Optimization of Protein Hydrolysate from Earthworms (*Perionyx excavatus*) of Angiotensin-I-Converting Enzyme Inhibitory Activity by One Variable at a Time

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Abstract: *Hypertension, characterized by elevated blood pressure, is commonly treated with angiotensin-I-converting enzyme (ACE) inhibitors. This study evaluated the ACE inhibitory activity (ACEIA) of protein hydrolysate from earthworms (*Perionyx excavatus*) by commercial proteases. The results showed that the protein hydrolysate of earthworms gave the highest ACEIA of 85.38 ± 2.31% (at the protein concentration of 2 mg/mL) and IC_{50} value of 844.64 μg/mL when the earthworms were hydrolyzed with Flavourzyme® 500 MG under optimized conditions such as earthworm:phosphate buffer ratio of 1:6 (w/v), hydrolysis temperature of 50 °C, pH 7, enzyme:substrate ratio of 600 U/g protein and hydrolysis time of 2 h. The membrane further fractionated the hydrolysate, and the < 1 kDa fraction had the highest ACEIA with an IC_{50} value of 261.94 μg/mL. The stability of ACEIA was assessed under various conditions, including* in vitro *digestion, heat treatment at 100 °C for 180 min, and pH adjustments from pH 1 to 11. The < 1 kDa fraction maintained ACEIA activity at 133.34, 76.25, and 84.95%, respectively, after these treatments. These results suggest that earthworm protein hydrolysates, particularly the <1 kDa fraction, exhibit strong stability and could be promising candidates for the development of functional foods or pharmaceuticals targeting hypertension.*

Keywords: *ACE; angiotensin-I-converting enzyme; hypertensive;* Perionyx excavatus

■ INTRODUCTION

According to the World Health Organization, about 1.13 million people worldwide had high blood pressure in 2015, mostly in low- and middle-income countries. Hypertension (also known as high blood pressure) is a chronic medical condition in which the blood pressure against the artery walls rises, and its effects increase the risk of cerebral vascular accidents, stroke, heart failure, kidney failure, and other problems [1]. The angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II in the blood pressure regulation system, causing vasoconstriction and increased blood pressure. Hence, ACE inhibitors contribute to vasodilation, adequate blood volume, and blood pressure reduction. Synthetic medications used to suppress ACE, such as captopril and enalapril, frequently cause hazardous side effects such as angioneurotic edema, skin rashes, and altered taste. ACE inhibitors that are both cost-effective and non-toxic are a promising trend, and peptides are one of those types of compounds [2]. The biologically active peptides typically contain between 3 and 20 amino acid residues, and the peptides from *Juglans regia* [3], *Ginkgo biloba* seeds [4], longan seeds [5], and chicken skin [6] were reported to decrease high blood pressure. Usually, the compound capable of inhibiting ACE has a carbonyl group to form an ionic bond with the cation radical of ACE. This functional group can form a hydrogen bond with ACE and an ionizable functional group to bind to the Zn^{2+} of ACE [7].

The enzymatic hydrolysis method is the most prevalent process of forming ACE inhibition proteins and peptides. Because proteases cleave peptide bonds at specific amino acids, highly biologically active peptides can be produced. In addition, hydrolysis occurs under standard reaction conditions, so there is little denaturation of proteins and peptides [3-5]. Alcalase, flavourzyme, neutrase, and protamex are commercial enzymes typically used to make protein hydrolysates exhibiting ACE inhibitory activity (ACEIA) [4-6]. Short-chain peptides that inhibit ACE often contain acidic amino acids (Asp and Glu) to form a bond with Zn^{2+} of ACE. Peptides containing amino acids such as Tyr, Phe, Lys, Ile, Val, Leu, and Arg also have high ACEIA [8]. The ACEIA of peptides is also related to the N- and C-terminal amino acid sequences. The N-terminus of ACE-inhibiting peptides often contains hydrophobic amino acids (Gly, Ile, Leu, and Val). In contrast, the C-terminus often contains cyclic or aromatic amino acids (Tyr) [9].

Earthworms (*Perionyx excavatus*) is a common earthworm that plays an essential role in soil fertility and is an excellent source of protein (55–70% of the protein in earthworms on a dry matter basis) [10-11]. Earthworms are the most widely used in traditional medicine systems. Earthworms are used in cardiovascular diseases, blood pressure, and diabetes [12]. In numerous studies in Vietnam, earthworms have been demonstrated to help support immunity and cardiovascular health [13]. The protein of earthworms contains amino acids such as Leu (3.74% in dry weight basis), Asp (3.18% in dry weight basis), Ile (2.74% in dry weight basis), Met (1.2% in dry weight basis), Arg (0.4% in dry weight basis), and Lys (0.13% in dry weight basis) should be able to potential produce peptides with ACEIA [14].

Hence, this study uses commercial proteases to prepare protein hydrolysates from earthworms to develop ACEIA. The hydrolysis conditions such as a hydrolytic enzyme type, earthworm:phosphate buffer ratio (w/v), temperature, pH, enzyme:substrate (E:S) ratio, and hydrolysis time were investigated to obtain the hydrolysate expressing the highest ACEIA. Peptide fractions by molecular weight were obtained by ultrafiltration membranes (30, 10, 3, and 1 kDa) to find the fraction with the highest ACEIA. The stability of the ACEIA of the hydrolysate and fractions against thermal, pH, and simulated gastrointestinal digestion treatments was assessed.

■ EXPERIMENTAL SECTION

Materials

Earthworms (*P. excavatus*) were purchased from the Biotechnology Center, which is located in Ho Chi Minh City. Later on, they were crushed and preserved in polyethylene bags at −20 °C before testing. The chemical composition of earthworms contained 80.99 ± 0.53% moisture, 69.92 ± 0.23% crude protein, 6.97 ± 0.10% crude lipid, 12.78 ± 0.18% crude carbohydrate, and 10.25±0.10% ash (based on weight without moisture content).

ACE from rabbit lung (2.0 units/mg protein), hippuryl-histidyl-leucine (HHL), and captopril were supplied by Sigma Chemical Co. (located in St. Louis, MO, USA). The enzyme preparations, including Alcalase® 2.5 L, Neutrase® 0.8 L, Protamex®, and Flavourzyme® 500 MG, were purchased from Novozymes Co. (Bagsvaerd, Denmark) and AB enzymes (Darmstadt, Germany). All reagents were at analytical grade.

Instrumentation

UV-vis 1800 (Shimadzu), centrifuges (Mikro 200), and incubator (Memmert) are the devices used for this study. Ultrafiltration membranes (30, 10, 3, and 1 kDa) were purchased from Pall Laboratory Co., USA (Macrosep, Pall Laboratory, USA).

Procedure

Preparation of protein hydrolysates

With slight modifications, the method described by Vo et al. [15] was used to hydrolyze earthworm protein. The 0.02 M phosphate buffer was mixed with ground earthworm with an appropriate ratio for the pH to reach the required value for hydrolysis and then heated at 90 °C for 10 min to deactivate endogenous enzymes. After that, the pH was adjusted again with 1 M HCl or 1 M NaOH solution, while the hydrolysis temperature was set before adding the prepared enzyme with an appropriate E:S ratio. At a set time, the enzyme

in the hydrolysate was deactivated at 90 °C for 10 min. The hydrolysates were centrifuged at 5,000 rpm for 15 min to collect the supernatant. The Lowry method was used to determine soluble protein content in the hydrolysate. The obtained supernatant was then lyophilized and stored at −20 °C for further use.

Effect of hydrolysis condition on the ACEIA of protein hydrolysate from earthworms

Alcalase (55 °C, pH 8), flavourzyme (50 °C, pH 7), neutrase (50 °C, pH 7), and protamex (55 °C, pH 6.5) were selected for protein hydrolysate, with an E:S ratio of 500 U/g protein, a hydrolysis time of 4 h, and earthworm:phosphate buffer ratio of 1:6 (w/v). Other hydrolysis conditions were examined with the hydrolytic enzyme that gave the highest ACEIA.

The earthworm:phosphate buffer ratio (w/v), temperature, pH, E:S ratio, and hydrolysis time are five factors on the ACEIA of a protein hydrolysate that was examined using a single factor test method. In greater detail, one factor was investigated at different levels, with the other four factors staying the same.

Determination of ACEIA

The method of evaluating ACEIA was described in a previous study with slight modifications [16]. First, 50 µL of hydrolysate (2 mg protein/mL) was pre-incubated at 37 °C for 10 min with 50 µL of ACE solution (25 mU/mL). The mixture was incubated for 30 min at the same temperature with 150 µL of substrate solution (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3). After that, 1 M HCl (250 µL) was added to terminate the reaction, and 500 µL ethyl acetate was used to extract the resulting hippuric acid. Then, 200 µL of the upper layer (ethyl acetate) was placed into a glass tube after centrifugation (3,000 rpm, 10 min) and dried at 60 °C for 30 min. Finally, 2 mL of distilled water was redissolved and mixed, and the mixture's absorbance was measured at 228 nm using a spectrophotometer.

The Eq. (1) was taken to calculate the ACEIA of the protein hydrolysate;

$$\text{ACEIA } (\%) = \frac{(A_1 - A_2) - (A_3 - A_4)}{A_1 - A_2} \times 100\% \quad (1)$$

where A_1 is the absorbance of the control (the mixture containing enzyme and substrate, without hydrolysate or peptide fraction). A_2 is the absorbance of the control blank (the mixture with substrate, no enzyme, hydrolysate, or peptide fraction). A_3 is the absorbance of the tested sample (the mixture containing enzyme, substrate, and hydrolysate or peptide fraction). A_4 is the absorbance of the sample blank (the mixture containing substrate, hydrolysate or peptide fraction, and no enzyme).

Logarithmic regression analysis was applied to determine the half inhibitory concentration (IC_{50}, the inhibitor's concentration to inhibit 50% of enzyme activity) for ACEIA by the earthworm protein hydrolysates and their five peptide fractions. The positive control was captopril at 14, 16, 18, 20, and 22 nM concentrations.

Fractionation of protein hydrolysate from earthworms

The protein hydrolysate from earthworms was further fractionated using ultrafiltration centrifugal devices of 30, 10, 3, and 1 kDa (Macrosep, Pall Laboratory, USA). Five peptide fractions, including > 30, 10–30, 3–10, 1–3, and < 1 kDa, were prepared and evaluated for their ACEIA.

In vitro *digestion stability of protein hydrolysate from earthworms and their fractions*

The stability of the ACEIA against *in vitro* digestion treatments of the fraction and the hydrolysate was measured following a previous report with minor modifications [17]. The hydrolysate and their fractions with a protein concentration of 5% (w/v) were calibrated to pH 2 with 6 M HCl solution and incubated at 37 °C. After, the hydrolyzed progress was done by pepsin at a condition in E:S ratio of 4% (w/w), 37 °C, and shaken at 230 rpm for 60 min. Subsequently, its pH was adjusted to pH 7.5 with a 6 M NaOH solution. The hydrolyzed progress by pancreatin at an E:S ratio of 4% (w/w) at 37 °C and shaken at 230 rpm for 2 h. The mixture was heated under the condition of 90 °C for 10 min to inactivate the digestive enzymes. During *in vitro* digestion, samples were collected to determine its ACEIA.

Thermal and pH stability of protein hydrolysate from earthworms and their fractions

The method outlined by Sripokar et al. [18] was employed to assess the thermal and pH stability of the protein hydrolysates and their peptide fractions. For stability of the ACEIA against thermal treatments of the hydrolysate and the fraction, 5 mL of sample solutions (40 mg protein/mL) were heated to 100 °C at 15, 30, 45, 60, 90, 120, 150, and 180 min, respectively. After that, the solutions were brought to room temperature with cold water. The solutions were then diluted with deionized water to the final volume of 10 mL. The ACEIA was determined from all samples.

For the stability of ACEIA against pH treatments and the hydrolysate and the fraction, 5 mL of sample solutions (40 mg protein/mL) were treated at pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 by using 6 M HCl or 6 M NaOH solution for 30 min at room temperature. After that, all samples were altered to pH = 7.0 by 1 M phosphate buffer and then diluted to the final volume of 20 mL using deionized water. The ACEIA was determined from all samples. The thermal and pH stability of the sample was measured in terms of relative activity (%), representing the percentage of bioactivity observed in the treated sample in comparison to the control (untreated sample).

Statistical analysis

All experiments were performed in triplicate. The data demonstrated as mean ± standard deviation were processed using the Microsoft Excel application. The software SPSS (IBM SPSS Statistics 20) determined the statistically significant differences.

■ RESULTS AND DISCUSSION

ACEIA of the Earthworm Hydrolysate under the Impact of Certain Hydrolysis Conditions

Effect of hydrolysis enzyme type on the ACEIA of protein hydrolysate from earthworms

The biological activity of the hydrolysate depends on the enzyme used in the hydrolysis process because different enzymes will cleave the protein chain to create peptides with different amino acid compositions and chain sequences [19]. The flavourzyme hydrolysates expressed the highest ACEIA of 74.36 ± 0.44%, which were 1.09, 1.55, and 1.79 folds higher than that of alcalase, protamex, and neutrase hydrolysates, respectively (Fig. 1(a)). Among all the enzyme hydrolysates in proteolytic assays, flavourzyme showed effective ACE inhibition in another study [20-21].

Peptides of ACEIA often contain highly acid amino acids such as Lys, Ile, Leu, and Arg, especially Asp, to form a bond with Zn^{2+} of ACE [8]. The N-terminus of ACEIA peptides usually contains hydrophobic amino acids, especially those with aliphatic chains (such as Ile and Leu). In contrast, the C-terminus of these peptides usually contains amino acids with aromatic cyclic (Pro, Tyr, and Trp) [9]. Therefore, proteases that can cleave peptide bonds at those amino acid positions will produce ACEIA peptides. Among the proteases, flavourzyme usually cuts peptide bonds at amino acid positions such as Lys (P1, P1'), Asp (P1', P2'), Ile (P2), Leu (P2), and Arg (P2, P3, P3') [22]. Simultaneously, the protein of earthworms contains amino acids such as Leu, Asp, Ile, Arg, and Lys [14], which should be able to be cleaved to produce peptides with ACEIA. The match between the peptide bond cleavage site of flavourzyme and the amino acid composition of earthworms resulted in the hydrolysate by flavourzyme having the highest ACEIA. Therefore, flavourzyme is used in the hydrolysis process to maximize the efficient production of ACEIA peptides from earthworm protein.

Effect of earthworm:phosphate buffer ratio on the ACEIA of protein hydrolysate from earthworms

When increasing the ratio of earthworm:phosphate buffer ratio from 1:2 to 1:6 (w/v), the ACEIA gradually increased, and the highest ACEIA (74.24±0.76%) at the ratio 1:6 (w/v) (Fig. 1(b)). These results suggest that increased substrate concentration led to a higher viscosity, diminishing the enzyme-substrate interaction, thereby reducing the enzymatic activity associated with protein hydrolysis [23].

Upon further increasing the ratio of earthworm extract to phosphate buffer from 1:6 to 1:14 (w/v), ACEIA exhibited a declining trend (Fig. 1(b)). Excessive buffer concentration can decrease the frequency of collisions

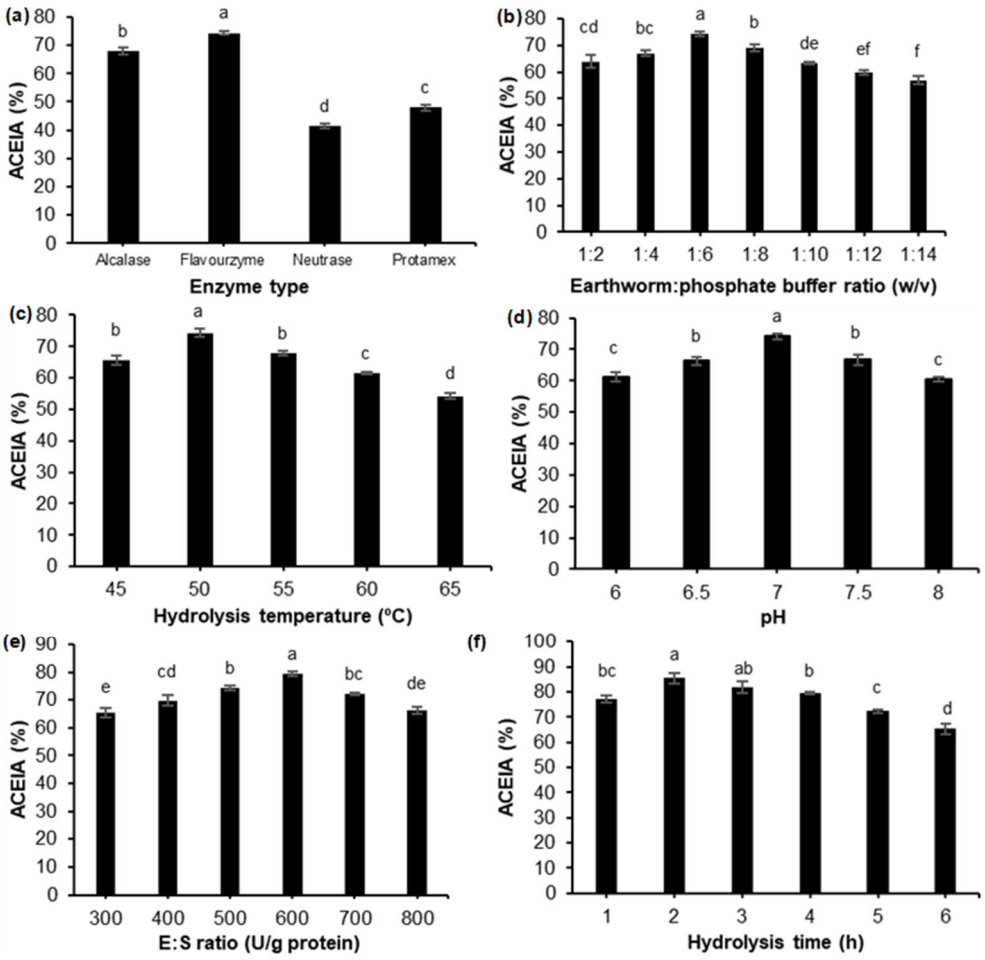

Fig 1. (a) Effect of hydrolysis enzyme type, (b) earthworm:phosphate buffer ratio, (c) temperature, (d) pH, (e) E:S ratio, and (f) hydrolysis time on the ACEIA of the earthworm protein hydrolysate. The bars in each chart indicate the significant differences in the conditions according to their belonging letter ($p < 0.05$)

between substrate molecules and the enzyme, thus impeding the hydrolysis process and lowering the enzyme's catalytic efficiency and bioactivity [24]. Consequently, the 1:6 (w/v) ratio of earthworm extract to phosphate buffer was selected for subsequent experiments to optimize the efficiency of protein hydrolysis and enzyme bioactivity.

Effect of temperature on the ACEIA of protein hydrolysate from earthworms

Experiments investigating hydrolysis temperature's effect on the ACEIA of hydrolysates have determined that hydrolysate has the highest activity (74.38 ± 1.14%) when the hydrolysis temperature is 50 °C (Fig. 1(c)). At lower temperatures (below 50 °C), both the protein and enzyme molecules exhibit reduced kinetic energy, which impedes the frequency of enzyme-substrate collisions, thereby limiting the formation of biologically active peptides during the hydrolysis process [25-26]. As the temperature increases from 55 to 65 °C, it can induce conformational changes in the enzyme's active site, resulting in a decrease in catalytic efficiency and a corresponding reduction in the yield of ACEIA peptides [26]. Therefore, a hydrolysis temperature of 50 °C will be selected for subsequent experiments to optimize the production of ACE-inhibitory peptides.

Effect of pH on the ACEIA of protein hydrolysate from earthworms

Experimental results showed that the hydrolysate at pH 7 exhibited the highest ACEIA (74.13 ± 0.86%) (Fig. 1(d)). This can be explained by the influence of pH on the charge distribution, structure of the enzyme and

substrate, and the enzyme-substrate complex. Specifically, pH affects the ionizable groups on the enzyme and substrate, altering their conformation and the ability to bind, impacting the hydrolysate's biological activity and its ACEIA [24,27]. Besides, pH affects the ionization ability of proteins, the solubility of proteins, and the spatial structure of enzymes, changing the reaction speed and ACEIA of the hydrolysate. At pH values that are too low or too high, the enzyme may become unstable, reducing its ability to bind to the substrate and thereby decreasing efficacy. However, pH 7 maintains the enzyme's stable conformation, optimizes activity, and provides an environment for maximal enzymatic efficiency [28]. In comparison to other pH values, pH 7 is superior in maintaining ACEIA. Therefore, pH 7 will be selected for subsequent hydrolysis experiments to maximize ACEIA.

Effect of E:S ratio on the ACEIA of protein hydrolysate from earthworms

When the E:S ratio was increased by 300 to 600 U/g protein, the ACEIA of the hydrolysate gradually increased. The highest ACEIA (79.39 ± 0.76%) at the E:S ratio of 600 U/g protein (Fig. 1(e)). This can be explained by the increased enzyme quantity, which accelerates the hydrolysis reaction, generating more biologically active peptides with ACE inhibitory activity. However, when the E:S ratio exceeds 600 U/g protein, hydrolysis occurs too rapidly, leading to the breakdown of many biologically active peptides. This may be due to the excess enzyme reducing the specificity of the hydrolysis process, causing the degradation of ACEIA peptides [29]. Therefore, an E:S ratio of 600 U/g protein is optimal for hydrolysis to obtain hydrolysate with ACEIA. Consequently, this E:S ratio will be used in subsequent hydrolysis experiments to generate the best biologically active peptides.

Effect of hydrolysis time on the ACEIA of protein hydrolysate from earthworms

The ACEIA of the protein hydrolysate from earthworm rose until reaching a maximum at a hydrolysis time threshold of 2 h (85.38 ± 2.31%) and declined afterward (Fig. 1(f)). It is possible that during the first hydrolysis stage, this enzyme breaks down the quaternary, tertiary, and secondary structures of intact earthworm proteins for releasing high ACEIA peptides into the hydrolysate [26,30].

However, when the hydrolysis time was extended from 2 to 6 h, ACEIA decreased due to the further degradation of active peptides into smaller fragments, reducing ACEIA. This may be attributed to excessive enzyme activity, which results in a loss of selectivity and a reduction in the production of high-activity peptides [31]. Therefore, 2 h will be used for the following hydrolysis experiments.

ACEIA of Peptide Fractions

The investigation of the ACEIA of the fractions showed that the smaller the fraction, the higher the ACEIA. The fraction with the highest ACEIA was the < 1 kDa fraction (IC_{50} μg/mL = 261.94), followed by the 1–3, 3–10, 10–30, and > 30 kDa fractions (Fig. 2). Peptides are low molecular and efficiently bind to the enzyme's active site, changing its configurations and blocking their substrate-binding sites [8]. Furthermore, ACE has a tiny groove that prevents large polypeptides from accessing the active site [5]. The results of this study are also consistent with many other studies, such as tuna (*Katsuwonus pelamis*) blood [30] and groundnut protein [32]. ACEIA of peptides usually has 2 to 12 amino acids [8] and polar containing some hydrophobic amino acids on the sequence [33].

Besides molecular weight, the ACEIA of peptides depends on the peptide's amino acid composition. Earthworm protein with amino acid composition can produce peptides with high ACEIA [8-9,14], thus, the peptide fractions all have ACEIA with inhibition rates varied to the peptides with different molecular weights. According to previous studies, amino acids (Leu, Ile, and Met) contained in ACEIA peptides [34] are also contained in earthworm proteins [14]. In some cases, the amino acid composition has a more significant influence than the length of the peptide on ACEIA. For example, some net negatively charged amino acids can create stable bonds with Zn^{2+}, such as Glu (the second most abundant amino acid in the amino acid composition of earthworms) [8,14].

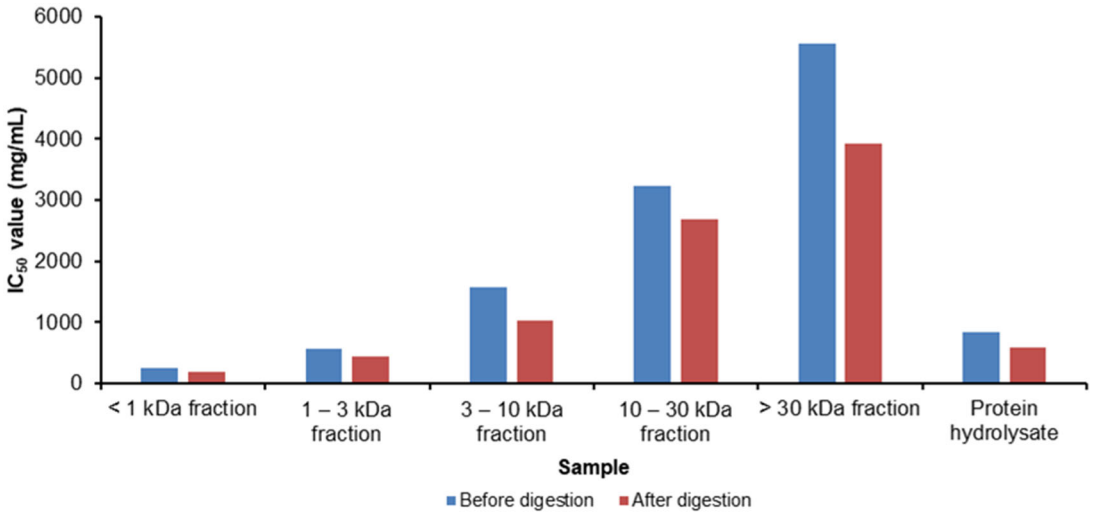

Fig 2. ACEIA is demonstrated with the half inhibitory concentration, which is IC_{50} (μg/mL), of the earthworm protein hydrolysate and their five peptide fractions before and after the simulated digestion

Captopril is a thiol-containing competitive inhibitor of the angiotensin-converting enzyme [35], and it is widely used in the treatment of hypertension as well as as a control in trials investigating ACE inhibitor activity [4-5]. In this study, the highest ACE inhibitory activity was the < 1 kDa fraction (IC_{50} value of 261.94 μg/mL); this activity was lower than Captopril (IC_{50} = 0.004 μg/mL). Consequently, the < 1 kDa peptide fraction from the earthworm protein hydrolysate is possibly a potential antihypertensive contributor.

Stability of ACEIA of the Fraction and the Hydrolysate

The biological activity of the peptide is closely related to the number and sequence of amino acids, especially the amino acids in the chain. Digestive enzymes, pH in the digestive system, and temperature can destroy peptide bonds. Therefore, investigating the activity stability of peptides under *in vitro* digestion conditions, pH, and temperature is an indispensable study when investigating the biological activity of peptides [36]. After treatment (*in vitro* digestion, heat, and pH), the hydrolysate and peptide activity can be stabilized or decreased in activity [5,16].

In vitro *digestion stability of ACEIA of the hydrolysates and their peptide fractions*

After the *in vitro* digestion, the highest increase in ACEIA of the sample was observed at 1.21–1.55 folds (Fig. 2). The results of this study are consistent with the *in vitro* digestibility studies of the hydrolysates and fractions in ingredients such as squid skin collagen [37]. Alemán et al. [37] determined that *in vitro* digestion increased short-chain peptide content and ACEIA. Pepsin has broad cleavage specificity and prefers peptide bonds between aromatic or carboxylic amino acids. For example, amino acids such as Phe or Leu at the C-terminal are essential for the ACE-inhibitory capacity of peptides produced by pepsin cleavage [8]. Pancreatin usually cleaves amino acids, including Phe, Tyr, Ala, Val, Arg, Lys, Trp, and Leu [38]. Among those, Leu, Lys, and Arg are present in the protein composition of earthworms [14], so the activity of earthworm protein hydrolysate and its fractions have increased ACEIA after *in vitro* digestion.

pH stability of ACEIA of the hydrolysates and their peptide fractions

At pH 7 and 8, the hydrolysate and their fractions showed the best stability of their ACEIA (Fig. 3(a)). Protein has a low net electrostatic repulsive energy, making it only stable in the range of neutral pH. This state could minimize the swelling and unfolding of protein molecules, accordingly, maintaining their bioactivity [39]. The extraordinarily high or low values of pH change the charges of peptides, especially with the

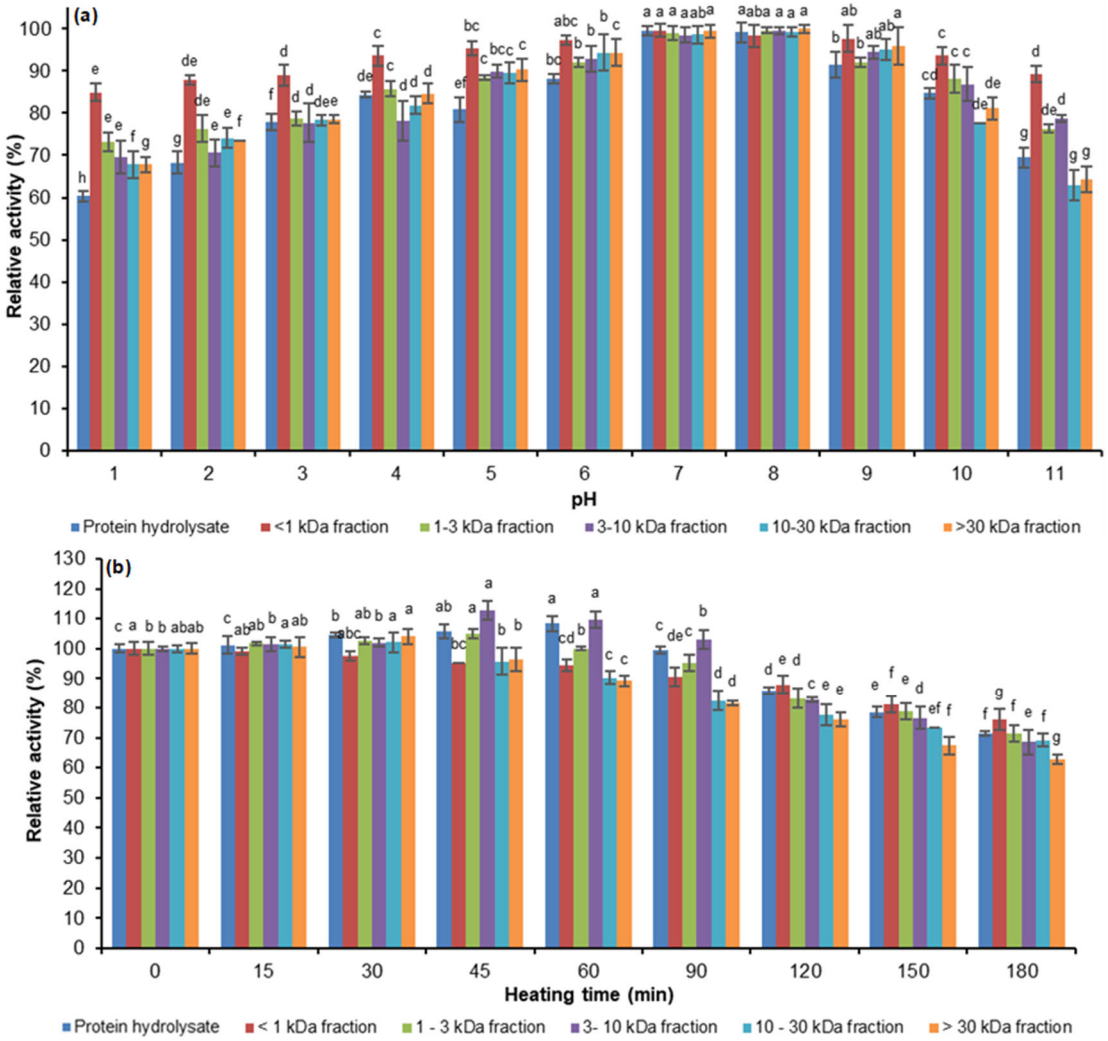

Fig 3. (a) pH stability and (b) thermal stability of ACEIA of the earthworm protein hydrolysate and their five peptide fractions. The bars in similar color with different letters indicate the significant differences ($p < 0.05$)

N- and C-terminus [36], changing the electrostatic interactions and hydrogen bonds and modifying their solubility and secondary structures, leading to the decrease of bioactivity [40-41].

Thermal stability of ACEIA of the hydrolysates and their peptide fractions

During heat treatment, the ACEIA reduction rate of the hydrolysate and fractions increased with heat treatment time (Fig. 3(b)). High temperatures break down non-covalent interactions, such as hydrophobic interactions, electrostatic interactions, and hydrogen bonds, and increase the conformational entropy of protein molecules, causing protein instability and, therefore, loss of biological activity [39].

The presence of amino acids that enhance heat stability, such as Ile, Leu [39,42], so that the ACEIA of the samples remained unchanged in the first heat treatment period the first heat treatment period. However, when the heat treatment time is continuously prolonged, it will reduce the biological activity of the peptide by causing denaturation, aggregation, and degradation of amino acids or breaking the bonds of the peptide and causing protein aggregation [40] or random reformation of intramolecular disulfide bonds [43]. Changes in the structure of amino acids, such as deamination (Asn and Gln) or oxidation (Cys, Met, Trp) at high temperatures, reduce the activity of protein hydrolysis and fractions [39].

The larger the molecular mass of the fraction, the greater the rate of activity reduction (Fig. 3). Low molecules will be more stable than larger molecules when agglomerated at high temperatures [39], and large peptides will quickly form clusters, so they do not bind to the active site of ACE [44]. The earthworm protein hydrolysates and their fractions stayed above 70% of ACEIA even with wide-range pH treatment or being heated at 100 °C for 180 min, which suggests that these samples can be applied to various food or nutraceutical products.

■ CONCLUSION

The study determined that the hydrolysis conditions to obtain earthworm protein hydrolysate with ACEIA were using Flavourzyme® 500 MG, earthworm:phosphate buffer ratio of 1:6 (w/v), hydrolysis temperature of 50 °C, pH 7, E:S ratio of 600 U/g protein, hydrolysis time of 2 h. However, further studies are required, such as isolating the ACE inhibitor peptide from the most active fraction (fraction < 1 kDa). This study determined that the highest ACEIA was in the < 1 kDa fraction and the stability of this fraction after *in vitro* digestion, pH, and heat. Therefore, the antihypertensive activity of earthworms will be further elucidated by measuring the hydrolysate/peptide activity after simulating *in vivo* digestion in living organisms. Despite that, to be possibly applied in future reality use, extended analysis towards their active components, exploration of the characterization and the potential mechanisms of the peptides and their action within a living organism, *in vivo* tests, allergy assays, or clinical trials are necessary.

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■ CONFLICT OF INTEREST

The authors have no conflict of interest to declare regarding this study.

■ AUTHOR CONTRIBUTIONS

Phong Thanh Bui, Linh Le Phuong Tran, and Oanh Kim Nguyen conducted the experiment. Phong Thanh Bui, Truc Anh Hoang, and My Thi Thuy Pham conducted the calculations. Phong Thanh Bui, Oanh Kim Nguyen, and Truc Anh Hoang wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

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