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BASIC INVESTIGATION

An extract from the earthworm Eisenia fetida non-specifically inhibits the activity of influenza and adenoviruses

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Abstract

OBJECTIVE: To test the in vitro antiviral activity of a crude tissue extract (CTE) from the earthworm Eisenia fetida, determine any effective components in the CTE, and elucidate possible mechanisms of action.

METHODS: A CTE was made by homogenizing earthworms, followed by treatment with ammonium sulfate, then thermal denaturation. Inhibition of virus-induced cytopathic effect (CPE) was used to assess antiviral activity. Chromatographic analysis was used to identify effective components in the CTE.

RESULTS: The CTE inhibited viral CPE at non-cytotoxic concentrations. Chromatography indicated that antiviral components corresponded to three active peaks indicative of proteases, nucleases and lysozymes. For adenoviruses, reduction in viral activity occurred for 100 μg/mL CTE. The reduction in adenoviral activity for four fractions was 100% , 91.8%, 86.9%, and 94.7%. For influenza viruses, reduction in viral activity of 100%, 86.6%, 69.1% and 88.3% was observed for 37 μg/mL CTE. In addition, three active fractions mixture had stronger antiviral activity (98.7% and 96.7%) than three fractions alone.Gel electrophoresis results indicated that nucleases from E. fetida could degrade the genome of influenza viruses and adenoviruses.

CONCLUSION: The earthworm CTE displayed non-specific antiviral properties, possibly mediated by a combination of proteases, nucleases and lysozymes. Nucleases likely participate in the antiviral process, and degrade the genome of the virus thereby preventing further replication.

Key words: Oligochaeta; Complex mixtures; Influenza a virus, H1N1 subtype; Adenoviruses

INTRODUCTION

One of the characteristics of Traditional Chinese Medicine (TCM) is the use of insects and other terrestrial arthropods, along with their natural derived products, as drugs. These products are believed to be an important source of new chemical substances with potential therapeutic effects. Much work on the bioactivity and use of plants, insects and their active components in TCMs has been reported.[1-3]

Earthworms play an important role in TCM and are commonly assumed to be safe for medical use. The Compendium of Materia Medica, written by Shizhen Li in 1578 AD, recorded the medicinal uses of earthworms. Earthworm extracts have been used in many TCMs to treat various diseases such as decubital necrosis, osteomyelitis, and ecthyma.[4-7]

Earthworms are invertebrates that protect themselves with innate immunity as opposed to adaptive immuni-

ty.[7] Their preferred diet is composed of many kinds of microorganisms that are digested and thoroughly absorbed. Earthworms rarely die because of microbial infections, even if they reside in waste or refuse.[8] Therefore there must be powerful molecular systems comprising many non-specific factors that can inhibit and degrade surrounding microbes in their environment. Molecules such as esterases, lysozymes, antibacterial peptides, and proteases have been previously derived from earthworms.[9-13] Despite their popular use in TCM, the scientific basis for the use of earthworms is unclear. To the best of our knowledge, few studies regarding in vitro antiviral activity of earthworm extracts have been conducted.

The aim of our study was to test the in vitro antiviral activity of an extract from the earthworm Eisenia foetida. We also sought to identify any effective components in the extract, and elucidate possible mechanisms of antiviral activity.

MATERIALS AND METHODS

Extract preparation

Samples of E. fetida were obtained from the Beijing Shuangqiao farm, China. Experiments reported in this study were carried out in accordance with current guidelines for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986). All efforts were made to minimize the number of animals used and their suffering.

Earthworms were washed with sterile water, then anaesthetized at 4°C for 4 h. They were then homogenized with a tissue blender, with earthworm tissue suspended in 20 mM Tris-HCl (pH 8.0) at a volume 2.5-fold greater than the sample. Samples were centrifuged (13 000 g, 20 min, 4°C) and the precipitate separated from the supernatant. Ammonium sulfate was slowly added to the supernatant fraction to make a final concentration of 30%, then stirred at 4°C for 2 h. For the precipitate fraction, ammonium sulfate was added to achieve a final concentration of 70%. The precipitated proteins collected by centrifugation were resuspended in 20 mM Tris-HCl (pH 8.0), dialyzed to remove the ammonium sulfate, and then passed through a filter membrane (MW cutoff=50 kDa). The filtrate was heated at 65°C for 10 min, then centrifuged at 2500 g for 20 min, with the resulting supernatant designated as the crude tissue extraction (CTE). The CTE was lyophilized and then used in chromatographic analysis, and in assays to determine antiviral activity.

Virus and culture

For the antiviral assays, we used an influenza virus (H1N1, A\PR\8\34 strain) grown in African green monkey kidney cells (Vero; American Type Culture Collection, Rockville, MD, USA). We also used an adenovirus (type 35, holden strain) that was grown in Madin-Darby canine kidney cells (MDCK; American Type Culture Collection).

DEAE sepharose fast flow chromatography

The CTE was further purified by loading on a DEAE Sepharose Fast Flow column (2.6 cm×10 cm) that had been equilibrated with 20 mM Tris-HCl (pH 8.0). After loading the sample, the column was washed with the equilibration buffer to remove unabsorbed material (designated as F1). Bound proteins were then eluted with a linear gradient of NaCl (0 M-0.5 M) at a flow rate of 4.0 mL/min. Eluted fractions were collected separately, desalted, lyophilized and tested for antiviral activity.

CM sepharose fast flow chromatography

The F1 fraction was applied to a CM Sepharose Fast Flow cation exchange column (2.6 cm × 10 cm) that had been equilibrated with 50 mM NaAc-HAc buffer (pH 4.0). The column was washed with the equilibration buffer, and the absorbed protein eluted with a linear gradient of NaCl (0.5-1 M) at a flow rate of 2 mL/min. All fractions were pooled, desalted, lyophilized and tested for antiviral activity.

Protein assays

Protein concentrations were measured using the Bradford method,[14] with bovine serum albumin (BSA) used as the standard.

Cytotoxic antiviral assays

Cells were seeded in 96-well plates (10^5 cells/well) and incubated at 37°C for 24 h. Culture medium was then replaced with media containing serial dilutions of CTE, with quadruplicate wells for each dilution. Three days later, the number of viable cells was determined by staining with 1% crystal violet in ethanol. Cytotoxicity of the extract was evaluated as the CC_{50}, corresponding to the concentration required to reduce the number of viable cells to 50% of the control.[15]

Inhibition of CPE

The inhibitory effect of various fractions on viruses was monitored by observing inhibition of virus-induced CPE in cell cultures. Cells were seeded in 96-well plates (10^5 cells/well) and incubated at 37° C. After serial dilutions, 10 μL of various concentrations of fractions were mixed with equal volumes of virus at a multiplicity of infection (MOI) of 0.02. Then virus-fraction mixtures were then incubated for 1 h at room temperature. Once cultures were confluent, monolayers were infected with the virus-fraction mixtures. Cells infected with virus only acted as controls. After adsorption for 0.5-1 h, culture medium was added and plates incubated at 37° C. Each treatment was repeated four times. When control cultures, inoculated with virus only, were no longer viable (usually 3 days post-inoculation), the number of viable cells was deter-

mined by staining with 1% crystal violet in ethanol. Following the 2-3 min staining period, excess dye was removed by washing cultures three times with phosphate-buffered saline (PBS). Plates were then left to dry upside down, and the amount of dye bound was determined directly by inspection of the cells against a white background. Plaques were counted and the percent plaque inhibition calculated. The concentration of the fractions required to inhibit virus-induced CPE to 50% of that observed in controls was designated the 50% effective concentration (EC_{50}). Virus titers were determined by plaque inhibition assays.

Determination of DNase activity

DNase activity was measured through the formation of acid-soluble DNA products. The standard reaction mixture (1 mL) contained 50 μg of calf DNA in 100 mM NaAc/HAc buffer (pH 5.2) and appropriately diluted fractions (D5, C1 and C3). Reactions were terminated after 15 min by adding 10% trichloroacetic acid. DNase activity was detected as an increase in the absorbance at 260 nm (A260) in the supernatant. One unit of DNase activity was equivalent to an A260 increase of 0.001.

Determination of RNase activity

Reaction mixtures (0.75 mL) contained 1.25 mg of RNA in NaAc/HAc buffer (pH 5.2) and appropriately diluted fractions (D5, C1 and C3). The reaction was initiated by the addition of RNA followed by incubation at 37° C for 15 min. Reactions were stopped through the addition of 0.25 mL of MacFadyen's reagent (0.75% (w/v) uranyl acetate in 25% (v/v) perchloric acid). The mixture was left on ice for 10 min and the precipitate removed by centrifugation (2500 g, 15 min). Supernatant (0.1 mL) was diluted with 2.5 mL of distilled water and the A260 measured. One unit of RNase activity was equivalent to an A260 increase of 0.001.

Agarose gel electrophoresis

Adenovirus and influenza virus were incubated at 37°C for 20 min with the C1 fraction, whose nuclease activity was the highest among the D5, C1 and C3 fractions. Reactions were terminated by the addition of 2 μL of loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 12 mM EDTA). Samples were then subjected to electrophoresis on 1% (w/v) or 1.5% (w/v) agarose gels. Products were visualized by staining with ethidium bromide.

Protease activity assays

Protease activity was measured in accordance with the Folin's methods standard of the People's Republic of China (SB/T10317-1999).

Lysozyme activity assays

The lyophilized cell wall of Micrococcus lysodeikticus was used as a substrate. Fractions (D5, C1and C3; 10-100 μL) were added to 3 mL of the substrate suspension in 0.1 M phosphate buffer (pH 7.0). The optical density was adjusted to 1.0 at 540 nm, and reductions in absorbance measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that caused a decrease of 0.1 absorbance units at 540 nm during a 3 min incubation at 25°C.

Statistical analysis

Values are expressed as mean ± standard deviation (mean±SD). Data from different group were compared using Student's *t*-test. Differences were considered statistically significant when the *P*-value was less than 0.05. All data were analyzed using SPSS 17.0 (SPSS Inc.).

RESULTS

Inhibitory activity of CTE against viruses

The EC_{50} was designated as the effective concentration of the test fraction required to inhibit virus-induced CPE to 50% of control levels. The earthworm extract showed high levels of inhibitory activity against both viruses. The CC_{50} of the extract was greater than 200 μg/mL for both cell lines. Based on the high CC_{50} value, the extract did not appear to exert cytotoxicity at low concentrations. The selectivity index (SI) was calculated as the ratio of CC_{50} to EC_{50}. For the anti-influenza virus assay, the CTE demonstrated a higher SI (15.9) than for the anti-adenovirus assay (6.9; Table 1).

Table 1 EC_{50} and CC_{50} for the earthworm CTE

	Cytotoxic effect CC_{50}[a](μg/mL)		Antiviral activity (μg/mL)			
			EC_{50}[b]		SI[c]	
	Vero	MDCK	Adenovirus	Influenza virus	Adenovirus	Influenza virus
CTE	323	296	48.6	18.9	6.9	15.9
Ribavirin	>100	>100	9.2	6.8	>10	>12

Notes: EC_{50}: 50% effective concentration; CC_{50}: cytotoxicity of the extract; CTE: crude tissue extraction; SI: selectivity index; MDCK: Madin-Darby canine kidney; Data are mean±SD from four independent experiments. [a]Concentration required to reduce the number of viable cells to 50% of the control; [b]Effective concentration of the extract required to inhibit virus-induced CPE to 50% of control; [c]The selectivity index was defined as the ratio of CC_{50} to EC_{50}.

Chromatographic analysis of the CTE (Figure 1)

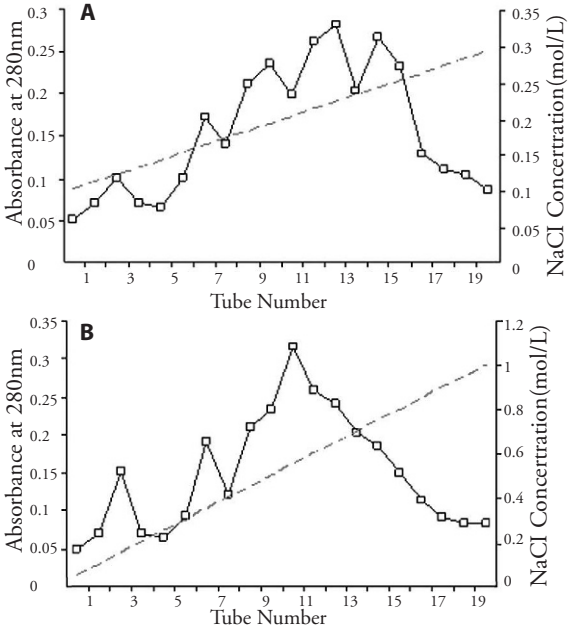

Figure1 Purification of antiviral proteins from the CTE by DE-AE cellulose (A) and CM Sepharose Fast Flow (B).

CTE: crude tissue extraction; Antiviral activity was used as an index for purification. (A) Five peaks were evident in the CTE (D1-D5) and were eluted using an NaCl gradient (0 mM-0.5 mM). Fraction D5 and unabsorbed material (F1) demonstrated antiviral activity, with the D5 fraction used for further analyses. The F1 fraction was applied to CM Sepharose Fast Flow chromatography. (B) Three peaks (C1-C3) were evident. Proteins were eluted using an NaCl gradient (0.5 M-1 M). Only the C1 and C3 fractions demonstrated any antiviral activity, and were used for further study.

Antiviral Activity of the CTE And Obtained Fractions (Table 2)

We tested all obtained fractions and the CTE for antiviral activity. Our results indicated that the CTE had the best antiviral activity (100%, $P<0.05$; Table 2). Fractions D5 and C3 possessed lower activities against adenoviruses (91.8% and 93.6%, respectively; $P<0.05$) and influenza viruses (86.6 and 88.3%, respectively; $P<0.05$). Fraction C1 also exhibited antiviral activity (86.9% and 69.1%; $P<0.05$). No significant reduction in CPE was observed when we applied fractions D1, D2, D3, D4 and C2 to infected cell cultures.

Protease, nuclease and lysozyme activity (Tables 3 and 4)

We detected three kinds of enzyme activity, corresponding to three active peaks in our chromatographic analysis. The CTE fraction possessed protease, nuclease and lysozyme activity. The principal activity of the D5 fraction was associated with proteases. The main activities of C1 and C3 were associated with nucleases and lysozymes, respectively.

Fraction C1 degrades adenovirus and influenza virus genomes

Figure 2 shows that the genome of both adenoviruses and influenza viruses were digested following incubation with the C1 fraction for 20 min.

Table 2 In vitro antiviral activity of each fraction and CTE on Vero and MDCK cells

Fractions	Virus titer (pfu/mL)[a]		Percentage (%)[d]	
	Adenovirus[b]	Influenza virus[b]	Adenovirus	Influenza virus
Control	$(6.65\pm0.14)\times10^{6}$	$(6.73\pm0.11)\times10^{6}$	-	-
CTE	0[a]	0[c]	100	100
F1	$(2.35\pm0.15)\times10^{5c}$	$(4.33\pm0.14)\times10^{5c}$	96.5	93.6
D1	$(6.58\pm0.11)\times10^{6}$	$(6.63\pm0.14)\times10^{6}$	1	1.4
D2	$(6.55\pm0.13)\times10^{6}$	$(6.65\pm0.14)\times10^{6}$	1.5	1.1
D3	$(6.70\pm0.12)\times10^{6}$	$(6.68\pm0.13)\times10^{6}$	-0.7	0.7
D4	$(6.46\pm0.14)\times10^{6}$	$(6.56\pm0.11)\times10^{6}$	2.8	2.5
D5	$(5.42\pm0.14)\times10^{5c}$	$(8.96\pm0.11)\times10^{5c}$	91.8	86.6
C1	$(8.67\pm0.14)\times10^{5c}$	$(2.08\pm0.11)\times10^{6c}$	86.9	69.1
C2	$(6.60\pm0.14)\times10^{6}$	$(6.65\pm0.14)\times10^{6}$	0.7	1.1
C3	$(4.26\pm0.14)\times10^{5c}$	$(7.86\pm0.14)\times10^{5c}$	93.6	88.3
D5+C1+C3	$(8.46\pm0.12)\times10^{4c}$	$(2.16\pm0.14)\times10^{5c}$	98.7	96.7

Noters: CTE: crude tissue extraction; F1, D1, D2, D3, D4, D5, C1, C2, C3: diluted fractions; [a]Values are mean±SD from four independent experiments. [d]Values are % reduction in virus yield. [b]For anti-adenovirus, the concentration of each fraction was 100 μg/mL; for anti-influenza virus, the concentration of each fraction was 37 μg/mL. [c]$P<0.05$ vs. control, as determined by Student's *t*-test.

Table 3 Protease and lysozyme activity for each active peak

Active Fraction	Total protein(mg)	Total activity		Specific activity	
		Protease activity (U)	Lysozyme activity (U)	Protease Activity (U/mg)	Lysozyme activity (U/mg)
CTE	432.6	22780	44.87	52.66	96.4
D5	26.8	5321	16	198.56	0.6
C1	15.6	5.46	23	0.35	1.5
C3	27.6	3.5	29714	0.13	1076.6

Notes: CTE: crude tissue extraction; D5, C1, C3: diluted fractions.

Table 4 DNase and RNase activity for each active peak

Active Fraction	Total protein(mg)	Total activity		Specific activity	
		DNase activity (U)	RNase activity (U)	DNase activity (U/mg)	RNase activity (U/mg)
D5	21.2	12.72	0.21	0.6	0.01
CTE	432.6	140421.96	85957.62	324.6	198.7
C1	15.6	19762.08	10526.88	1266.8	674.8
C3	27.6	40.296	26.50	1.46	0.96

Notes: CTE: crude tissue extraction; D5, C1, C3: diluted fractions.

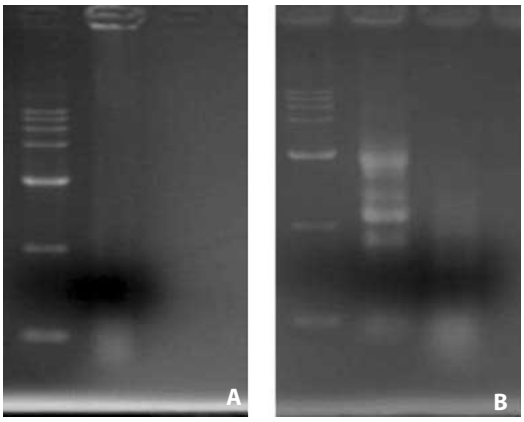

Figure 2 Digestion of adenovirus (A) and influenza virus (B) genomes with the C1 fraction
CTE: crude tissue extraction; (A) Lane 1: marker D-15000 (15 000, 1000, 7500, 5000, 2500, 1000, and 250 bp); 2: adenovirus; 3: adenovirus incubated with the C1 fraction. (B) Lane 1: marker D-15000 (15 000, 1000, 7500, 5000, 2500, 1000, and 250 bp); 2: influenza virus; 3: influenza virus incubated with the C1 fraction.

DISCUSSION

Immune system of the earthworm

The immune systems of earthworms are innate, natural, non-specific, non-anticipatory and non-clonal.[7] Earthworms live on unicellular organisms, digesting them thoroughly. It is thought that many enzymes play important roles in this process. The natural habitats of earthworms suggest that there are strong antimicrobial molecules or antiviral substances in the earthworm itself. In previous work, the existence of antibacterial molecules in earthworms has been shown.[16-18] In our present study, antiviral activity of the a CTE derived from the earthworm E. fetida was confirmed. The antiviral activity we observed was non-specific, corresponding with characteristics of innate immunity.

Inhibition of virus-induced CPE

In our work, the CTE demonstrate high and non-specific antiviral activity against DNA or RNA viruses, with or without envelopes (influenza virus and adenovirus). Further, the CC_{50} of these extracts was several orders of magnitude higher than the effective concentrations inhibiting plaque formation by 50%, indicating that the extract was safe (Table 1 and Table 2).

To determine the active antivirus substance(s) in the CTE we used DEAE Sepharose Fast Flow chromatography. We obtained five separate components from the CTE (D1-D5). Fractions D5 and F1 displayed antiviral activity. Further separation of the F1 fraction was carried out, with three peaks (C1-C3) evident. Of these, C1 and C3 showed antiviral activity (Table 2).

Using conditions in which the protein concentrations remained similar, the CTE displayed 100% inhibition of virus-induced CPE for both tested viruses. Protein concentrations were 100 μg/mL for adenoviruses and 37 μg/mL for influenza viruses. Inhibition of CPE due to the separated fractions was lower than that for CTE (Table 2). This is indicative of a loss in inhibition activity during purification. It is possible that this loss in activity could be due to reactions between various components in the fractions However, an attempt to restore maximum antiviral activity by pooling several fractions failed. We did manage to recover antiviral activity up to 98.7% and 96.7% (Table 2).

The antiviral activity assays indicated that fractions D5, C1 and C3 inhibited CPE. Fractions D5, C1 and

C3 also possessed protease, nuclease and lysozyme activity, respectively (Tables 3 and Table 4). These results suggest that the three activities might be closely related to antiviral processes. Only weak antiviral activity was exhibited by other fractions, but this could probably be considered as background residual activity. Previously, lysozyme has been purified from many invertebrates, including E. fetida.[10] Lysozyme has been shown to have antiviral properties,[19] directly acting on alkaline viral proteins. With respect to the envelope of a virus, lysozyme can attack the envelope and decrease the aggressiveness of the virus.

Nakajima reported that earthworm proteases have better stability, catalytic function and stronger tolerance to organic solvents than other proteases.[20] Nobuyoshi et al. cloned and characterized six proteases from earthworms. They also hypothesized that these proteases have broad substrate specificity and play a role in breaking down ingested proteins.[21] It is widely known that viral proteins are often strongly resistant to many proteases, but it is possible they may be susceptible to those of earthworms.

Nucleases were detected while we were attempting to extract antiviral substances. It was found that these nucleases have broad substrate specificity, and are able to degrade supercoiled plasmid DNA, and linear λDNA (data not shown). We have shown that the detected group of nucleases could digest the genome of the two viruses we investigated. This implies that nucleases participate in antiviral processes, degrading the genetic material of the virus.

It is possible that the three types of enzyme we purified cooperate with each other to attack and degrade viruses. Proteases and lysozymes play an important role in the breakdown of virus proteins. For enveloped viruses, lysozyme possibly destroys the lipid envelope, and is particularly active against enveloped viruses such as influenza virus. Nucleases degrade the genomes of viruses in combination with other proteases, and lysozyme. Obviously, the virus genome is very important in replication of the virus. There is not enough evidence as yet to show that naked viral nucleic acid is not infective. Therefore, even if we completely degrade the virus envelope and other proteins, the genetic material can still infect host cells and replicate. Thorough degradation of viral genetic material is the key in determining if a virus is non-viable. Further investigation of the mechanism(s) of action for all of the extracts we purified are necessary.

Implications for traditional treatments and innate immunity

TCM is widely used and is recognized as an alternative to conventional medicine. Generally speaking, traditional medicines, including TCM, are effective. However, they also have shortcomings; often, the active molecules or constituents, and the mechanisms underlying their effectiveness are not always clear. In this study we have tried to identify the active components in earthworms used for the treatment of inflammatory diseases by TCM, and elucidate the associated mechanisms. Lysozymes and proteases have been widely studied and are known to be involved in the innate immunity of invertebrates. However, there are few reports about the relationship between innate immunity and nucleases. It is worth noting that the digestion of the genetic material of an organism is more thorough and effective than restraining metabolism. It is evident from our work that nucleases are a major component of the innate immune system.

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