has a DNase function under acidic conditions. Why does this enzyme function as both a protease and a DNase? We suggest that *Ef*P-III-1 digests both proteins and nucleic acids in the alimentary tracts of earthworms, based on the following reports. (i) Many authors have reported that the earthworm protease is located in the alimentary canal [4]. (ii) Frédéricq and coworkers have shown that the protease is secreted from the alimentary tract in earthworms [40]. (iii) We have previously shown that *Ef*P-III-1 is located in the alimentary epithelial cells of the crop, gizzard and intestines [13]. (iv) *Ef*P-III-1 administered orally can be transported into the blood circulation through the intestinal epithelium and its biological function is still partially maintained [14]. (v) The *Ef*P-III-1 precursor has a signal peptide at its N-terminal end that targets the precursor to the endoplasmic reticulum and through the secretory pathway [15]. Thus, at very least, these reports show that *Ef*P-III-1 works as an enzyme to help earthworms digest food from the environment.

*Ef*P-III-1, unlike the Potyviral NIa protease, does not have an absolute requirement for Mg^{2+} in the degradation of DNA. However, the DNase activity of the earthworm enzyme increases 6-fold in the presence of 5 mM Mg^{2+}. It is notable that the concentration of Mg^{2+} that increases DNase activity by one fold is 1 mM while that for Na^{+} is 100 mM (Fig. 2).

The DNase activity of *Ef*P-III-1 requires acidic conditions; and proteolytic activity requires alkaline conditions (Panel B, Fig. 3) [13]. Most interestingly, both the DNase and protease activities here were also present (30% compared with the highest activity) at pH 7.0. The earthworm *E. fetida* can tolerate a wide range of environmental conditions with pH ranging from 5 to 11 [41]. The pH dependence of *Ef*P-III-1 activity may be a response to the complex living environment of earthworms, and its dual function may play an important role in helping the earthworm adapt to its environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.120.

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