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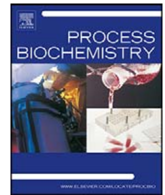

Purification and characterization of a new serine protease (EF-SP2) with anti-plant viral activity from *Eisenia foetida*: Analysis of anti-plant viral activity of EF-SP2

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ABSTRACT

Previously, we have reported that purification and characterization of an anti-plant viral serine protease (EF-SP1) from *Eisenia foetida*. In this study, a new serine protease (EF-SP2) showing strong antiviral activities against cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) was purified from the coelomic fluid of the earthworm *E. foetida*. The activity of EF-SP2 was suppressed by various known serine protease inhibitors, suggesting that the EF-SP2 is a serine protease. Its molecular weight was estimated to be 26,000 by SDS–PAGE, and its optimal pH and temperature were pH 9.5 and 60 °C, respectively. N-terminal amino acid sequence of EF-SP2 was the same as those of *E. foetida* serine proteases (EFE-d and EFE-e) with fibrinolytic activity, but different from that of EF-SP1. The enzymatic properties of anti-plant serine proteases (EF-SP1 and EF-SP2) and fibrinolytic enzymes (EFE-d and EFE-e) were similar to each other, e.g., substrate specificity, molecular weight, and effect of inhibitors. Our results suggest that EF-SP2 as well as EF-SP1 can be also applicable as a potential antiviral factor against CMV, TMV, and other plant viruses.

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1. Introduction

Viruses represent significant threats to modern agriculture, and their biological control remains a challenge in the twenty-first century [1]. In their simplest form, viruses consist of a segment of DNA or RNA encoding the genetic information required for their own multiplication in host cells. Such apparent simplicity belies the difficulties of studying and controlling these obligate intracellular parasites. Plant viruses are the vast majority of the viruses consisting of non-enveloped positive-stranded RNA viruses [2–4]. They show generally less diverse than their animal counterparts. Endowing host plants with resistance to viral infection is one of the most exciting issues in plant virology. Resistance to infection by plant viruses operates at various levels. Fraser summarized three general mechanisms for resistance: (1) a positive mechanism in which the host mounts defense against a pathogen; (2) a negative mechanism in which the host lacks factors that are required for susceptibility; and (3) nonspecific mechanisms in which the host is protected by physical or chemical barriers against the initial site of viral infection [5]. However, viruses continue to be a major threat in the production of vegetable and ornamental crops worldwide, underscoring the need for safer and more effective agents.

We have screened antiviral substances, which can be applicable as an environmental-friendly agrochemical, in microorganisms, insects, and soil animals. Recently, we have found that the earthworm *Eisenia foetida* could be a unique source of various enzymes with useful characteristics [6–8]. Cold-adapted raw starch-digesting amylases were isolated from the cell free extract of *E. foetida* [6]. An anti-plant viral protein was also isolated from the same crude extract, and the protein was shown to exhibit protease activity (EF-SP1) at wide pH range and at low temperature [7] (Table 1). EF-SP1 was characterized as a serine protease. Its molecular weight was estimated to be 27,000 by SDS–PAGE. The enzyme was most active at pH 9.5 and 40 °C. The activity was suppressed by various known serine protease inhibitors. In the present paper, we describe isolation, purification and characterization of a new anti-plant viral serine protease (EF-SP2) from *E. foetida*.

2. Materials and methods

2.1. Materials

Enzymes (bovine pancreas α-chymotrypsin, trypsin, subtilisin Carlsberg, and ficin) and chromogenic substrates [*N*-benzoyl-L-Arg-*p*-nitroanilide (BANA),

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Table 1
Comparison of enzymatic characterization of *Eisenia* anti-plant viral serine proteases and fibrinolytic enzymes.

	EF-SP1 [7]	EF-SP2 (this study)	EFE-d [15]	EFE-e [15]
Molecular weight	27,000	26,000	24,201	24,170
Optimum pH	9.5	9.5	ND	ND
pH stability	4.0–10.0	7.0–11.0	ND	ND
Optimum temperature	40 °C	60 °C	ND	ND
Thermo stability	50 °C	60 °C	ND	ND
Substrate specificity	*N*-Succinyl-Ala-Ala-Pro-Phe-pNA > *N*-Glutaryl-Phe-pNA	*N*-Succinyl-Ala-Ala-Pro-Phe-pNA > *N*-Succinyl-Ala-Ala-Pro-Leu-pNA	*N*-Succinyl-Ala-Ala-Pro-Phe-pNA	*N*-Succinyl-Ala-Ala-Pro-Phe-pNA
Anti-plant viral activity	+	+	ND	ND
Fibrinolytic activity	ND	ND	+	+

ND: not determined, +: the enzyme has anti-plant activity or fibrinolytic activity.

N-succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide (Suc-AAPL-*p*NA), *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA), and *N*-glutaryl-L-Phe-*p*-nitroanilide (Glt-F-*p*NA)] were obtained from Sigma (St. Louis, MO, USA). Protease inhibitors [soybean trypsin inhibitor (SBTI), ε-aminocaproic acid (e-ACA), phenylmethylsulfonyl fluoride (PMSF), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK), 2,2-bipyridyl, chymostatin, 1,10-phenanthroline, ethylenediaminetetraacetic acid (EDTA), and E-64] were purchased from Wako Pure Chemical (Osaka, Japan). Polyvinylidene fluoride membrane was purchased from Bio-Rad Laboratories Japan (Tokyo, Japan). The molecular weight marker kit, Daiichi II was purchased from Daiichi Pure Chemical (Tokyo, Japan). Sephacryl S-200 and Resource Q (6 ml) were purchased from GE healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). DEAE-TOYPEARL 650M was purchased from Tosoh Co. (Tokyo, Japan). All other reagents were of analytical grade and were from Nakalai Tesque (Kyoto, Japan).

2.2. Animal strain, coelomic fluids extrusion and homogenation

Earthworm *E. foetida* at the same age group within this purification was obtained from Nagane Industry (Sapporo, Japan). They were washed and covered in wet filter paper, followed by starvation for 24 h at 20 °C. Then, they were freeze-dried and pounded in mortar, and the resulting powder (30.1 g) was suspended in 50 mM Tris–HCl buffer, pH 7.0. The above procedures were done to promote the extrusion of coelomic fluid through epidermal dorsal pores. The suspension was homogenized by mixing gently in a reciprocal shaker for 1 h at 4 °C.

2.3. Virus and host plants

An isolate of pepo-cucumber mosaic virus (CMV) propagated in tobacco (*Nicotiana tabacum* cv. Xanthi) was purified as described [9]. A local lesion host for CMV, cowpea (*Vigna sesquipedalis* cv. Kuromame-sanjaku), was grown in a growth chamber at 25 °C. Cowpea plants with two primary leaves were used for the assay of viral infection. An isolate of tobacco mosaic virus (TMV) propagated in tobacco (*Nicotiana tabacum* cv. Xanthi) purified according to the method of Steere and Acker [10].

2.4. Assay for the inhibitory effects of E. foeida protease against CMV and TMV infection

The inhibitory effect of the crude extract and purified protein from *E. foeida* against CMV and TMV infections to cowpea plants were examined using local lesion assay according to the method of previously reported [11].

2.5. Enzyme assay and protein determination

Protease activity was determined by the method of previously reported [12]. After enzyme (0.5 ml) reaction with the substrate (1.5 ml of 1.14% Hammarsten casein in 0.1 M glycine–NaOH buffer, pH 9.5) at 37 °C for 10 min, 2.0 ml of 0.44 M trichloroacetic acid was added, and then the mixture was centrifuged at 15,000 × *g* for 10 min. The supernatant (0.5 ml) was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of the Folin reagent and allowed to stand at 37 °C for 20 min, and then the absorbance was measured at 275 nm. One unit of the enzyme activity was defined as the amount that liberates 1 μg of tyrosine per ml of the reaction mixture per min. Protein was determined by the Bradford method [13] with bovine serum albumin (BSA, Nacalai Tesque, Kyoto, Japan) as the standard.

2.6. Purification of anti-viral serine protease enzyme

The homogenized suspension of *E. foetida* described above was centrifuged at 27,000 × *g* for 30 min, and the clear supernatant was used as the starting material for purification. All purification steps were done at 4 °C. The protease was precipitated from the supernatant with ammonium sulfate (80% saturation) and allowed to stand overnight followed by centrifugation at 27,000 × *g* for 20 min. The precipitate was collected, dissolved in 50 mM Tris–HCl buffer, pH 7.0, and dialyzed against 20 mM Tris–HCl buffer (buffer A), pH 7.0 for 2 days. The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650 M column (Tosoh, Tokyo, Japan) [2.5 cm (inner diameter) × 27 cm] equilibrated with buffer A. The enzyme bound to the gel was eluted with a NaCl linear gradient from 0 to 1 M. The protein contents in the effluents were monitored with absorption at 280 nm. It was shown that the peaks containing anti-plant-viral activity contained also protease activity. The active fractions were precipitated with ammonium sulfate of 80% saturation and allowed to stand overnight, followed by centrifugation at 27,000 × *g* for 20 min. Then, the active fractions were applied to a Sephacryl S-200 column (GE healthcare, Little Chalfont, Buckinghamshire, UK) [2.5 cm (inner diameter) × 91 cm] equilibrated with buffer A containing 0.2 M NaCl, and eluted with the same buffer. Proteolytically active fractions were precipitated with ammonium sulfate of 80% saturation and allowed to stand overnight, followed by centrifugation at 27,000 × *g* for 20 min. The precipitate was collected, dissolved in 50 mM Tris–HCl buffer (pH 7.0) and dialyzed against buffer A (pH 7.0) for 2 days. The dialyzed enzyme solution was loaded onto a 6 ml of Resource Q column (GE healthcare) equilibrated with buffer A. Elution was done using a NaCl gradient from 0 to 0.5 M (Fig. 1). After Resource Q column chromatography, fraction I and fraction II with the anti-plant viral and protease activity were obtained. As fraction I was homogeneous state by SDS–PAGE (Fig. 2), fraction I was designated as EF-SP2. The yield and purity of the protease at each purification steps are summarized in Table 2. EF-SP2 was purified by 8.0-folds with a recovery of 2.43% to homogeneity as examined by SDS–PAGE.

2.7. Effects of pH and temperature on protease activity

Protease activity of the purified enzyme was measured using a synthetic substrate, Suc-AAPF-*p*NA, at 37 °C in glycine–HCl buffer (pH 2.5–3.5), sodium acetate buffer (pH 3.5–6.0), phosphate buffer (pH 6.0–8.0), Tris–HCl buffer (pH 8.0–9.0), and glycine–NaOH buffer (pH 9.0–11.5) with 4% DMSO. All buffers were of 0.1 M. The effect of temperature on protease activity was examined at 10–80 °C.

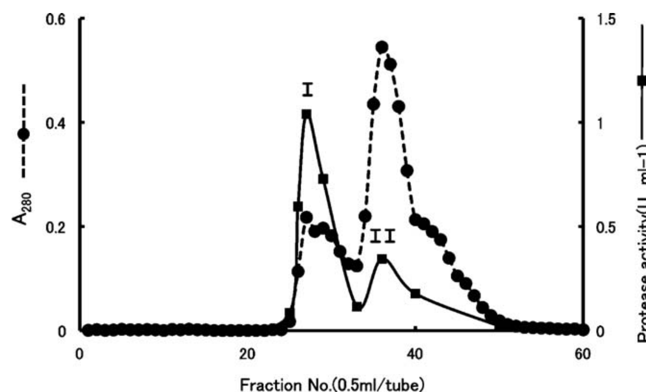

Fig. 1. Resource Q column chromatography. The dialyzed enzyme solution after Sephacrly S-200 gel filtration column chromatography was loaded onto a Resource Q column equilibrated with buffer A. The bound protein was eluted from 0 to 0.5 M NaCl solution. Fractions I and II with the anti-plant and protease activity were obtained, which fraction I was homogeneous state by SDS–PAGE. Fraction I was designated as EF-SP2. Symbols: (●) absorbance at 280 nm; (■) protease activity. I: fraction I, II: fraction II.

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