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Activating effects on trypsin, α-chymotrypsin, and lipase and inhibitory effects on α-amylase and α-glucosidase as provided by low-molecular-weight compounds in the water extract of the earthworm *Eisenia fetida*

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ABSTRACT

A fraction (designated as U3EE) with molecular mass under 3 kDa from the water extract of the earthworm *Eisenia fetida* was prepared and its effects on mammalian digestive enzymes were examined. U3EE itself showed no relevant enzyme activities. However, it increased the activities of trypsin, α-chymotrypsin, and lipase, while decreased those of α-amylase and α-glucosidase. The trypsin activation and α-amylase inhibition were analyzed precisely by enzyme kinetics. The former was solely dependent on increase in the molecular activity k_{cat} without any change in the Michaelis constant K_m, and the latter was mainly dependent on decrease in K_m with a slight change in k_{cat}. Effects of the treatments of U3EE with freezing-and-thawing, heat, acid, and hexane were examined. The treated U3EE showed the effects on the enzymes with the same potencies as those provided by the non-treated one, indicating that U3EE was enough stable toward the harsh treatments to hold the modulating effects on the enzyme activities. U3EE was also shown to be highly hydrophilic. The results obtained in this paper suggested that U3EE could be applicable as a novel constituent for pharmaceuticals and functional foods.

1. Introduction

Earthworms have been known to play important roles in agriculture by mixing soils through their movement and by giving soil nutrition from their casts [1]. They take and digest microorganisms and bio-organic materials in soil as nutrients, and this activity has been utilized for turning garbage into compost [2]. They are known to be nutritionally favorable for feed, and have been used widely as fishing bait and feed for livestock and cultured fish [3,4]. Use of earthworms for human food has not been reported openly. Recently, the Food and Agricultural Organization (FAO) of the United Nations appealed the promotion of utilization of insects for food and feed [5]. The utilization of earthworms seems to be out of the FAO's scope, although it should be considered to be on the same vector as that of insects as long as regarding our long and wide experience in the use of earthworms for feed.

On the other hand, earthworms have been known for therapeutic potentials to improve various diseases for tens of centuries especially in Asian countries including China, India, Korea, Japan, etc. In these countries, the decoction of dried earthworms and earthworm extracts or powders have been used for oral administration as traditional medicine or supplements with antipyretic, antispasmodic, detoxic, diuretic, antihypertensive, antiallergic, antiasthmatic, spermatocidal, antioxidative, antimicrobial, anticancer, antiulceral, and anti-inflammatory effects [6–9]. Despite these various biological activities, biochemical studies of earthworms have not been sufficient, and only a few biologically-active compounds have been identified.

Some enzymes from earthworm species such as *Eisenia fetida*, *Lumbricus rubellus*, etc. have been reported. Especially, serine proteases (termed lumbrokinases) isolated from *Lumbricus rubellus* were shown to possess strong fibrinolytic activity in vitro, and their in vivo application has been also expected [10–12]. Lumbrokinases on this line have been extensively studied, although there might be a lot of problems remained not to be solved for their therapeutic application. Actually, no evidence has been provided for the stability of lumbrokinases in human gastric tracts (stomach and intestines), their transfer from intestine to blood, their stability and activity in blood, etc. Otherwise, we reported cold-adapted α-amylases and cellulases from *E. fetida* [13–15] and a unique protease having antiviral activities against plant viruses [16],

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suggesting that these enzymes could be useful in biotechnology, food science, and plant biology. It is noted that a crude extract of earthworms was used as a biocatalyst for various organic syntheses such as aldol, Mannich, Henry, Biginelli, Diels-Alder reactions in organic media with high reaction yields [17]. The enzymes related with these reactions were not characterized, although it was obvious that the enzymes were stable and active even under the drastic reaction conditions used.

Biologically-active compounds with low molecular weights have been also screened in earthworms. Various types of antimicrobial peptides have been reported from the body extracts, coelomic fluids, and skin secetions [18]. Interestingly, a class of surface-active metabolites (dialkylfuransulfonates) termed as drilodefensins, which was found in earthworm gut, was shown to protect earthworms against plant polyphenols contained richly in earthworm diets by counteracting the inhibitory effects of polyphenols on earthworm gut enzymes [19]. Recently, we reported that the low-molecular-weight fraction (< 10 kDa) in earthworm (*Eisenia fetida*) extracts showed inhibitory activities against angiotensin-converting enzyme (ACE) [20] and dipeptidyl peptidase IV (DPPIV) [21], suggesting that the fraction could be useful for treatment of hypertension and diabetes. Based on these lines of evidence, we have supposed that the earthworm extract might contain various compounds which could affect, modulate, or regulate biological and enzyme activities of animals, plants, microorganisms, etc., and have been examining the effects of the earthworm extract on these activities.

In the present study, we describe the effects of the fraction under 3 kDa prepared from *E. fetida* water-extract (hereinafter designated as U3EE) on several mammalian digestive enzymes, such as trypsin, α-chymotrypsin, lipase, α-amylase, and α-glucosidase. We report here that U3EE shows interesting effects on the enzymes examined. Namely, U3EE activates trypsin, α-chymotrypsin, and lipase, and inhibits α-amylase and α-glucosidase. Kinetic analyses on the activation and inhibition are also described. A possibility of U3EE as novel useful constituents for pharmaceuticals and functional foods could be suggested.

2. Materials and methods

2.1. Materials

Cultured living earthworms (*E. fetida*) were harvested from the earthworm farm of Waki Pharmaceutical Co. (Nara, Japan). Materials especially concerned are described in each subsection below. All other chemicals were of analytical grade and purchased from Nacalai Tesque (Kyoto, Japan). Except for that especially mentioned, water used for preparation and biochemical characterization of U3EE was deionized-and-distilled water made using a Yamato WG1000 water purifier (Tokyo, Japan).

2.2. Preparation of the dried earthworm powder

The dried earthworm powder used as a source of U3EE was prepared by the method described previously [22]. Briefly, 30 kg of cultured earthworms was washed with tap water and immersed in 5% (w/v) sodium bicarbonate for 1 h to eliminate the coelomic fluid. They were washed again with water followed by homogenization with a Nantsune M-22 meat-chopper (Osaka, Japan). The homogenates were packed into a plastic bag and sealed, and subjected to 100 MPa at 60 °C for 16 h with a Shinada SHP-100-50A high-hydrostatic-pressure processor (Niigata, Japan). The resulting products were centrifuged at 17,000 rpm with a Tomoe 160AP ASM-tubular-centrifuge (Tokyo), and the supernatant was lyophilized. The lyophilized extracts were dried at 80 °C for 6 h. Finally, 4 kg of the dried powder was obtained with the yield of 13% (w/w) to the initial weight.

2.3. Preparation of U3EE from the dried earthworm powder

The dried earthworm powder (5 g) was suspended in distilled water (50 mL) and stirred for 10 min, followed by centrifugation at 16,100 × *g* for 10 min. The supernatant was applied to ultrafiltration with a Vivaspin 20-3 K filter (GE Healthcare, Little Chalfont, UK), and the filtrate was named as the U3EE fraction. It was lyophilized, powdered, and stored at -20 °C until use. UV–vis spectra of U3EE were measured with a Shimadzu UV mini-1240 spectrophotometer (Kyoto). The protein was determined using the Takara Bradford protein assay kit (Otsu, Japan) with bovine serum albumin (BSA) as standard [23].

2.4. Stability of U3EE

The stability of U3EE against various treatments was examined by evaluating the effects on trypsin and α-amylase activities. The treatments are as follows. Freezing-and-thawing: U3EE dissolved in a buffer for enzyme assay (or enzyme-assay buffer; see below) was frozen over night at -80 °C in a deep freezer (MDF-U384, Sanyo, Osaka), and thawed on water, followed by incubation at 37 °C for 15 min before the assay; Heat: U3EE dissolved in an enzyme-assay buffer was heated for 10 or 30 min at 100 °C in a dry thermo-unit (FTU-1B, Titec, Tokyo) and then incubated at 37 °C for 15 min in a capped tube; Acid: U3EE dissolved in 10 mM HCl was incubated for 3 h at 37 °C, and then sequentially, neutralized with 10 mM NaOH, freeze-dried, and re-dissolved in an enzyme-assay buffer; and Hexane: U3EE dissolved in an enzyme-assay buffer was vortexed with an equal volume of the solvent for 5 min at 25 °C and then the aqueous layer was recovered after a centrifugation at 10,000 × g for 5 min.

2.5. Trypsin assay

Trypsin activity was evaluated by its hydrolytic activity toward α-*N*-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA) [24,25]. Increase in a substrate *p*-nitroaniline (PNA) was measured as increase in absorbance at 405 nm (A_{405}) to determine the reaction rate (v). For an assay, 75 μL of 100 mM Tris-HCl buffer at pH 8.0 (buffer A) and 15 μL of water were put into a well of a 96-well microplate (BioLite 96-well Multidish, 130188, Thermo Scientific, Rochester, NY), and 10 μL of 30 μg/mL bovine pancreatic trypsin (24.0 kDa; Lot: SLBG6452 V, Sigma, St. Louis, MO) dissolved in 1 mM HCl was added. As an enzyme blank, 1 mM HCl was used instead of the trypsin solution. U3EE (20, 10, 5, 2, and 1 mg/mL) dissolved in buffer A was used instead of the buffer (75 μL) for examining the effect of U3EE on trypsin activity. After 5-min incubation of the microplate at 37 °C, 50 μL of 1.0 mg/mL BAPNA mono-hydrochloride (398.4 Da; Lot: BCBN0139 V, Sigma) dissolved in water was added to start reaction. Besides, 2.0, 1.5, 1.25, 0.75, and 0.50 mg/mL BAPNA were used for trypsin kinetics. All reagents except trypsin were pre-incubated at 37 °C. The reaction was measured with a microplate reader (xMark, Bio-Rad Laboratories, Hercules, CA) by monitoring A_{405} at every minute over 15 min. After subtracting the blank value from the sample one at every measurement time, the change in A_{405} per minute (ΔA_{405}/min) was obtained from the range where A_{405} increased linearly. The v was calculated using ΔA_{405}/min and the following parameters: the light-path length (0.458 cm) of the microplate well with 150 μL of the reaction mixture; and the molar absorption coefficient (ε_{405} = 9.5 mM^{-1} cm^{-1}) of PNA at pH 8.0 [26]. Throughout this study, each measurement was done in triplicate and the data were expressed as mean ± standard deviation (SD).

For analyzing the effect of U3EE on trypsin activity, the v was plotted against the BAPNA concentration ([S]). The plotted data were fitted to the Michaelis-Menten (M-M) equation [$v = V_{max}$ [S]/(K_m + [S])] with a nonlinear-least-squares regression method using Microsoft Excel 2013 (Redmond, WA) to draw the best-fitted curves, and the maximum velocity (V_{max}) and Michaelis constant (K_m) were determined. The V_{max} and K_m were also determined by Lineweaver-Burk