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Research article

Preparation and therapeutic biological activities of hydrolysate mixture produced from frog (*Rana tigrina*) skin using protease

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Inhibitory angiotensin I-converting enzyme (ACE-I) activity

Abstract

Importance of the work: Nowadays, a noncytotoxic hydrolysate containing multiple biological activities is an ideal product for discovering vital bioactive peptides for use in the production of nutraceuticals and pharmaceuticals and for developing alternative treatments for metabolic syndrome.

Objectives: To determine the biological activities and bioactive peptides involved in the hydrolysate mixture (HFS) from frog (*Rana tigrina*) skin.

Materials and Methods: The FHS was investigated for its 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activities, its angiotensin I-converting enzyme (ACE) inhibitory (ACE-I) activity, antimicrobial activities and toxicity to fibroblast (HS 68) cells. Connections of peptides with the ACE in the HFS were determined using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and the docking method.

Results: The HFS confirmed the DPPH and ABTS scavenging activities. Notably, the HFS inherited ACE-I activity at a significant half-maximal inhibitory concentration value of 85.47 mg/mL but did not involve antimicrobial actions or inhibit the growth of HS 68 cells at contents of 0.5 mg/mL or 10 mg/mL. Four peptides (F1, F2, F3, F4) in the HFS were determined for their amino acid sequences using HPLC-MS/MS. The docking results between these peptides with the ACE model improved insights into the structure-activity relationship of the ACE inhibitory peptides. Notably, F1 contained vital residues for forming the most hydrogen bonds, and a Pi-alkyl interaction resulted in effective ACE-I activity based on its binding energy (-10.9 kcal/mol) compared to captopril (-5.4 kcal/mol).

Main finding: All results suggested effective enzymatic hydrolysis had produced HFS containing potential value for its biological activity and non-toxic ability; the novel bioactive peptides found in the HFS could be applied as scaffolds for more intensive research on therapeutic peptides against ACE and contribute to treating hypertension.

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Introduction

Current public health diseases of concern, such as diabetes and hypertension, are increasing and are considered dangerous for human health (Manzoor et al., 2022). Therefore, many health issues have focused on exploring bioactive peptides from distinct food sources, including seeds and meat, to evaluate and collect potential therapeutic peptides (Manzoor et al., 2022; Koirala et al., 2023).

Angiotensin-I-converting enzyme (ACE) is one of the most vital enzymes that control hypertensive blood pressure in humans (Suo et al., 2022; Hu et al., 2023). Thus, a presumed antihypertensive effect is the inhibition of ACE (Official Journal of the European Union, 2015). Compared with pharmacological agents, peptides derived from natural food sources contain high ACE-inhibitory (ACE-I) activity and are preferred for their safety (Feng et al., 2016; Ngo et al., 2016). Researchers have explored the ACE mechanism based on molecular docking methods and experimental validation of ACE (PDB: 1O8A) and *in silico* peptides (Suo et al., 2022; Zheng et al., 2022). Specifically, the molecular docking results of peptides to the ACE structure displayed metal-acceptor binding and conventional hydrogen bonding in the interaction of peptides with ACE (Zhang et al., 2023). The observed findings at the binding site provide essential data for examining the structure-binding ability of ACE-I peptides and designing novel peptides with enhanced inhibitory activity (Zhang et al., 2019).

Frog skin is a natural resource that contains many active peptides with antibacterial, antioxidant and wound-healing activities (Conlon, 2004; Yang et al., 2012; Wang et al., 2016; Yang et al., 2016). Numerous studies have assessed the biological activities of frog skin hydrolysates, harvested based on different hydrolytic reactions. For example, a hydrolysate mixture of frog skin (*H. guentheri)* made using flavourzyme had a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity with a half-maximal inhibitory concentration (IC_{50}) of 9.94 ± 0.13 mg/mL; however, the DPPH radical scavenging activities of hydrolysate mixtures of bullfrog skin treated with two enzymes (bromelain and pancreatin) produced no significant differences compared to the untreated hydrolysate mixture of bullfrog skin, except in the treatment with collagenase that produced a slight increase (7%) in the DPPH scavenging effect (Huang et al., 2012; Gu et al., 2014). Notably, among hydrolysate mixtures of bullfrog skin, only hydrolysate treated with collagenase inherited the ACE-I activity, as opposed to hydrolysate mixtures released using bromelain and pancreatin or untreated hydrolysate mixtures not involving ACE-I activity. Therefore, peptides hydrolyzed from one type of enzyme had multifunctional biological activities that have garnered scientific interest for comprehensive studies to understand their potential actions better and to gather important information for commercialization in the future (Manzoor et al., 2022).

For years, based on the contribution of the abundant peptides in hydrolysates, it has been recognized that peptides with a molecular weight of approximately 1.4 kDa had a higher antioxidant activity than peptides with lower molecular weights (900 Da and 200 Da) (Huang et al., 2012; Wu et al., 2013), which contrasts with the two dipeptides with smaller sizes (259.1607 Da and 293.1446 Da) in the hydrolysates of frog (*Hylarana guentheri*) skin that were also reported to have antioxidant capacity (Gu et al., 2014). On the other hand, one peptide (SSYYPFK) from globulin hydrolysates and another (YVVF) from soybean hydrolysate, with different variations in residue composition, were found to be involved in ACE-I activities (Zheng et al., 2020; Xu et al., 2021). In addition, another study screened the ACE-I activity of 70 peptides in naked oat globulin hydrolysates *in silico*, with one particular peptide (G. SSYYPFK. G) having ACE-I activity, with an IC_{50} value of 91.8 mM (Zheng et al., 2020).However, to date, there has not been any valid assessment of the different variations in the residue composition of hydrolysates to clarify the relationship between the presence of critical residues in the peptides responsible for both their antioxidative function and activity.

Owing to these health-promoting properties, hydrolases have great potential for developing alternative treatments for metabolic syndrome and the production of nutraceuticals and pharmaceuticals (Koirala et al., 2023). In addition, while the antioxidants and antibacterial peptides involved in the hydrolysates of frog skin have been studied (Pan et al., 2012; Zheng et al., 2020; Xu et al., 2021), there has been limited reported results on using hydrolysate mixtures from frog skin, especially containing multiple biological activities as a antioxidant or for their ACE-I activity and cytotoxic effects on normal cells(Liu et al., 2010; Huang et al., 2012; Gu et al., 2014). To overcome these limitations, the current study used sources of frog skins that are discarded and not utilized in food-processing technologies on farms that have cultured frog species (*Rana tigrina*) in Dong Nai Province, Vietnam. The current study aimed to produce and harvest frog skin hydrolysate (HFS) of hydrolyzed frog skins using protease to evaluate the potential biological activities of the HFS.

The HPLC-MS/MS method was applied to determine the peptide number inside the HFS beforehand assessing the interactions between these peptides and the ACE molecule. This study should provide an initial evaluation of the dominant biological activities and non-toxic abilities of HFS. Furthermore, the occurrence of four peptides and their typical amino acid components in the HFS make it ideal for elucidating which vital residues are superior for their bioactive or therapeutic properties.

Materials and Methods

Materials and reagents

The frog skin samples were obtained from commercially raised frogs at a cultured frog (*Rana tigrina*) farm in the Trang Bom district, Dong Nai Province, Vietnam. After harvesting, the samples were immediately transported to the laboratory under cold conditions (4°C). Next, the frog skin samples were washed to remove impurities before being ground and dried at 40-50 ± *2°C until a constant weight was achieved to produce a fine powder*. The protease enzyme (SEBneutral PL) was provided by Hung Thinh Corp. (Ho Chi Minh City, Vietnam). This enzyme is produced through the fermentation of non-genetically modified *Bacillus species*. It is an endoprotease capable of hydrolyzing proteins at internal peptide bonds, releasing polypeptides and peptides of varying lengths. Four standard bacterial strains (*Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* B96.5, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923) were provided by the Institute of Applied Materials Science (Vietnam) to investigate antibacterial activity. The materials consisting of a peptide (hippuryl-histidyl-leucine; HHL), pepsin (10 international units (10 U), from porcine stomach mucosa), chymotrypsin (5 U, from bovine pancreas), trypsin (10 U, from bovine pancreas) and the angiotensin-I-converting enzyme (ACE; 5 U, from rabbit lung) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other reagents used in this study were reagent grade.

Effects of enzyme/frog skin powder ratio, incubation time and pH on frog skin hydrolysis reaction

The effects of the protease-to-frog skin powder ratio (E:S), incubation time and pH on frog skin hydrolysis were determined according to the method of Bui et al. (2021). First, frog skin powder was mixed with protease enzyme in the E:S ratios of 0%, 0.02%, 0.04%, 0.06%, 0.08% or 0.10% in separate 250 mL Erlenmeyer flasks. Each reaction was conducted at room temperature (25°C) for 20–180 min, with different pH levels (5.5, 6.0, 6.5, 7.0, 7.5 or 8.0), followed by heating at 90°C for 15 min and then centrifugation at 13,000 revolutions per minute (rpm) for 15 min to collect the hydrolysate mixture from the frog skin (HFS). The experiments were repeated three times. The resulting HFS samples were evaluated using the ninhydrin method. Briefly, a mixture of 3 mL of hydrolysis solution and 1 mL of ninhydrin solution was prepared and the components were mixed and boiled for 20 min to allow the reaction to occur. Then, the reaction mixture was cooled quickly. Finally, 1 mL of ethanol (40% volume per volume; v/v) was added to the color reaction. The absorbance of each reaction sample was measured using a spectrophotometer (Genesis 10S ultraviolet-visible light (UV-Vis); Thermo Fisher Scientific; Waltham, MA, USA) at 570 nm using glycine as the standard. Next, the hydrolyzed product was freeze-dried and used as a sample for subsequent experiments.

Determination of degree of hydrolysis

The (HFS) content was measured using a ninhydrin colorimetric assay, according to Gao et al. (2020), with slight modifications. Briefly, ninhydrin (1.5 g) was prepared in 10 mL of acetate buffer, followed by the addition of 60 mL of ethylene glycol, 15 mL of n-propanol and 15 mL of n-butyl alcohol. The mixture was prepared by adding 3 mL of the hydrolysate mixture to 1 mL of the ninhydrin solution, followed by boiling for 20 min. After the reaction, the mixture was placed in an ice bath and combined with 1 mL of ethanol solution (40% v/v) to develop the color reaction. The quantity of hydrolysate was determined by measuring absorbance at 570 nm, with glycine used as the standard. The degree of hydrolysis (DH%) was calculated using Equation 1:

$$DH\% = [(A2 - A1) / A2] \times 100\% \quad (1)$$

where A2 is the hydrolysate product concentration (in micro moles) and A1 is the control sample concentration (in micro moles).

The experiments were conducted in triplicate.

Total phenolic and flavonoid contents of frog skin hydrolysate

The total phenolic content (TPC) was determined using the method described by Molole et al. (2022). First, a reaction mixture was prepared in a glass test tube, containing 250 µL of HFS and 1 mL of 10% Folin-Ciocalteu reagent; next, the mixture was homogenized and incubated for 3 min. After pre-incubation, 2 mL of 7.5% (weight per volume) Na_2CO_3 was added to the mixture, mixed thoroughly and subsequently incubated for an additional 30 min. The absorbance of the mixture was measured at 765 nm using the spectrophotometer. The total phenolic content was calculated using a gallic acid standard curve. The experiments were repeated three times. The TPC results were expressed as micrograms of gallic acid equivalents (GAE) per 1 milligram of sample, according to Equation 2:

$$Cph = C1GA \times V / m \quad (2)$$

where Cph is the total phenolic content (in micrograms of GAE per milligram), C1GA is the gallic acid concentration obtained from the standard curve (in micrograms per milliliter), V is the volume of the sample diluent (in milliliters) and m is the mass of HFS (in milligrams).

The total flavonoid content (TFC) was determined using the colorimetric method (Ghasemi et al., 2009). A sample (500 µL) of HFS was mixed with 0.1 mL of 10% aluminum chloride, 1.5 mL methanol, 0.1 mL of 1 M potassium acetate and 2.8 mL distilled water. The solution was incubated at room temperature for 30 min. After incubation, the absorbance of the reaction mixture was measured at 415 nm wavelength. The experiments were repeated three times. The TFC result was expressed as micrograms of quercetin equivalents (QE) per 1 mg sample, according to Equation 3:

$$Cfl = C1QE \times V / m \quad (3)$$

where Cfl is the flavonoid content, C1QE is the quercetin concentration obtained from the standard curve (in micrograms per milliliter), V is the volume of the sample diluent (in milliliters) and m is the mass of HFS (in milligrams).

Production of spray-dried hydrolysate powder from frog skin hydrolysate

The spray-drying method produced spray-dried hydrolysate powder (SHP) from the HFS hydrolysate solution by thoroughly mixing HFS and maltodextrin, before placing the mixture in a spray dryer (Labplant; Model SD-Basic; Labplant; Model SD-Basic; North Yorkshire, United Kingdom) at an inlet air temperature of 160°C, an outlet air temperature of 80°C and pumping speed of 6 mL/min. The SHP in a smooth white state was created after 4 min and had a moisture content of 3.42%. The SHP samples were stored in airtight bags until the start of the experiment. Then, an SHP sample was dissolved in double-distilled water at a ratio equal to the HFS content in the following tests to evaluate the biological activities of SHP.

Analysis of in vitro 2,2-diphenyl-2-picrylhydrazyl scavenging activity of frog skin hydrolysate and spray-dried hydrolysate powder (Qian et al., 2020)

The DPPH free radical-scavenging activity was determined using a previously reported method (Qian et al., 2020). A 0.076 mM DPPH solution was prepared by first dissolving 0.003 g DPPH in 100 mL of methanol and then homogenizing the mixture until the DPPH mixture was completely dissolved. The reaction mixture consisted of 1.0 mL of HFS or SHP at various concentrations in distilled water (20, 40, 60, 80 or 100 mg/mL) with 0.076 mM (4.0 mL) dissolved in methanol. After shaking, the mixture was incubated in the dark at 25°C for 30 min. The absorbance of the reaction mixture was measured at 517 nm using the UV-Vis spectrophotometer. The percentage of radical DPPH scavenging activity (RSA) was calculated using Equation 4:

$$RSA = [(A1 - A2) / A1] \times 100 \quad (4)$$

where A1 is the absorbance of the control sample (DPPH solution without HFS), and A2 is the absorbance of the test sample.

Standard curves were used to determine the free radical scavenging activity at different ascorbic acid concentrations used to replace HFS or SHP. The experiments were repeated three times. IC_{50} was defined as the concentration that scavenged 50% of the free radicals.

Analysis of in vitro 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) of frog skin hydrolysate and spray-dried hydrolysate powder (Centenaro et al., 2011)

The 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical-scavenging activity was determined based on Centenaro et al. (2011). First, a free radical solution ($ABTS^{\bullet+}$) was prepared by mixing solutions A and B in a 1:1 ratio (solution A contained 7 mM ABTS and solution B

contained 2.45 mM sodium persulfate) in 250 mL. The mixture was homogenized and incubated in the dark for 16 h at 25°C. After that, the ABTS stock mixture was diluted with distilled water twice until the color absorbance reached a value of 0.70 ± 0.02 at a wavelength of 734 nm. The reaction mixture consisted of 100 µL of HFS or SHP at various concentrations in distilled water (20, 40, 60, 80 or 100 mg/mL), combined with 3 mL $ABTS^{\bullet+}$ solution and was incubated in the dark for 6 min. The absorbance was measured at 734 nm. The percentage of radical ABTS scavenging activity (RSA) was calculated using Equation 5:

$$RSA = [(A3 - A4) / A3] \times 100 \quad (5)$$

where A3 is the absorbance of the control substance ($ABTS^{\bullet+}$ solution without HFS) and A4 is the absorbance of the test sample.

Standard curves were used to represent the free radical scavenging activity at the different ascorbic acid concentrations used to replace HFS or SHP. The experiments were repeated three times.

Angiotensin-I-converting enzyme inhibitory activity of frog skin hydrolysate

The ACE-I activity of HFS was determined according to Wang et al. (2008) and Zheng et al. (2020), with slight modifications. First, dissolve 1.8 mg of HHL was mixed with 500 µL sodium borate buffer (50 mM; pH 8.3) to prepare the reaction substrate (HHL) solution. Next, 50 µL of each HFS at five different concentrations (50, 100, 150, 200 or 250 µg/mL) were added in separate 150 µL HHL solutions and placed in a 1.5 mL Eppendorf tube. Then, the mixture in the tube was homogenized before incubation for 5 min at 37°C. After the mixture had been preincubated, 50 µL of ACE solution (25 mU/mL) was added, homogenized and incubated for 60 min at 37°C. After incubation, the reaction was stopped by adding 250 µl of 1.0 M HCl before continuously adding 1.5 mL of ethyl acetate. The supernatant was collected using centrifugation at 3,000 rpm for 10 min at 25°C. Next, 1.0 mL of the supernatant was added to a new Eppendorf tube and evaporated entirely for 3 hr at room temperature. Finally, the release of hippuric acid by ACE was determined using photometric measurements at a wavelength of 228 nm. The percentage of ACE-I activity was calculated using Equation 6:

$$\text{ACE-I activity} = [(A6 - A7) / (A6 - A5)] \times 100 \quad (6)$$

where A6 is the absorbance of the mixture without HFS, A7 is the absorbance of the reaction mixture and A5 is the absorbance of the buffer solution.

A standard curve was constructed using a series of captopril concentrations to replace HFS for ACE inhibition. The experiments were performed in triplicates.

Antibacterial activity

The antibacterial activity was evaluated using the agar disk diffusion method (Zhang et al., 2015), with slight modifications. First, each test tube containing 5 mL of Luria-Bertani (LB) broth was supplemented with 50 µL of one of the experimental bacteria. Then, the solution was incubated at 37°C for 180–240 min to activate the bacterial growth until the optical density at 600 nm (OD_{600nm}) value reached approximately 0.08–0.1 (according to the 0.5 McFarland standard). After activating the bacteria in LB at 37°C, a sterilized cotton swab was dipped in the bacterial solution and streaked onto the prepared Mueller Hinton agar (MHA) surface. The lid of the agar plate was gently opened for approximately 3–5 min in an incubator to dry the surface and agar wells (diameter of 6 mm) were punched. In addition, HFS was passed through a 0.45 µm sterile filter membrane. After preparing the agar plate containing the bacterial solution, 100 µL of filtered HFS was pipetted at different concentrations (80, 90 or 100 mg/mL) into the separate wells on the agar plate. The plates were incubated at 5–10°C for 120 min, then increased to 37°C for 24 hr. Based on the growth of bacteria on the contact area between the agar and HFS sample, the diameter of the inhibition zone was determined using a technical ruler. Gentamycin (100 mg/mL) was used as a positive control.

Cytotoxicity of frog skin hydrolysate to fibroblast (HS 68) cell line

The fibroblast (HS 68) cell line was cultured in 5% CO_2 at 37°C in complete growth Dulbecco's modified Eagle's medium (DMEM; Gibco; Waltham, MA, USA) containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Gibco, Waltham, MA, USA). Fibroblasts (HS 68) (5×10^3/well) were seeded into 96-well plates and incubated in 5% CO2 at 37°C. After 24 hr, the cells in each well were treated with 100 μL fresh medium containing either 0.5 mg/mL HFS or 10 mg/mL HFS. After a further 48 hr, cell viability was measured using a (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) assay.

Absorbance was recorded using an Agilent BioTek Cytation 5 Cell Imaging Multimode Reader (Santa Clara, CA, USA) at a wavelength of 450 nm. All assays were performed in triplicate. The negative control wells received double-distilled water, with Vinblastine (VBN) 0.25 µM, serving as the positive control wells.

Purification and determination of amino acid sequence using high-performance liquid chromatography-tandem mass spectrometry

The amino acid sequences of the HFS were analyzed using the HPLC-MS/MS system at a flow rate of 0.3 mL/min and the indicated gradient. The mobile phase consisted of two solvents (A and B), where A was 0.1% formic acid v/v (FA; Fisher Scientific; city, state abbreviation, USA) in miliQ-H2O (Merck; Merck; Darmstadt, Germany). In contrast, solvent B was acetonitrile (ACN; Fisher Scientific; Fisher Scientific; Hampton, USA), containing 0.1% FA (v/v). The elution gradient was performed by varying the solvent B ratio from 10% to 90% over 37 min. An XBridge C18 3.5 µm 2.1 × 150 mm column on a Triple TOF 6600+ mass spectrometer (AB SCIEX; SCIEX; Marlborough, MA, USA) used with the Analyst TF 1.8 software (SCIEX; Marlborough, MA, USA). The search engine Protein Pilot (SCIEX; Marlborough, MA, USA) was used to analyze the MS/MS spectra.

Molecular docking

The crystal structure of the ACE enzyme (PDB: 1O8A, resolution 2.0 Å) was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The molecular structure of captopril was downloaded from the PubChem database (http://pubchem.ncbi.nlm.nih.gov) and the structure of the simulated peptide sequences was built using the PyMOL 2.5.5 software (Schrödinger, NY, USA). Molecular docking was conducted using the AutoDock Vina software (The Scripps Research Institute, La Jolla, CA, USA) to evaluate the binding free energy and several physical interactions. The docking results were analyzed using the PyMOL 2.5.5 software. The suitability of docking between the peptides and the active site of ACE was evaluated based on binding energy. In addition, hydrogen bonds and distances were calculated.

Data processing methods

Raw data were pre-processed using Microsoft Excel 365 software (Redmond, WA, USA). The experiments were performed in triplicate, and the results were presented as mean ± SD. Single- and two-factor analyses of variance were performed, with the graphs and standard curves constructed using the Stat Graphics Centurion XVI (Statgraphics Technologies, The Plains, VA, USA) and Prism 8.3 (GraphPad Software, Boston, MA, USA. In the cytotoxicity assay a Duncan's test was used to evaluate the differences in values with significance tested at $p < 0.05$.

Result

Effects of protease enzyme-to-skin powder ratio, pH and reaction time on hydrolysis reaction

The high product contents present in hydrolysate is an index used to determine the best parameter in each hydrolysis reaction condition at the same reaction temperature of 25°C. Therefore, the constituted product content on the hydrolysis reactions was assessed using the different influences on the hydrolysis degree (DH) of six E:S ratios (0%, 0.02%, 0.04%, 0.06%, 0.08% or 0.1%), pH levels (5.5, 6.0, 6.5, 7.0, 7.5 or 8.0) and reaction times (0 min, 20 min, 40 min, 60 min, 80 min, 100 min, 120 min, 140 min, 160 min and 180 min), as shown in Fig. 1. In detail, Fig.1A shows that the amount of hydrolysis product in the hydrolysate fluctuated independently

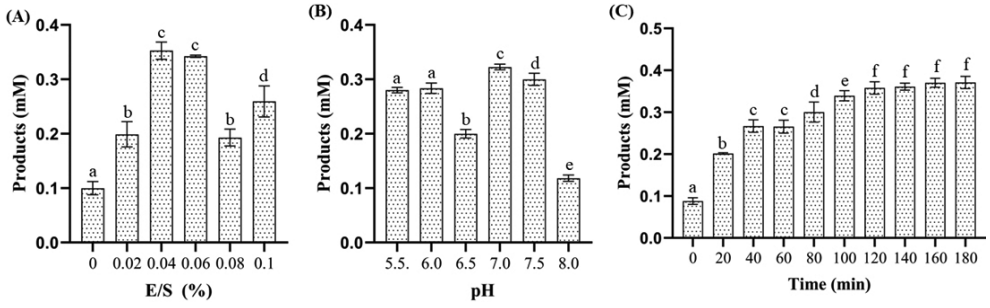

Fig. 1 Effects on hydrolysis products of: (A) protease-to-frog skin powder ratio (E:S); (B) pH level; (C) reaction time, where columns with different lowercase letters are significantly ($p < 0.05$) different and error bars indicate ± SD

or non linearly for all six E:S ratios for a reaction time of 180 min, with the highest levels of hydrolysis product (0.352 mM and 0.352 mM) occurred at ratios of 0.04% and 0.06%, respectively ($p > 0.05$), against the lowest level observed at ratios of 0.2% and 0.08% ($p < 0.05$). Similar to the effects of the E:S ratio, the hydrolysis product contents at pH values of 5.5–8.0 were significantly different (Fig. 1B). The lowest amount of hydrolysis product was at pH 8.0, while the highest amount was observed at pH 7.0. Next, Fig. 1C shows a gradual increase in the hydrolysis product from 0 min to 120 min, until it approached saturation amounts sequentially. The product content increased proportionally with an increase in reaction time from 20 min to 120 min, reaching the highest level of 0.358 mM ($p < 0.05$), and then there was no significant change for three sequential times (140 min, 160 min and 180 min). Based on these results, hydrolysis conditions of an E:S ratio of 0.04%, pH of 7.0 and reaction time of 120 min were the best for the hydrolysis of frog skin with protease enzyme to obtain the highest HFS content of 0.358 mM. Finally, the calculated DH% value was 54.70% for these optimal hydrolysis conditions. This DH% result indicated a much higher level of hydrolysis efficiency than that for the hydrolysate of frog skin (*Quasipaa spinosa*) using a two-step hydrolysis method (papain combined with protease), which only achieved a hydrolysis efficiency of 30% (Wu et al., 2024).

Total phenolic and total flavonoid contents of frog skin hydrolysate

Vishwakarma et al. (2017) reported that phenolic compounds were essential for antioxidant activity. The TPC estimated in the current study was 15.99 ± 0.2 µg GAE/mg in HFS (Fig. 1SA). The total phenolic content in the HFS was much higher than the contents in other products, such as reported by Asha et al. (2016) in hydrolysates from oysters (*C. madrassas*) with papain and pepsin enzymes (0.19 *µg GAE/mg* and 0.2 µg GAE/mg, respectively). In contrast, the TFC (Fig. 1SB) in the HFS of 1.84 µg QE/mg was approximately 10 times lower than the TPC value of 15.9 µg GAE/mg. These results were in good agreement with Asha et al. (2016) that determined the role of phenolic groups in aromatic amino acids that were responsible for their antioxidant activity.

Antioxidant activities of frog skin hydrolysate and spray-dried hydrolysate powder

The results of the DPPH radical scavenging activities of HFS and spray-dried hydrolyzed powder (SHP) at different concentrations are displayed in Fig. 2, demonstrating a linear relationship between the DPPH radical scavenging ability and HFS concentration. Specifically, HFS produced the highest DPPH radical scavenging activity (78.84 ± 2.09%) at a concentration of 100 mg/mL (Fig. 2A), while the activity in the SHP was 74.17 ± 1.25% at a concentration of 250 mg/mL (Fig. 2B). These results were similar to those of hydrolyzed products from the skin of another frog (*Rana catesbeiana*) having a DPPH radical antioxidant ability of approximately 80% (Huang et al., 2012; Gu et al., 2014). The DPPH free radical-scavenging activity is shown by the IC_{50} values in Figure 2C, where the lower the IC_{50} value, the stronger the ability to scavenge free radicals. Based on these results, the HFS had an IC_{50} value of 69.15 ± 2.69 mg/mL, while the SHP had an IC_{50} value of 128.7±5.57 mg/mL, indicating that the SHP had a significantly lower ability to scavenge DPPH-free radicals than the HFS.

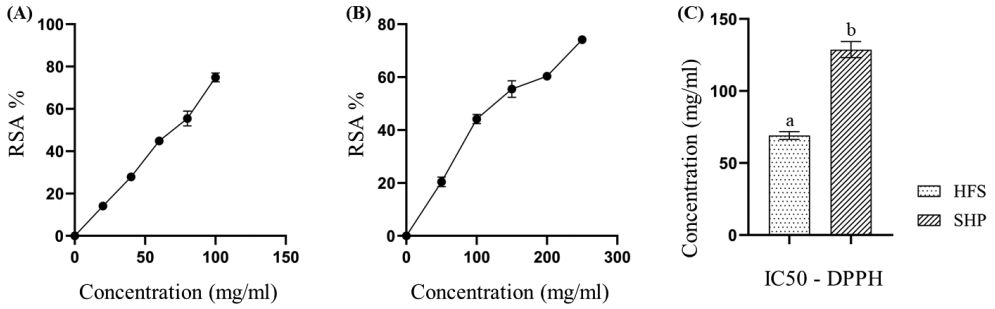

Fig. 2 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (RSA) of: (A) hydrolysate mixture (HFS) at different concentrations; (B) spray-dried hydrolysate powder (SHP) at different concentrations; (C) half-maximal inhibitory concentrations of HFS and SHP to DPPH scavenging activity ((IC50-DPPH)). Values are mean ± SD of three replicates and columns with different lowercase letters are significantly ($p < 0.05$) different.

Based on the results of the ABTS scavenging activities of HFS (Fig. 3), the ABTS free radical scavenging activity grew following the gradual increase in concentration of HFS. Specifically, the HFS at a concentration of 100 mg/mL had the highest activity (70.88 ± 5.00%), while the SHP had the greatest activity (79.66 ± 1.41%) at 250 m/mL (Fig. 3A and 3B, respectively). Similar to the DPPH test, the HFS had an IC_{50} value of 57.91 ± 8.27 mg/mL—less than half of that with SHP (117.6 ± 13.69 mg/mL), as shown in Fig. 3C. The radical scavenging activity levels of DPPH and ABTS in this study showed the spray-dried powder products (SHP) in the powder state significantly reduced antioxidant activity compared to those in the original hydrolysate mixture (HFS). This difference could be explained by the SHP containing maltodextrin, which caused the lower scavenging of free radicals of the SHP than of the HFS for the same concentration range. Therefore, HFS was chosen to investigate the sequential assessment.

Angiotensin I-converting enzyme inhibitory activity of frog skin hydrolysate

The ACE-I activities were compared of five different concentrations of HFS with a series of concentrations of captopril, which acted as an ACE inhibitor (Fig. 4A). All the HFS concentrations showed significant ACE-I activity with increasing concentrations of HFS. The highest ACE-I activity was 85.47 ± 1.42% at a concentration of 100 mg/mL compared to the ACE-I activity of the hydrolysate of oysters (*Crassostrea gigas*) of 75.92% (Zhang et al., 2015). Regarding the ACE-I activity, the IC_{50} value of HFS was approximately 45 mg/mL (Fig. 4B). Thus, HFS was selected for the effective digestion of frog skin by protease, significantly proving its ACE-I activity.

Antibacterial activity of frog skin hydrolysate

The antibacterial activity of the HFS was identified based on the inhibition zone of bacterial growth on MHA media of cultured food-related bacteria (*E. coli* ATCC 25922, *S. aureus* ATCC 25923, *B. subtilis* ATCC 6051, and *P. aeruginosa* B96.5). All the tests supplemented with one of the five different HFS concentrations and the negative tests showed explicit inhibitory rings, as opposed to the presence of clear white rings in the positive tests (Fig. 5).

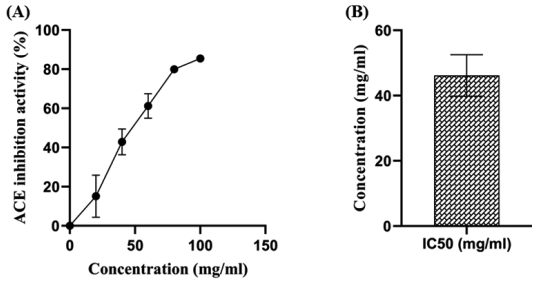

Fig. 4 Effect of hydrolysate mixture concentration on angiotensin I-converting enzyme (ACE-I) inhibitory activity. Values are mean ± SD of three replicates.

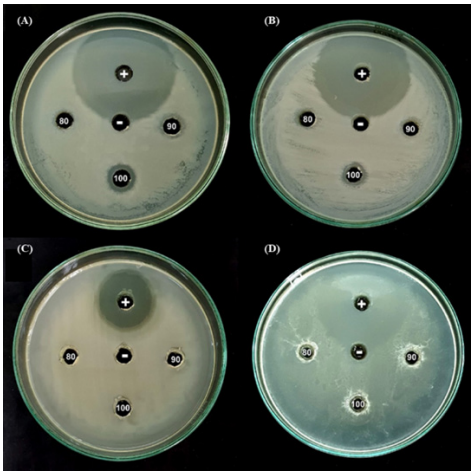

Fig. 5 Evaluation of antibacterial activity of frog skin hydrolysate against four bacterial strains: (A) *Bacillus subtilis*; (B) *Pseudomonas aeruginosa*; (C) *Escherichia. coli;* (D) *Staphylococcus aureus.* '+' = gentamycin antibiotic (positive control and '-' = distilled water (negative control).

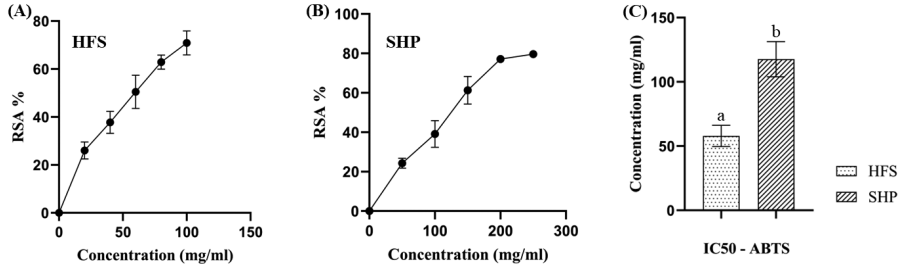

Fig. 3 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging activity RSA) of: (A) hydrolysate mixture (HFS) at different concentrations; (B) spray-dried hydrolysate powder (SHP) at different concentrations; (C) half-maximal inhibitory concentrations of HFS and SHP to $ABTS^{\cdot+}$ scavenging activity (IC50-ABTS). Values are mean ± SD of three replicates and columns with different lowercase letters are significantly ($p < 0.05$) different.

Based on these results, none of the HFS concentrations interfered with the growth of the four treated bacteria. The antibacterial characteristics of HFS in this study were similar to the results of research on hydrolyzed products from green mussels (*Perna canaliculus*) in New Zealand, where there was also no antibacterial activity against *E. coli* (ATCC 25922), *Bacillus cereus* (ATCC 10702), *S. aureus* (ATCC 6538) or *Candida albicans* (Jayaprakash and Perera, 2020). However, another hydrolyzed product from oysters (*C. gigas*) from another study produced effective antibacterial activity levels to *Vibrion parahaemolyticus* and *Vibrio harveyi* based on antibacterial diameters of 13.44 and 10.76 mm, respectively, while not causing any inhibition or weakness against the bacterial strains *E. coli* and *S. aureus* (Zhang et al., 2015). These comparative results indicated that the HFS was safe and did not inhibited any of the four treated bacterial strains.

Cytotoxicity assay of frog skin hydrolysate

The cytotoxic activity of HFS on HS68 cells was evaluated using an MTT assay after 48 hr of treatment. As shown in Fig. 6, HFS significantly affected the viability of the HS68 cells. Both concentrations of HFS (0.5 mg/mL and 10 mg/mL) significantly increased cell viability compared to the negative control and the vinblastine treatment ($p < 0.05$). The increased viability in the presence of HFS suggests a potential protective or proliferative effect, which may be attributed to the antioxidant properties of the compounds within HFS, which can reduce oxidative stress and prevent cell damage (Shahidi and Ambigaipalan, 2015). The data also suggest that, while HFS does not exert cytotoxic effects on normal cells, it could be of interest for further research into its potential applications.

Purification results and amino acid sequence of hydrolysate mixture based on high-performance liquid chromatography-tandem mass spectrometry

With the currently available knowledge regarding peptide specificity, extensive investigation of biological activity and sequencing is essential for screening and harvesting both naturally active peptides and HFS based on enzymatic hydrolysis. The current study identified four peptides inside the HFS (Table 1 and Fig. 2S). Among the four peptides, only the peptide with the sequence (TERGYSF) and coded as F1 had a small molecular weight (858.3872 Da) and a hydrophobic amino acid content of approximately 28.57% in the HFS, which contrasted with three remaining peptides with higher molecular masses of 1258.73–2164.11 Da. Two peptides (F3 and F4) had approximately equal molecular masses; however, there were notable differences in the composition of their hydrophobic amino acids. The molecular mass of peptide F2 and the hydrophobic amino acid content were the highest. Analyses of the peptide sequences and sizes of the four peptides in the HFS revealed a close correlation to the characteristics of bioactive peptides that are frequently present in the hydrolysates produced from frog skin (Huang et al., 2012; Gu et al., 2014).

Molecular docking

The molecular docking method was used to simulate the interaction between peptides and ACE molecules and to evaluate the interaction energy between these molecules (Huang et al., 2012). The binding energies and bonds occurring between the peptides or captopril and ACE in Table 2 showed that the best pose for binding the F1 peptide was at the active site of ACE, with a binding energy of –10.9 kcal/mol compared to the higher binding energies of the three other peptides (F2, F3 and F4).

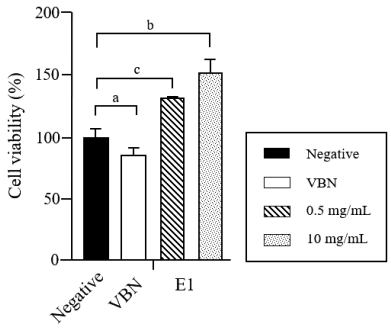

Fig. 6 Effects of frog skin hydrolysate (HFS) on cell viability in fibroblast (HS68) cells using MTT assay after treatment, where treatments included vinblastine (VBN, a known cytotoxic agent) and HFS at two concentrations of 0.5 mg/mL and 10 mg/mL, where columns with different lowercase letters are significantly ($p < 0.05$) different and error bars indicate ± SD.

Table 1 Peptide sequences in frog skin hydrolysate determined using high-performance liquid chromatography-tandem mass spectrometry

Symbol	Peptide sequence	Molecular mass (Da)	Hydrophobic residue content (%)
F1	TERGYSF	858.3872	28.57
F2	GQKDSYVGDEAQSKRGILTL	2164.1123	30.00
F3	GFAGDDAPRAVFPS	1405.6626	42.86
F4	RVAPEEHPVLL	1258.7034	45.45

Table 2 Binding energies and bonds occurring between peptides or captopril and angiotensin I-converting enzyme

Peptide sequence	Binding energy (kcal/mol)	Interaction between amino acids and hydrogen and distance (Å)
TERGYSF	–10.9	Asn66 (2.35), Ser516 (2.52), Asp358 (2.24), Tyr62 (2.31), Arg124 (2.55), Tyr51 (2.64), Lys118 (2.99), Glu123 (3.08)
GQKDSYVGDEAQSKRGILTL	–8.3	None
GFAGDDAPRAVFPS	–10.0	Tyr360 (2.82), Ser355 (2.85), Asn66 (2.06), Trp220 (2.76), Arg124 (1.73), Glu123 (1.93)
RVAPEEHPVLL	–8.9	Gly404 (2.66), Phe570 (3.57), Glu403 (1.93), Arg124 (2.20), Asn70 (2.86), Trp357 (2.70), Asp358 (1.94), Ala356 (3.74)
Captopril	–5.4	Gln281 (2.35), Glu376 (2.91), Lys511 (2.60), His353 (3.63)

In addition, based on the two-dimensional and three-dimensional images, examination of the assembled structure revealed that van der Waals forces and hydrogen bonding were the main binding forces in the interaction between F1 and ACE (Fig. 7 and Fig. 3S). The peptide F1 interacted with the ACE active site to form eight hydrogen bonds with amino acids N66, S516, D358, Y62, R124, Y51, K118 and E123 (Fig. 7A). Pan et al. (2012) reported that the formation of multiple hydrogen bonds could increase ACE inhibition; thus, peptide F1 should have a strong interaction force with ACE. Only the F2 peptide showed no connection between residue sequences and ACE. For the F3 and F4 peptides, the main interactions observed between both peptides and ACE were van der Waals forces and hydrogen bonds (Fig. 7B and Fig. 7C, respectively). Specifically, the peptide F3 comprised six hydrogen bonds with ACE: Tyr360, Ser355, N66, W220, R124 and E123, while the peptide F4 comprised G404, F570, E403, R124, N70, W357, D358 and A356 as the eight hydrogen bonds formed with ACE. The binding energies peptides F3 and F4 were –10.0 and –8.9 kcal/mol, respectively, providing clear evidence for their binding ability with ACE in the two complex structures (Table 2). The synthesis inhibitor captopril had a binding energy of –5.4 kcal/mol by forming three hydrogen bonds (Fig. 3S-D). These results indicated that three peptides (F1, F2 and F4) could bind to ACE to form complexes with a more stable combination than captopril inhibitors (Fig. 7 and Fig. 3S).

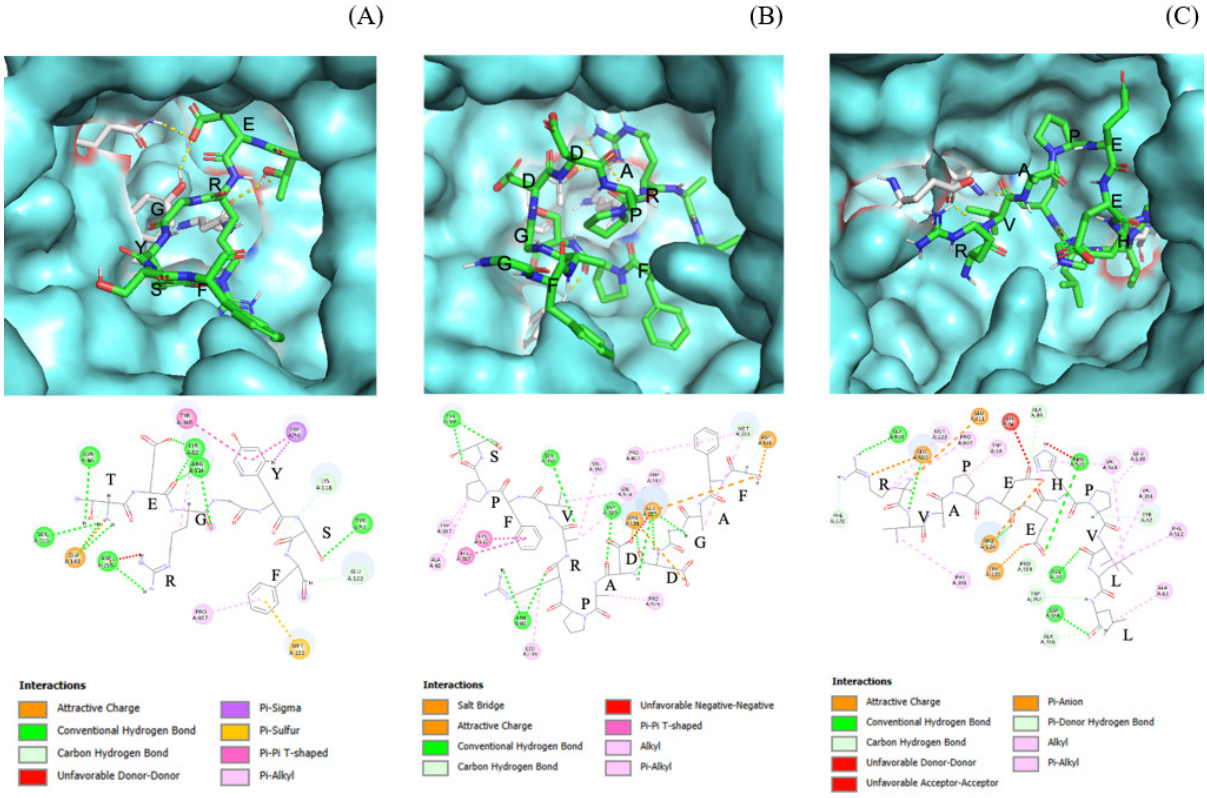

Fig. 7 3D (Up) and 2D (Down) images simulating molecular docking results of four peptides (F1, F2, F3 and F4) with angiotensin I-converting enzyme. (A) Interaction of F1 (TERGYSF); (B) Interaction of F3 (GFAGDDAPRAVFPS); (C) Interaction of F4 (RVAPEEHPVLL)

Discussion

Potential bioactivities and discovering novel peptides in frog skin hydrolysate produced using single hydrolysis process

Novel peptides that can assume bioactive properties from hydrolysate containing multiple biological activities and noncytotoxic have been considered ideal products for applications in the production of nutraceuticals and pharmaceuticals due to their health-promoting properties (Koirala et al., 2023). Comparative analyses of the FHS in the current study were undertaken with one hydrolysate from frog skin (*Hylarana guentheri*) produced by flavourzyme (FPH) (Gu et al., 2014) and four hydrolysates from bullfrog skin (*Rana catesbeiana*) consisting of three produced by bromelain (RSGH-Bm), pancreatin (RSGH-Pc) and collagenase (RSGH-Cg) and one hydrolysate without enzyme hydrolysis (RSGH). Based on the comparative results, the DPPH free radical scavenging of the HFS was lower than those of the five other hydrolysates, but with greater inherited biological activity than the other five (Huang et al., 2012). Compared to the hydrolysate from frog skin (*Hylarana guentheri*) produced by flavourzyme (FPH), although FHP had better DPPH activity than the FHS (based on the former's IC_{50} value of 9.94 ± 0.13 mg/mL), the ACE-I activity of FPH has not yet been examined. Notably, only two peptides (Leu/Ile-Lys and Phe-Lys) have been determined in all three fractions of FPH (Gu et al., 2014), while four longer peptides were found in the FHS. Further comparisons with the four hydrolysates from the bullfrog skin indicated that the DPPH scavenging activity levels of the three enzyme-treated hydrolysates and the one without enzyme-treated hydrolysate all had similar IC_{50} values (3.44–3.75mg/mL). Notably, among the hydrolysates, only RSGH-Cg had ACE-I activity, with an IC_{50} value of 0.024 mg/mL, while there have been no published details regarding the peptide composition of any the four hydrolysates of the bullfrog skin (Huang et al., 2012). In summary, the FHS produced in the current study using one optimal hydrolysis process with protease not only determined biological activity but also identified the putative peptides responsible for this bioactivity (Huang et al., 2012; Sheng et al., 2023; Wu et al., 2024).

Notable outstanding roles of hydrophobic amino acids involved in four peptides in frog skin hydrolysate

Currently, the outstanding roles of hydrophobic amino acid (methionine, histidine, phenylalanine, leucine, isoleucine, lysine and glycine) residues are known to be mainly responsible for increasing their antioxidant activities (Saito et al., 2003; Dong et al., 2008; Memarpoor-Yazdi et al., 2013; Tanaka et al., 2016; Zheng et al., 2020). In addition, two dipeptides (leucine-isoleucine-lysine or phenylalanine-lysine residues) can interact to prevent the formation of free radicals (Wu et al., 2003; Gu et al., 2014), while both glycine and serine residues can reduce the unpaired electrons or free radicals by donating their protons (Bischoff and Schlüter, 2012; Qian et al., 2020; Anh et al., 2023). In particular, among these residues, phenylalanine with the presence of an hydroxyl group on the aromatic rings has antioxidant and anticancer activities through hydroxyl substitutions on the aromatic rings and double bonds in its phenol ring (Koşar, 2017). Based on the above literature, the presence of phenylalanine, leucine, serine and glycine residues at the end of all four peptides (Table 1), combined with the high total phenolic content in the HFS (Fig 1.SA), provided scientific evidence regarding the antioxidant capacities of all four of the peptides found in the HFS.

The binding capacities of hydrophobic amino acids at the active site of the ACE with peptide residues are essential for understanding the ACE-I activity of peptides (Pan et al., 2012; Zhang et al., 2019). In addition, the existence of hydrogen bonds with short hydrogen-bond distances was evidence of good connection and structural stability of the ligands to their protein molecules (Rudolph et al., 2017). Therefore, F1, having the most hydrogen bonds and a Pi–alkyl interaction, along with residues at the active site of ACE, can effectively inhibit ACE activity compared with the remaining peptides. The F3 and F4 peptides, with highly hydrophobic amino acid composition (42.86% and 45.45%, respectively), along with the participation of Lys or Ser at one peptide end in both F3 and F4, suggests that these too can be proposed as vital residues to readily bind to the active sites of ACE via 6–8 hydrogen bonds (Pan et al., 2012; Zheng et al., 2020; Xu et al., 2021). These combined results determined that critical residues in the four peptides of FHS play meaningful roles in their antioxidant and ACE-I activities.

Biosafety of frog skin hydrolysate

Nowadays, there is a greater emphasis on the safety requirements regarding synthetic antioxidants; thus, natural antioxidants obtained from edible materials and by-products have been of increasing interest in recent years (Shahidi and Ambigaipalan, 2015). While reference hydrolysates have not yet been assessed for their resistance to bacterial strains

or their impact on HS68 cells, the HFS in the current study had no effect on the direct microbe-killing effects in all four HFS-treated bacterial strains and the non-cytotoxic activities of HFS in the generation of HS68 cells (Fig. 6) at both studied concentrations (0.5 mg/mL or 10 mg/mL). Based on these outcomes, HFS and the novel bioactive peptides found in HFS may be a valid potential non-toxic material for use in scaffolds for in-depth research into therapeutic peptides against ACE, as well as contributing to the treatment of hypertension and other pathologies.

Conclusion

HFS containing biological activities and non-toxic abilities was produced using effective enzymatic hydrolysis upon the defined DH value of 54.70%. At the same time, the HPLC-MS-MS and molecular docking results suggested that the critical residues of the four peptides identified in the HFS (such as Val, Leu, Iso, Gly, Ser, Lys, and Phe) responded to free radical scavenging activities and ACE-I activity. The HFS and its peptide sequences could provide valuable scaffolds for the in-depth screening, evaluation and development of therapeutic peptide products against ACE that could contribute to treating hypertension and other pathologies. Finally, the findings of the current study are consistent with the initial potential bioactivities assessment of novel peptides. In the future, supplementary detailed evaluation of the distinct activities of the isolated peptides should be evaluated based on clinical trials. Furthermore, different doses and durations of each peptide in the supplementation study would be essential to measure multiple biological activities. Hence, ongoing research will be crucial in providing valuable evidence to help understand the properties of each peptide before designing novel peptides with enhanced biological activities.

Conflict of Interest

The authors declare that they have no conflicts or competing interests concerning this article.

Acknowledgements

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Author contributions

Huyen Thi Tran conceived, designed, and analyzed the data. Anh Thi Hoang Phan, Son Hoang Pham, and Nam Minh Nguyen performed the experiments and prepared figures and tables. Tuan Huu Ngoc Nguyen performed the bibliographic research. Huyen Thi Tran provided the scope, and guidance, and critically reviewed and approved the final draft. All authors provided final approval for publication and agreed to be held accountable for the work performed.

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