Research article

Characterization and molecular docking of tetrapeptides with cellular antioxidant and ACE inhibitory properties from cricket (*Acheta domesticus*) protein hydrolysate

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ARTICLE INFO

ABSTRACT

Wide-ranging bioactivities of enzymatically digested insect protein to produce peptides have been targeted for functional food development. In this study, fractionated peptides obtained from cricket (*Acheta domesticus*) protein hydrolysate by alcalase digestion were identified and evaluated for their bioactivities. Peptide fractions F44, F45, and F46, isolated through size exclusion chromatography, demonstrated strong cytoprotective effects on SH-SY5Y and HepG2 cells exposed to H_2O_2. This was evidenced by a 2-fold decrease in reactive oxygen species (ROS) accumulation in the cells and a 3-fold upregulation of genes encoding antioxidant enzymes. The F45 peptide fractions also showed chemical antioxidant activities ranging from approximately 290 to 393 mg trolox/g peptide, measured by DPPH, ABTS, and FRAP assays. Furthermore, F45 demonstrated the highest angiotensin-converting enzyme I (ACE) inhibitory activity, 57.93 %. F45 induced higher levels of *Nrf2, SOD1, SOD2, CAT, GSR*, and *GPx4* gene expression in SH-SY5Y and HepG2 cells compared to cells treated with H_2O_2 and no peptides ($p < 0.05$). Cells treated with H_2O_2 and F45 exhibited significantly increased antioxidant enzyme activity, including SOD, CAT, GSR, and GPx ($p < 0.05$). The F45B fraction from F45 was sequenced to obtain FVEG and FYDQ tetrapeptides. Molecular docking analysis revealed their high binding affinity to cellular antioxidant enzymes (SOD, CAT, GSR, GPx1, and GPx4), an antioxidant-related protein (Keap1), and ACE. These results suggest that the novel tetrapeptides from *Acheta domesticus* demonstrate important biological activities, establishing them as significant cellular antioxidant activities and a potential source of antihypertensive peptides.

1. Introduction

In recent years, consumption of edible insects has increased, in response to demand for alternative, more sustainable, and eco-friendly protein sources [1]. Crickets have a long history of being used as both food and feed. They are environmentally friendly to

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rear, which makes them an appealing option for human consumption [2]. Nowadays, *Acheta domesticus* (House cricket) has recently been approved as a novel food ingredient by Commission Implementing Regulation (EU) 2022/188. According to EU Regulation 2015/2283, the authorization for *A. domesticus* is detailed in EU Regulation 2022/188, which pertains to the licensing for marketing *A. domesticus* in frozen, dry, and powder forms [3]. Additionally, EU Regulation 2023/5 covers the licensing for marketing *A. domesticus* in partially defatted powder form [4].

Industrial-scale production of crickets in Thailand is being supported by the government both in terms of farming and processing [5]. The most popular cricket species are Sa-ding or House crickets (*A. domesticus*) and Field crickets (*Gryllus bimaculatus*). They are high-protein insects that contain essential amino acids and fats [6]. In addition to processing the insects into individually eaten forms or as cricket powder for a single food ingredient, crickets are an interesting source of protein that can be hydrolyzed into peptides with various biological properties [7]. Study of the bioactivity of insect peptides will support a clearer approach for utilization of insect proteins.

Protein hydrolysates obtained by enzymatic hydrolysis consist of short-chain peptides and free amino acids. Enzymatic modification of proteins is a powerful method to enhance their functional characteristics. Protein hydrolysis generates structural and functional property changes that depend on the size and length of the resulting amino acid peptide chains [8]. These peptides play an important role in regulating the function of various systems within the body and reducing the risk of certain diseases through their antioxidant, blood pressure reducing, anti-aging, anti-inflammatory, anti-diabetes, osteoporosis prevention and antimicrobial properties [9–11].

Peptides derived from insects have the capacity for metal-ion chelation and radical scavenging [12]. The antioxidant capacity of edible insects such as grasshoppers, silkworms, and cricket peptides is five times greater than that of orange juice [13]. Moreover, cricket protein hydrolysates have been shown to have bioactive effects and increased DPPH radical scavenging [7]. These investigations also revealed that the strongest antioxidant effects of DPPH and ABTS radicals are found in the lower molecular weight peptides. However, cellular antioxidant capabilities of cricket peptides have not yet been elucidated, especially for those with reactive oxygen species (ROS) scavenging capability. ROS are highly reactive molecules that are generated as byproducts of cellular metabolism. They can damage cellular components [14]. Ultimately, this damage can lead to various diseases and accelerated aging. Furthermore, peptides can act as angiotensin-converting enzyme I (ACE) inhibitors which are related to functions of the renin-angiotensin-aldosterone system (RAAS) for responsiveness to physiological and pathological circumstances. They convert inactive angiotensin I (ANG I) into vasoconstrictor angiotensin II (ANG II). High ACE activity results in the generation of greater levels of ANG II, which raises blood pressure and accelerates some physiological and pathological cardiovascular processes, including hypertension, and atherosclerosis [15]. The ideal ACE inhibitors are nontoxic, safe, and affordable, such as ACE inhibitors from food proteins and natural bioresources [16]. Peptides derived from field cricket (*G. bimaculatus*) hydrolysates have reported antioxidant activities [17]. Inhibition of the angiotensin-converting enzyme (ACE) peptides derived from black crickets (*G. assimilis*) and tropical banded field crickets (*G. sigillatus)* has also been revealed [18,19]. Most peptides that inhibit ACE are typically characterized by short sequences, ranging from 2 to 12 amino acids [20]. However, ACE inhibitory tetrapeptides from house cricket (*A. domesticus*) protein have not been reported. Therefore, this study aimed to purify, characterize, and evaluate tetrapeptides from house crickets for their cellular antioxidant activities against ROS and ACE inhibitory activity. Furthermore, molecular docking of the tetrapeptides onto antioxidant enzymes (SOD, CAT, GSR, and GPx), an antioxidant-related protein (Keap1), and ACE was conducted to assess their potential as cellular antioxidants and ACE inhibitors.

2. Materials and methods

2.1. Materials

Export-grade house cricket powder from JR Unique Foods, Ltd. (Thailand) was used in the current study. All chemical reagents were analytical grade and obtained from Sigma Aldrich, USA and Thermo Fisher Scientific, USA. Alcalase® (*Bacillus licheniformis*, 2.4 U/g, EC 3.4.21.62), angiotensin converting enzyme (ACE) from rabbit lung (ACE, ≥2 units/mg protein, EC 3.4.15.1), and N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) substrate were all purchased from Sigma Aldrich, USA.

2.2. Cricket protein hydrolysate preparation

Crude cricket protein was extracted from cricket powder by adding deionized water, adjusting the mixture pH to 12 and then incubating it at room temperature for 1 h. The mixture was then centrifuged at 4000 ×g for 20 min, the supernatant was collected, its pH adjusted to 4.0 and centrifuged at 7000 ×g for 20 min to precipitate protein. Protein pellets were collected, freeze-dried and kept at −20 °C for further use. Cricket protein hydrolysates were prepared using hydrolyzed crude cricket proteins with 5 % alcalase. The pH of the crude protein mixture and alcalase was adjusted to 9.0 and incubated at 50 °C for 3 h. Enzyme was inactivated by heating at 100 °C for 10 min. The mixture was then centrifuged at 10,000 ×g for 10 min [7]. The supernatant was collected, freeze-dried and stored at −20 °C for further analysis.

2.3. Cricket protein hydrolysate fractionation and purification

2.3.1. Size exclusion chromatography (SEC)
Cricket protein hydrolysate was dissolved with DI water and filtered through a 0.45 µm filter. Then, peptides were separated

according to their size using AKTA fast protein liquid chromatography (FPLC) (GE Healthcare, USA) with a gel filtration column (Superdex® 30 Increase 10/300 GL, 30 cm × 10 mm, 13 µm, GE Healthcare Life Sciences, USA), as previously described [21]. Samples (20 mg/mL) were loaded into the FPLC system with an injection volume of 500 µL of 0.1 % trifluoroacetic acid (TFA) in 30 % acetonitrile, which was used as the eluent with a flow rate of 0.5 mL/min. The absorbance of each fraction was measured at 280 nm. Fractionated samples were concentrated by freeze drying and then stored at −20 °C. All samples (100 µg/mL) were reconstituted in DI water for their biological activity assays of both antioxidant and ACE inhibition properties. The molecular weight of fractionated peptides was determined using aprotinin (6511 Da), LNLQDFR (905 Da), YPW (465 Da) and leucine (131 Da) as molecular weight standards.

2.3.2. Reverse phase chromatography (RPC)

The lyophilized fractions (1 mg/mL) from SEC separation with the highest antioxidant and ACE inhibitory activities were further purified on a Source™ 5RPC ST 4.6/150 C18 column (5 µm, 4.6 × 150 mm, Waters Inc., USA), at a flow rate of 1 mL/min [21]. Eluent A was water/0.1 % TFA, while eluent B was acetonitrile/0.1 % TFA. After 100 µL of the sample was injected into the C18 column, concentrations of eluent B were increased as 0–40 min, 0–100 % (v/v), and 40–50 min, 100 % (v/v). Each collected fraction was analyzed to determine its antioxidant and ACE inhibition activities. Peptide fractions exhibiting the highest potential for antioxidant and ACE inhibition were identified using liquid chromatography-mass spectrometry (LC-MS/MS) to determine peptide sequences.

2.4. Biological activity assay

2.4.1. Radical scavenging activity by DPPH assay

Determination of radical scavenging activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed as described by Ref. [22]. Briefly, 100 µL of a DPPH solution was added to a mixture of 20 µL of each peptide fraction with 80 µL Tris-HCl buffer pH 7.4 in a 96-well microplate. The samples were vigorously shaken and incubated under a dark condition at room temperature for 30 min. Trolox was used for standard curve preparation.

2.4.2. Radical scavenging activity determined using an ABTS assay

Radical scavenging activity by the 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS$^{•+}$) method was determined according to Ref. [23]. Briefly, the ABTS reagent was incubated with potassium persulfate for 12–18 h. In a 96-well plate, 200 µL of the ABTS working solution and 10 µL of the peptide fractions were added. Absorbance was measured at 734 nm. Trolox was used as a standard.

2.4.3. Ferric-reducing antioxidant power assay (FRAP)

The FRAP assay was done according to a previously described procedure [24]. Briefly, 10 µL of peptide fractions were dissolved in DI water and mixed with 180 µL of a fresh FRAP solution. Reaction mixtures were incubated at 37 °C for 5 min. After that, OD was measured at 593 nm and the peptide activity was compared with a Trolox standard curve.

2.4.4. Determination of angiotensin-converting enzyme (ACE) inhibition

ACE-inhibitory activity of peptide fractions was measured using N-[3-(2-Furyl) acryloyl]-Phe-Gly-Gly (FAPGG) as a substrate, as previously described [25]. In each assay, samples (10 µL; 0.1 mg/mL) were incubated with 10 µL of enzyme (0.1 U/mL) and 80 µL of FAPGG (0.5 mM in 50 mM Tris-HCl buffer containing 0.3 mM NaCl, pH 7.5) in a 96-well microplate. The OD of the mixture at 340 nm was recorded at 1-min intervals for 40 min at 37 °C using a microplate reader. DI water was used as a control. The capability of samples to inhibit ACE is expressed as %ACE inhibition.

$$\text{ACE inhibition } (\%) = \left(1 - \left(\left(\Delta A/\text{min }\right)_{\text{sample}} \Big/ \left(\Delta A/\text{min }\right)_{\text{control}} \left(\Delta A/\text{min }\right)_{\text{sample}} \Big/ \left(\Delta A/\text{min }\right)_{\text{control}}\right) \times 100\right) \times 100$$

2.5. Cellular antioxidant assay

2.5.1. Cell culture

Human neuroblastoma SH-SY5Y and human hepatoma HepG2 cell lines were obtained from the Laboratory of Cell-Based Assays and Innovations, Suranaree University of Technology. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1 % antibiotics (50 units/mL penicillin, 50 µg/mL streptomycin). These cells were cultured as monolayers at 37 °C in a humidified atmosphere under 5 % CO_2 in air.

2.5.2. Acute cytotoxicity determination

Cytotoxicity was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to obtain nontoxic concentrations of peptides on SH-SY5Y and HepG2 cells [26]. Cells were seeded in 96-well tissue culture plates (3×10^4 cells/well) and incubated overnight at 37 °C. The H_2O_2 concentration that killed 50 % of the cells within 24 h was identified. Acute cytotoxicity was evaluated by exposing cells to various concentrations of sample (1, 2.5 and 5 µg/mL for peptide fractions; 100-1000 µg/mL for pre-fractionated hydrolysate (PFH); 200–1000 µM for H_2O_2) for 24 h before adding 0.5 mg/mL MTT for a further 2 h followed by the addition of DMSO to dissolve the formazan crystals produced in viable cells. All MTT assays were performed in

triplicate. The reported results represent the mean values of three independent experiments.

2.5.3. Determination of cytoprotective effects on cell viability

The protective effects of peptide fractions against H_2O_2-induced cytotoxicity was evaluated by pre-treating cells with peptide fractions or PFH at the nontoxic concentrations obtained from Section 2.5.2 for 24 h before treating them with 500 μM H_2O_2 for a further 24 h [27]. All MTT assays were performed in triplicate. The reported results represent the mean values of three independent experiments.

2.5.4. Detection of intracellular reactive oxygen species (ROS)

Cellular ROS levels were quantified using the 2,7-dichlorofluorescein (DCFH) assay [27]. Briefly, SH-SY5Y and HepG2 cells (2 × 10^5 cells/well) were pre-treated with peptide fractions at the indicated concentrations for 24 h and then exposed to 500 μM H_2O_2 for 24 h. Then, a 10 μM DCFH solution was added to the wells and incubated for 30 min at 37 °C. The cells were washed twice with phosphate-buffered saline (PBS). Fluorescent images of each well were made using the green fluorescent protein (GFP) channel on an Eclipse 80i fluorescence microscope (Nikon). After making these images, cells were lysed in a RIPA (radioimmunoprecipitation assay) buffer (Sigma Aldrich, USA). ROS generation was evaluated using a Varioskan LUX fluorescence microtiter plate reader (Thermo Scientific, USA) at an excitation wavelength of 485 nm and emission wavelength of 530 nm. Fluorescence intensities were normalized with protein concentrations. These values were calculated as the relative intensity fold of DCF fluorescence per mg protein compared with a control.

2.5.5. Antioxidant enzyme encoding gene expression

SH-SY5Y and HepG2 cells (3 × 10^5 cells/well) were treated with selected concentrations of peptide fractions for 24 h and then with 500 μM H_2O_2 for a further 24 h at 37 °C. RNA was extracted using a total RNA extraction kit (Vivantis, Malaysia). Total RNA was reverse transcribed to cDNA from the oligo-(dT)18 primer with a Viva cDNA synthesis kit (Vivantis, Malaysia) [28]. The expression of genes encoding antioxidant enzymes was then performed using quantitative polymerase chain reaction (qPCR) assays on a CFX Opus 96 real-time PCR system (Bio-Rad, USA), with CAPITAL™ qPCR Green Mix (Biotechrabbit, Germany) and specific primers (Bionics, Korea). Specific primers for genes encoding antioxidant enzymes are presented in the Supplementary Materials as Table 1S *β-actin* was used as an internal control gene. The ΔΔCT method was employed for quantitative analysis of relative gene expression and reported as the relative fold change compared to the control.

2.5.6. Determination of antioxidant enzymatic activities

SH-SY5Y and HepG2 cells (3 × 10^5 cells/well) were treated with selected concentrations of peptide fractions for 24 h and then with 500 μM H_2O_2 for a further 24 h at 37 °C. Total protein was extracted using the CHAP lysis buffer. Each sample was collected for enzymatic activity evaluation. Antioxidant enzymatic activities were determined following the manufacturer's instruction as described below.

2.5.6.1. Superoxide dismutase (SOD) activity.
SOD activity was determined using the CheKine™ micro superoxide dismutase (SOD) activity assay kit (Abbkine Scientific, USA). The inhibitory effect of SOD was quantified using a colorimetric method at OD 450 nm. SOD activity results are expressed as units of SOD per mg protein.

2.5.6.2. Catalase (CAT) activity.
CAT activity was determined using the CheKine™ micro catalase (CAT) activity assay kit (Abbkine Scientific, USA). Absorbance of the resulting product was then measured at 540 nm. The OD value is directly proportional to the

Table 1
Scavenging activity against ABTS$^{\bullet+}$, DPPH$^{\bullet}$, and FRAP and inhibition of angiotensin converting enzyme (ACE) by cricket peptide fractions.

| | | Antioxidant activity | | | ACE |
| | | (mg trolox/g peptide) | | | inhibition |
	Fraction	DPPH	ABTS	FRAP	(%)
Size exclusion	F44	412.0 ± 9.8[b]	420.1 ± 7.9[c]	383.2 ± 8.6[c]	19.79 ± 3.31[a]
	F45	393.6 ± 3.7[b]	305.9 ± 3.5[b]	290.4 ± 6.7[b]	57.93 ± 3.62[b]
	F46	360.9 ± 8.6[a]	215.9 ± 2.7[a]	201.8 ± 8.4[a]	13.11 ± 4.12[a]
Reverse-phase	F45A	nt.	nt.	nt.	35.42 ± 2.95[B]
	F45B	117.8 ± 2.81	177.6 ± 4.83	254.4 ± 6.6	30.17 ± 3.89[B]
	F45C	nt.	nt.	nt.	35.42 ± 5.84[B]
	F45D	nt.	nt.	nt.	34.57 ± 1.74[B]
	F45E	nt.	nt.	nt.	18.81 ± 1.18[A]
	F45F	nt.	nt.	nt.	62.08 ± 2.95[C]
	F45G	nt.	nt.	nt.	31.25 ± 2.95[B]
	F45H	nt.	nt.	nt.	13.31 ± 1.66[A]

Results are reported as the percentage of inhibiting activity and are expressed as a mean of least triplicate (n = 3) determinations, with standard deviation. F44–F46 are fractions collected from the gel filtration column. F45A-F45H are fractions collected from the reverse phase C18 column. nt. = not detectable. Different lowercases and uppercases on the bars indicate significant differences (p < 0.05) among SEC fractions and RPC fractions.

catalase activity in the sample. CAT activity results are expressed as units of CAT per mg protein.

2.5.6.3. Glutathione reductase (GR) activity. GR activity was determined using the CheKine™ micro glutathione reductases (GR) activity assay kit (Abbkine Scientific, USA). The rate of NADPH dehydrogenation is assessed by measuring decreased absorbance at 340 nm, allowing for calculation of GR activity. GR activity results are expressed as units of GR per mg protein.

2.5.6.4. Glutathione peroxidase (GPx) activity. GPx activity was determined using the CheKine™ micro glutathione peroxidase (GSH-Px) activity assay kit (Abbkine Scientific, USA). The rate of NADPH dehydrogenation was quantified by monitoring decreased absorbance at 340 nm, allowing for determination of GPx activity. GPx results are expressed as units of GPx per mg protein.

2.6. De novo peptide sequencing

Peptide identification was performed by BiotechPack Scientific. The collected fraction with the highest antioxidant and ACE inhibition activities was subsequently analyzed using liquid chromatography-mass spectrometry (LC-MS/MS) with an Nanoflow UPLC: Easy-nLC 1200 (Thermo Fisher Scientific, USA) coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, USA) with an ESI nanospray source. Data acquisition conditions included ion spray voltage (2.2 KV) and capillary temperature (270 °C). First, the sample was reduced using 10 mM DL-dithiothreitol (DTT) at 56 °C for 1 h and alkylated with 55 mM iodoacetamide (IAM) at room temperature in the dark for 40 min. Peptides were resuspended in 0.1 % formic acid and analyzed using LC-MS/MS with an ESI nanospray to obtain a raw file of the original mass spectrometric results. The raw MS files were analyzed and compared against a protein database based on the species of the samples using PEAKS Studio10.6 (Bioinformatics Solutions, Inc., Waterloo, Canada). Only peptides identified with high confidence were chosen for protein identification.

2.7. Molecular docking of cricket tetrapeptides

The crystal structures of human ACE (PDB: 1O8A), Keap1 (PDB: 3ZGC), SOD (PDB: 6FOL), CAT (PDB: 1QQW), GSR (PDB: 3GRS), GPx1 (PDB: 2F8A) and GPx4 (7L8K) were derived from the RCSB Protein Data Bank (https://www.rcsb.org/). Two-dimensional structures of peptides were modeled using the ChemBioDraw Ultra 14.0 package and converted to 3D structures with an energy-minimized structure in the ChemBio3D Ultra 14.0 software. Docking simulations were performed using Autodock Vina as previously described [29]. The ligand with the most favorable binding energy within the protein's binding pocket was chosen as the optimal docking pose. The best-scoring pose analyzed by the Vina docking score was selected and visually analyzed in Discovery Studio
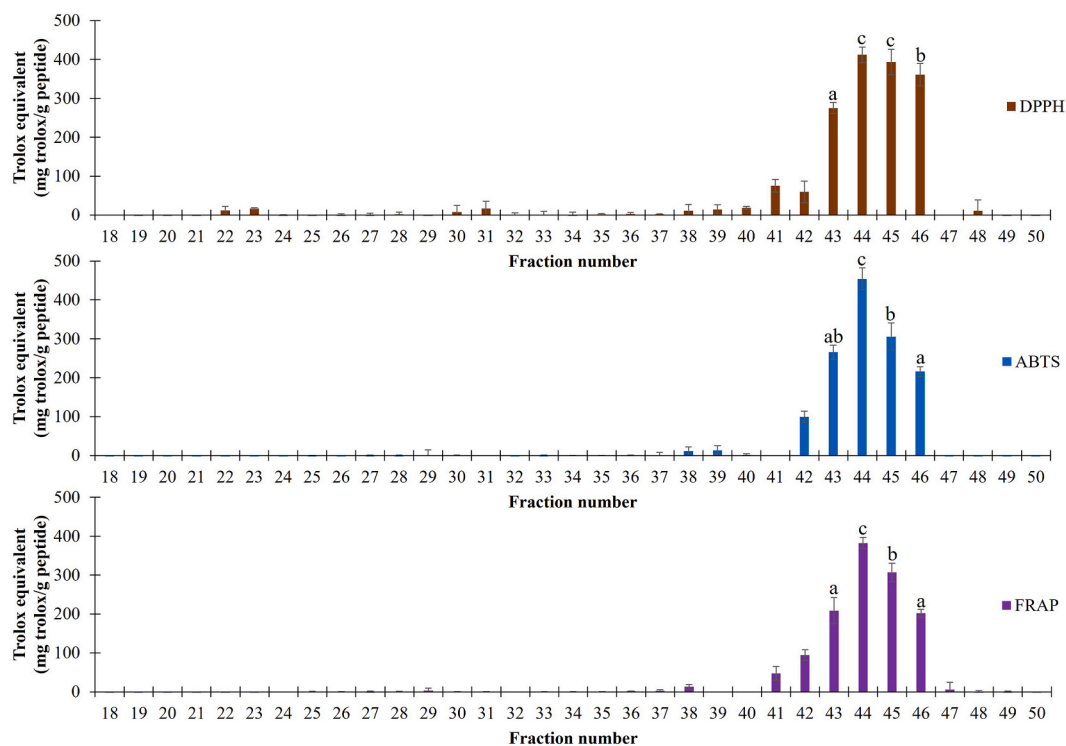
Fig. 1. Scavenging activity of cricket peptide fractions from SEC against ABTS$^{\bullet+}$, DPPH$^{\bullet}$, and FRAP. Different letters on the bars indicate significant differences (p < 0.05) among peptide fractions F43–F46.

Visualizer.

2.8. Statistical analysis

All experiments were conducted in triplicate. Mean values ± standard deviation are presented as the results. One-way ANOVA and Duncan's multiple-range test were applied for mean comparison using SPSS 22.0 (IBM Corp., NY).

3. Results

### 3.1. Cricket peptide hydrolysate (CPH) fractionation and chemical antioxidant activity

Sequential chromatography was used to separate the CPH, enabling identification and characterization of bioactive peptides. Alcalase proteolysis produced a cricket protein hydrolysate (CPH) consisting mostly of peptides with sizes smaller than 1 kDa. Fractions F18–F50 were collected and lyophilized for bioactivity screening, which included assessments of chemical antioxidant and ACE inhibitory activities. The antioxidant activities of peptide fractions were evaluated for free radical scavenging activity using DPPH and ABTS assays as well as for metal ion chelation activity with a FRAP assay. The antioxidant activities of fractions F44 to F46 were 200–480 mg Trolox/g peptide greater than that of other fractions (Fig. 1). Peptide F45 was isolated by size exclusion chromatography (SEC), demonstrating the highest bioactivity in terms of chemical antioxidant activities (Fig. 1).

3.2. Angiotensin converting enzyme (ACE) inhibition capacity of fractionated peptides

Fractions F18 to F50 from SEC were screened for their ACE inhibition properties using FAPGG as substrate. As shown in Fig. 2, fractions F22 and F45 exhibited higher ACE inhibition. The properties of ACE inhibition peptides correlated with our F22 and F45 fractions, showing high ACE inhibition activity. Fraction F45 showed both strong antioxidant and ACE inhibition activities, acting as a multifunctional bioactive peptide. From these results, three fractions (F44–F46) were chosen for analysis of cellular antioxidant activity against ROS because they showed two potential biological activities.

3.3. Cytoprotective effects of fractionated peptides

The acute cytotoxicity effects of the selected F44–F46 fractions on SH-SY5Y and HepG2 cells were determined using the MTT assay (Supplementary Materials Fig. 1S). SH-SY5Y and HepG2 cells were treated with H_2O_2 to study the cytoprotective capabilities of antioxidant peptides. Cell viability for both cell lines was reduced to approximately 50 % in 24 h in response to a 500 μM H_2O_2 treatment. Based on cell viability (%) after treatment, 500 μM H_2O_2 is an optimal concentration for subsequent experiments. Pre-treatment of SH-SY5Y and HepG2 cells with 0.5 and 1 μg/mL of F44–F46 fractions could counteract the deleterious effects of 500 μM H_2O_2 in a concentration-dependent manner. No significant difference was observed among the various peptide fractions. Additionally, pre-fractionated hydrolysate (PFH) showed cytoprotective effects at a 100 μg/mL concentration. These results indicated that F44–F46 peptides exerted cellular antioxidant activities against H_2O_2 induced oxidative damage at lower concentrations compared with PFH. Therefore, a 1 μg/mL concentration of peptide fractions and 100 μg/mL of PFH was used for cellular antioxidant assays.
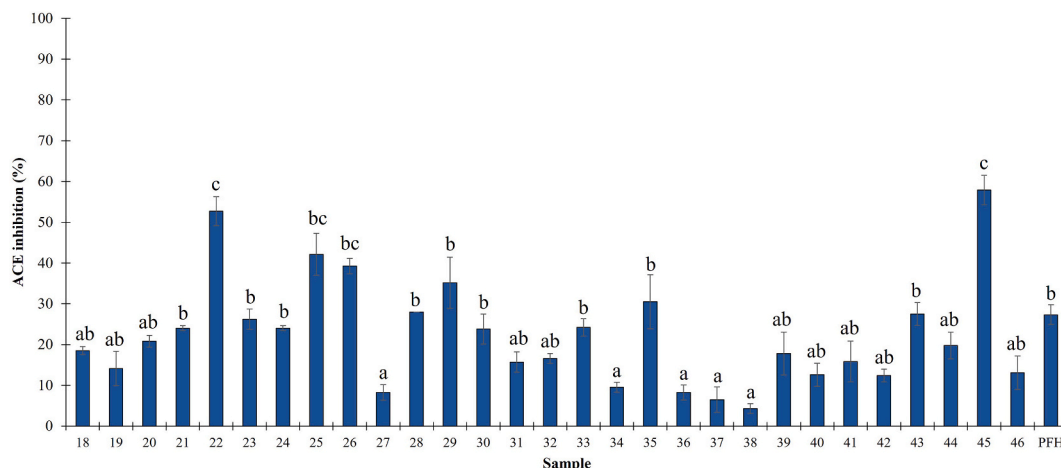
Fig. 2. Angiotensin-converting enzyme (ACE) inhibitory activity of SEC peptide hydrolysate fractions F18–F46 and PFH for 24 h. Cell viability was determined using an MTT assay and presented as the percent of cell viability compared to untreated cells. Different letters on the bars indicate significant differences ($p < 0.05$).

3.4. Effects of peptide fractions on H_2O_2 induced intracellular ROS accumulation

As shown in Fig. 3, exposure of both cell types to H_2O_2 led to a clear increase in ROS-induced fluorescence (Fig. 3A) with approximately a 3-fold greater fluorescence intensity (Fig. 3B). In contrast, pretreatment with fractions F44–F46 significantly reduced ROS production from H_2O_2 induction. Fractions F44 and F45 at 1 μg/mL reduced H_2O_2-induced ROS production to a greater degree than fraction F46 and PFH at 100 μg/mL. There was no difference between fractions F44 and F45 (Fig. 3B–p > 0.05). The results suggest that cricket peptide fractions containing low molecular weight hydrophobic amino acids sufficiently scavenged ROS produced in response to H_2O_2.

3.5. Effect of peptide fraction on Keap1-Nrf2 and antioxidant enzyme encoding gene expression

Peptides can regulate activation of antioxidant genes, which play vital roles in protecting cells from oxidative stress. Therefore, fraction F45 was further examined for antioxidant encoding gene expression, and the results shown in Fig. 4A. This was due to its powerful antioxidant activity (Fig. 1) and highest ACE inhibition (Fig. 2). Peptides are not normally recognized as transcription factors, however, they can indirectly modulate gene expression by influencing transcription factors through various mechanisms, including activating signaling pathways and modifying epigenetic changes [30]. As shown in Fig. 4A, exposing SH-SY5Y and HepG2 cells to H_2O_2 for 24 h resulted in significantly increased *Nrf2* gene activity in both cell lines. Cells treated with the F45 peptide fraction followed by H_2O_2 also exhibited activation of the *Nrf2* gene, indicated by a notable increase in its transcriptional targets, which were *SOD*, *CAT*, *GSR*, and *GPx*, when compared to the control (p < 0.05, Fig. 4A).

This indicated activation of the transcriptional regulator *Nrf2*, leading to stimulation of antioxidant enzymes such as SOD, CAT,
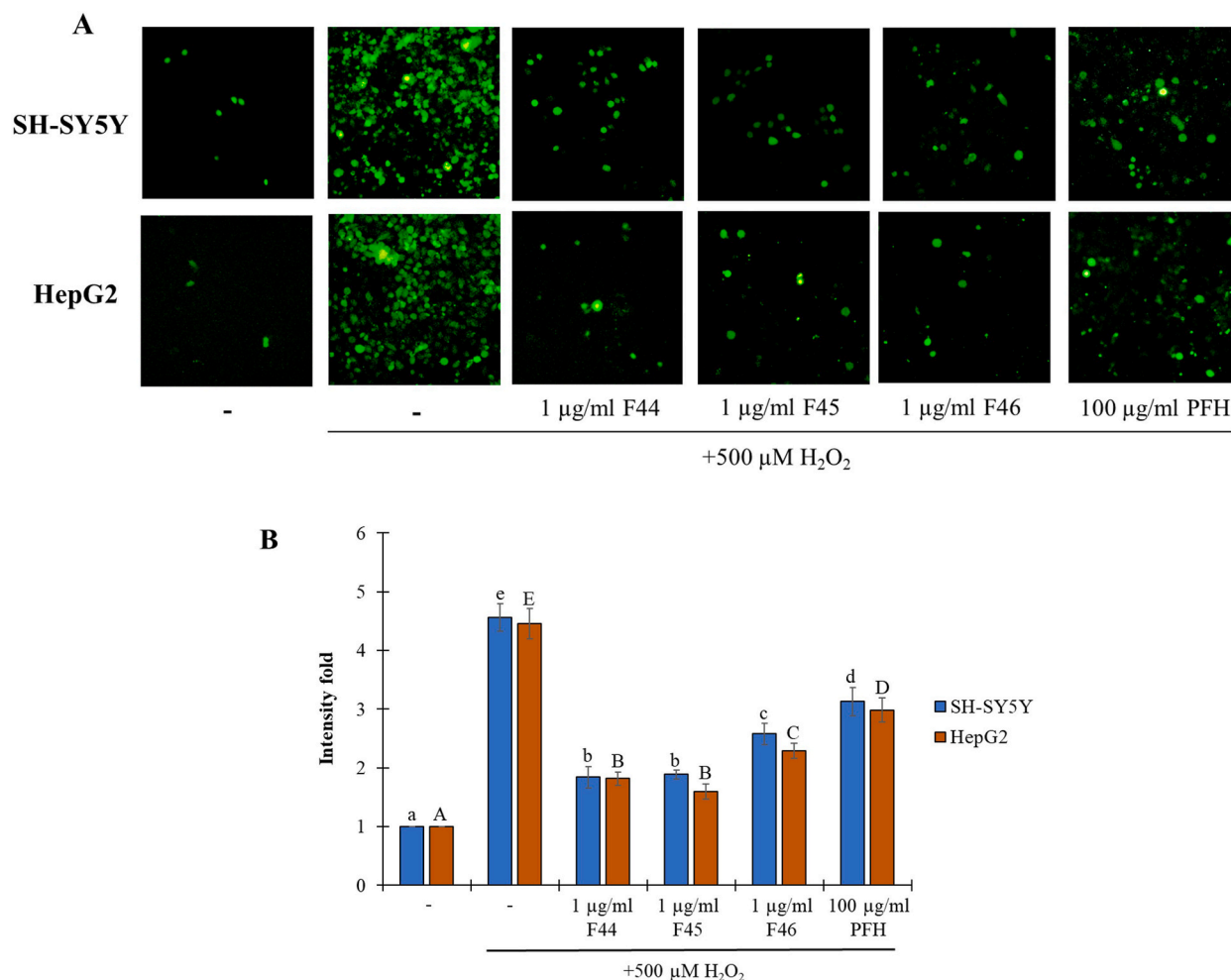
Fig. 3. Protective effect of antioxidant peptide fractions against ROS formation in SH-SY5Y and HepG2 cells. Representative fluorescent images were taken using a fluorescence microscope (A) and intensity quantification was done using a fluorescence microplate reader for intracellular DCF levels (B).
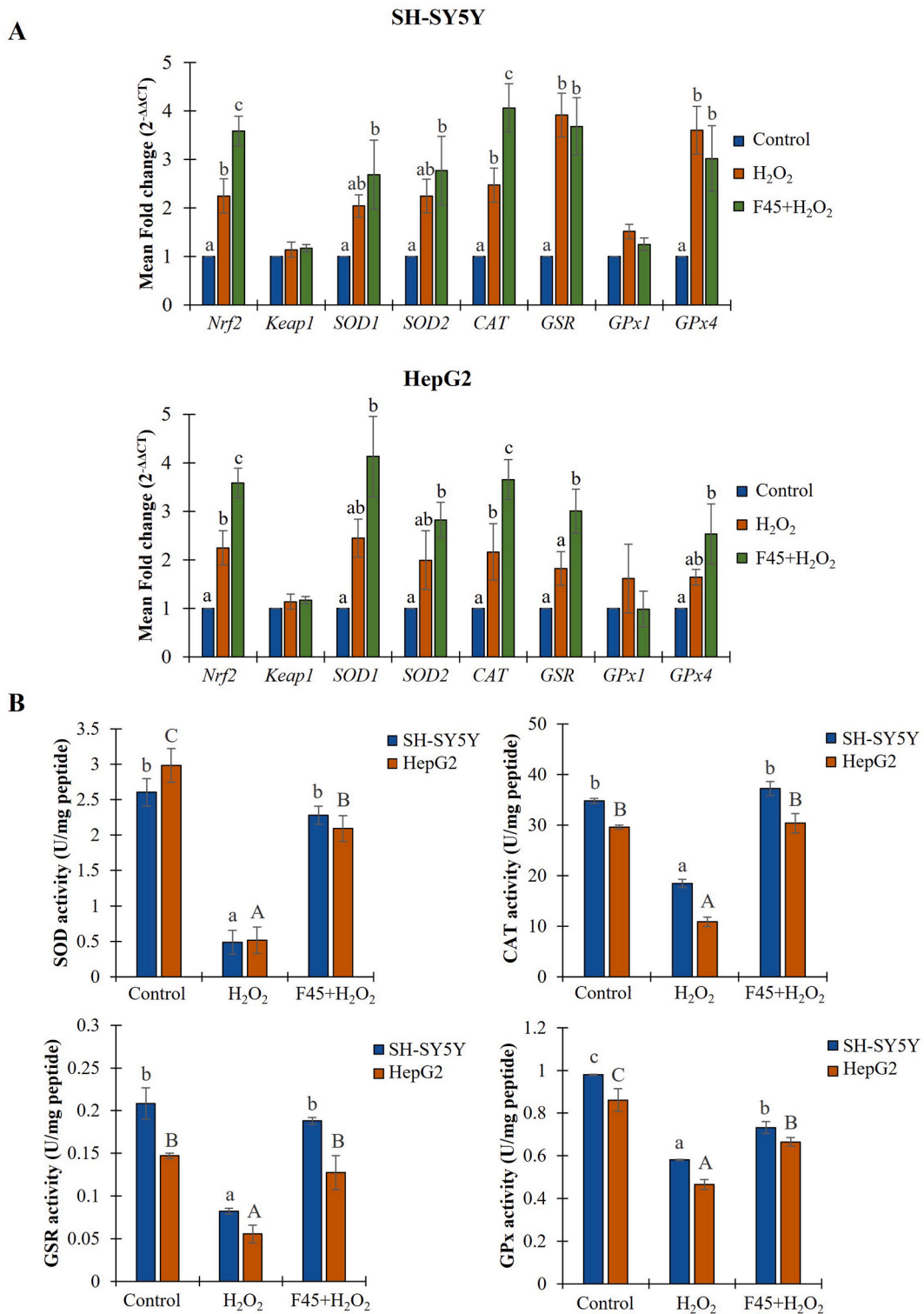
Fig. 4. Relative fold change determined by quantitative real-time PCR (qRT-PCR) analysis of antioxidant encoding genes in SH-SY5Y and HepG2 cells treated with F45 and H_2O_2 (A), and effects of peptide F45 on SOD, CAT, GSR and GPx activity on SH-SY5Y cells and HepG2 cells treated with 500 μM H_2O_2 (B).

GSR, and GPx, in response to H_2O_2-induced oxidative stress in both cell lines. However, there was no change in the mRNA levels of *Keap1* in either of the cell lines compared to the control (p > 0.05). Increases in *SOD1, SOD2, CAT,* and *GPx4* gene expression levels in SH-SY5Y and HepG2 were significantly upregulated after pre-treatment with F45 compared to an H_2O_2 treatment group (p < 0.05). Increased gene encoding for antioxidant enzyme expression can reduce oxidative stress in cells.

3.6. Effects of peptides on antioxidant enzyme activity

The activities of SOD, CAT, GSR and GPx enzymes were assessed in SH-SY5Y and HepG2 cells after adding F45 extracts and exposure to H_2O_2 (Fig. 4B). Antioxidant enzyme activity played a critical role in the system. Exposure of both cell lines to H_2O_2 led to a marked overexpression of the *SOD1, SOD2, GSR*, and *GPx4* genes (Fig. 4A). The F45 fraction demonstrated a significant increase in SOD, CAT, GSR, and GPx enzyme activity compared to the H_2O_2 treatment group (Fig. 4B, p < 0.05). Addition of F45 peptide fractions to SH-SY5Y and HepG2 cells, prior to treatment with H_2O_2, resulted in increased SOD, CAT, and GSR antioxidant enzyme activities (p < 0.05). These increases were greater than those observed in cells exposed to H_2O_2 with no peptide, effectively restoring enzyme activity to levels observed in normal cells (Fig. 4B, p > 0.05). From our results, the F45 peptide fraction exhibited antioxidant activity, effectively reducing ROS levels in cells after treatment with H_2O_2. This led to increased antioxidant enzyme activity, countering the negative effects of oxidative stress. These findings indicate that the F45 peptide fraction had antioxidant effects in H_2O_2-mediated oxidative stress and enhanced the activities of antioxidant enzymes in SH-SY5Y and HepG2 cells.

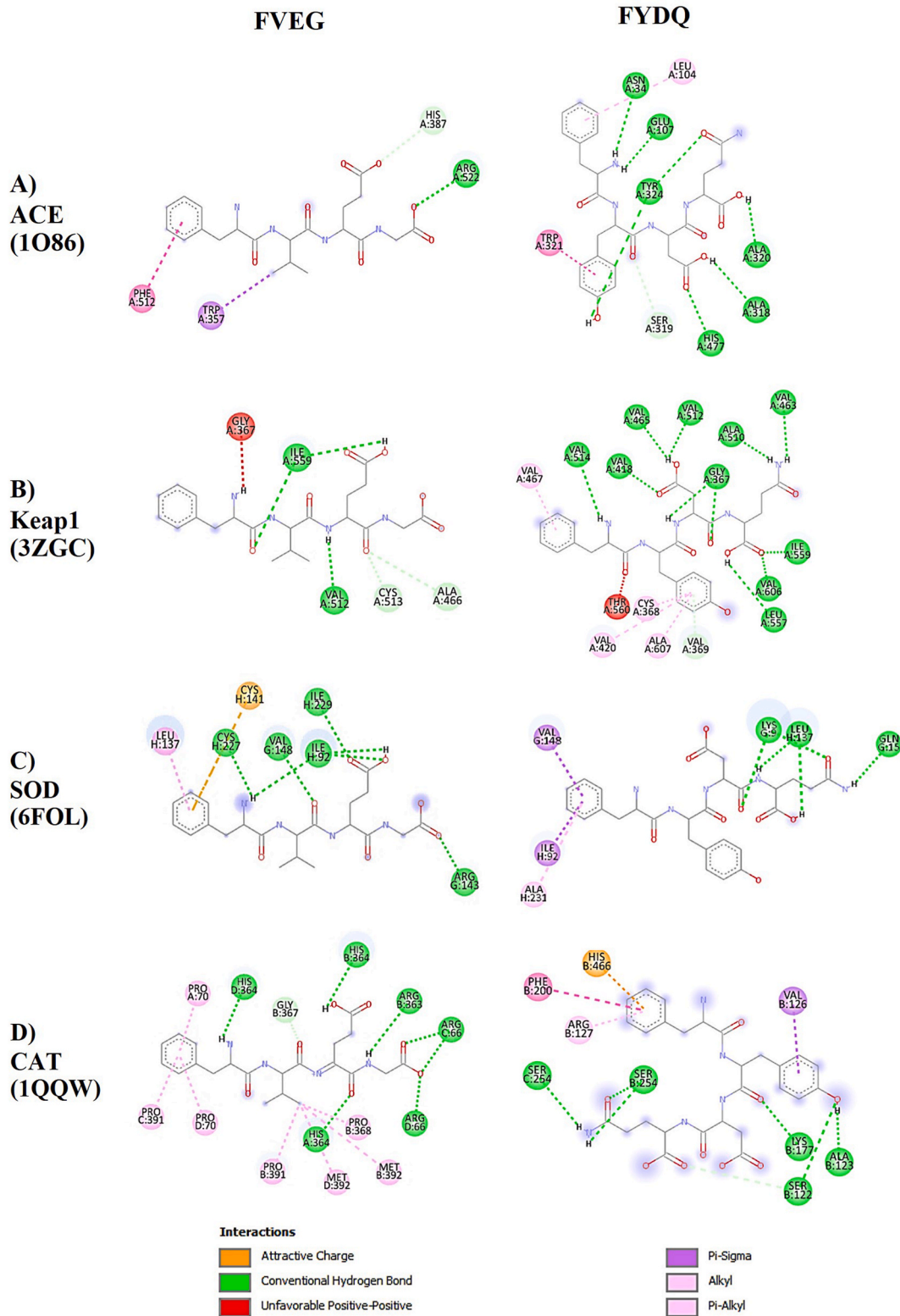### 3.7. Identification of cricket tetrapeptides and molecular docking analysis

The F45 fraction displayed the highest antioxidant activity, demonstrating 57.93 % ACE inhibition (Table 1). The F45 fraction was subjected to further separation using reverse-phase chromatography (RPC), resulting in separation of eight subfraction peaks, F45A, F45B, F45C, F45D, F45E, F45F, F45G, and F45H (Table 1). The F45B subfraction was chosen for peptide identification because it exhibited both antioxidant activity and ACE inhibition. Its identified peptides, ranging from 2 to 6 amino acids, showed higher scores (>90 %) for tetrapeptides compared to dipeptides (50–60 %) and tripeptides (60–70 %). Therefore, tetrapeptides were selected for evaluation of their binding affinity to target proteins. The identified tetrapeptides exhibited hydrophobic properties, with hydrophobicity compositions of approximately 50 % and 75 %. The tetrapeptides, FVEG (Phe-Val-Glu-Gly) and FYDQ (Phe-Tyr-Asp-Gln), demonstrated greater binding affinity to the target protein (Table 2).
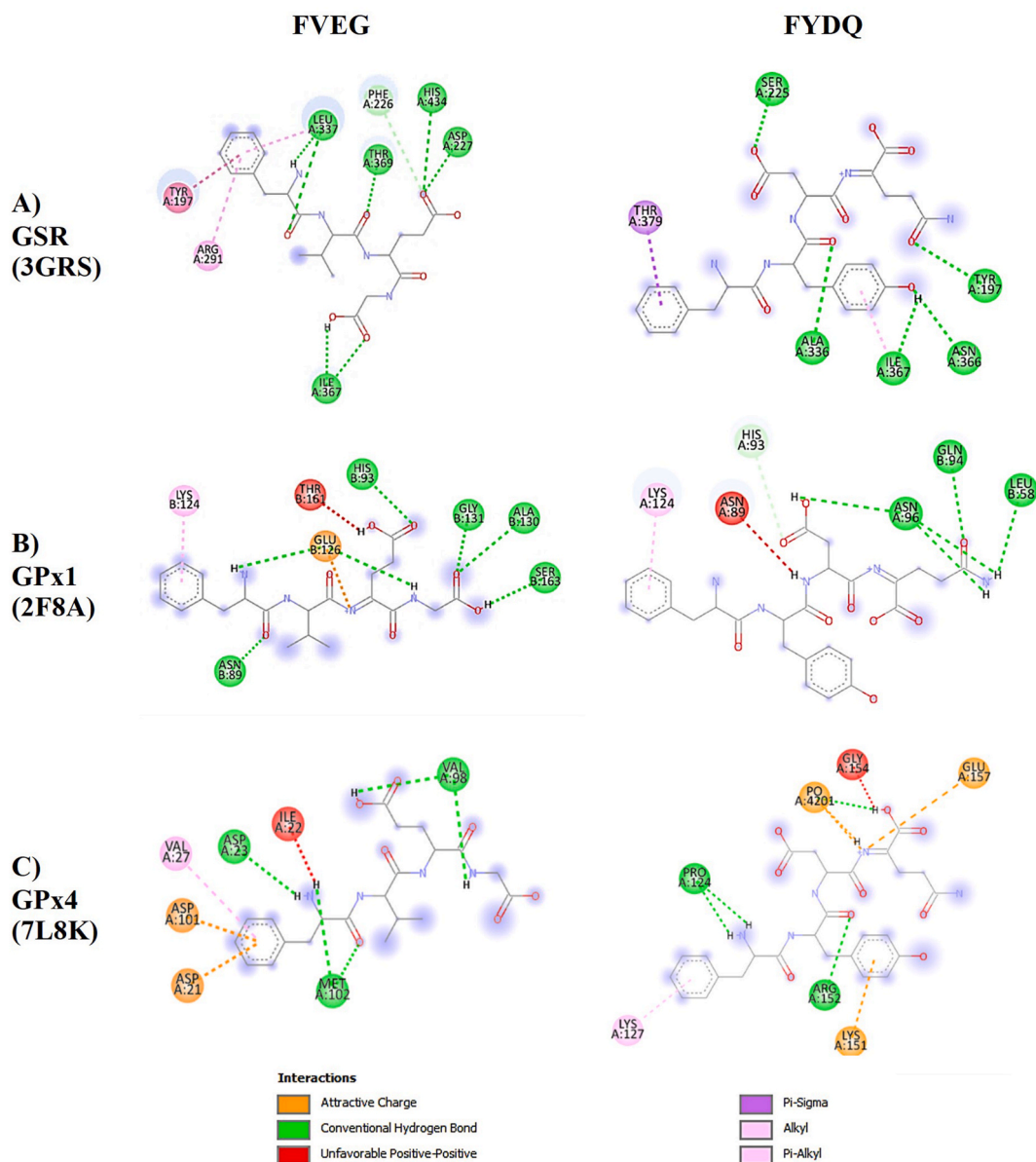
Currently, no identification of tetrapeptides exhibiting antioxidant and ACE inhibitory activity from crickets has been reported. This is the first report of these two peptides, which were found in cricket (*Acheta domesticus*) peptides. Both tetrapeptides, FVEG and FYDQ, contain hydrophobic and positively charged amino acids. They have good potential for antioxidant and ACE inhibitory properties. The FVEG and FYDQ peptide sequences were then chosen for further investigation of their interactions with target macromolecules. Molecular docking results, including binding affinity to ACE and antioxidant-related receptors, are summarized in Table 2. The molecular docking models and results are illustrated in Figs. 5 and 6. FVEG and FYDQ are novel peptides that are mainly stabilized by multiple H-bonds and van der Waals interactions with ACE residues (Fig. 5A). The stability of the enzyme-peptide complex was enhanced by the presence of hydrogen bonds between the peptides and ACE, significantly influencing the ACE inhibitory activity. Molecular docking analysis revealed that the amino acid residues, Phe, Gly, Tyr, Asp, and Gln, in the FVEG and FYDQ peptides formed interactions with the amino-acid residues in ACE. Although FVEG and FYDQ did not directly interact with amino acids at the active site of ACE, they engaged with nearby receptors through various intermolecular forces via hydrophobic interactions, van

Table 2
Molecular docking results of peptides with binding affinity to angiotensin converting enzyme (ACE) and antioxidant related receptors.

Peptides	Receptor	Binding affinity (kcal/mol)	Number of hydrogen bond	Binding sites
FVEG	ACE	−7.6	1	Trp357, His387, Phe512, Arg522
FYDQ		−8.8	7	Leu104, Asn34, Glu107, Trp321, Tyr324, Ser319, His477, Ala318, Ala320
FVEG	Keap1	−8.2	2	Gly367, Ala466, Val512, Cys513, Ile559
FYDQ		−9.1	11	Val467, Val514, Thr560, Cys368, Val420, Ala607, Gly367, Val418, Val465, Val512, Leu557, Val606, Ile559, Ala510, Val463
FVEG	SOD	−7.4	5	Ile92, Leu137, Cys141, Arg143, Val148, Cys227, Ile229
FYDQ		−8.3	5	Lys9, Leu137, Gln15
FVEG	CAT	−7.2	6	Arg66, Pro70, Arg363, His364, Gly367, Pro391, Met392
FYDQ		−8.4	6	Ser254, Lys177, Ala123, Ser122
FVEG	GSR	−7.3	5	Tyr197, Asp227, Arg291, Leu337, Ile367, The369, His434
FYDQ		−6.8	5	Ser225, Ala336, Ile367, Asn366, Tyr197
FVEG	GPx1	−6.4	5	Asn89, His93, Lts124, Glu126, Ala130, Gly131, Thr161, Ser163
FYDQ		−7.5	5	Asn96, Gln94, Leu58
FVEG	GPx4	−6.2	5	Asp21, Ile22, Asp23, Val27, Ala98, Asp101, Met102
FYDQ		−5.6	4	Pro124, Arg152

Fig. 5. Model of the interaction binding of tetrapeptide FVEG and FYDQ to angiotensin converting enzyme (ACE) (A), Keap1 (B), antioxidant enzyme SOD (C), and CAT (D).

Fig. 6. Model of the interaction binding of tetrapeptide FVEG and FYDQ to antioxidant enzyme GSR (A), GPx1 (B), and GPx2 (C).

der Waals forces, hydrogen bonds, π bonds, and electrostatic interactions. Therefore, the presence of a Phe residue in FVEG and Phe and Tyr residues in FYDQ may contribute to their ACE inhibitory activity. The Gly residue of FVEG mainly interacts with the Arg 522 through hydrogen bonding. Phe residues of the FVEG peptide are involved in π-interactions with the Phe 512 of ACE. Moreover, the Val residue of FVEG is stabilized by hydrophobic interactions with the Trp 357 residue of ACE. FYDQ was involved in hydrogen bonding and hydrophobic interactions with ACE to a greater degree than FVEG. FVEG interacts through six hydrogen bonds, with Asn 34, Glu 107, Ala 318, Ala 320, Tyr 324, and His 477. Phe and Tyr residues of the FYDQ peptide form π-interactions with Leu 104 and Trp 321, respectively.

The amino acid residues, Phe, Val, Glu, Gly, Try, Asp, Gln in the novel FVEG and FYDQ peptides were also docked with the amino-acid residues in Keap1, as shown in Fig. 5B. Keap1-Nrf2 signaling pathway activation can mitigate oxidative stress induced by adverse external stimuli. It has the capability to modulate expression of antioxidant enzymes, including SOD, CAT, GSR and GPx. The selected tetrapeptides with FVEG and FYDQ amino-acid residues were observed to form tight bonds with antioxidant enzymes, involving a significant number of hydrogen bonds or hydrophobic interactions. Tetrapeptides have been previously reported for their antioxidant properties. The binding energies of Keap1 with FVEG and FYDQ are −8.2 and −9.1 kcal/mol, respectively (Table 2). FVEG showed binding by forming two hydrogen bonds with Val 512 and Ile 559 amino acid residues. FYDQ forms hydrogen bonds with Keap1 and

the peptide residue of FYDQ also develops π-interactions between Phe with Val 467 and Tyr with Cys 368, Val 420 and Ala 607. FYDQ exhibits better binding affinity to Keap1 than FVEG. This is due to the high number of hydrogen bonds and π-interactions of FYDQ with Keap1 amino-acid residues.

The amino-acid residues of FVEG and FYDQ tightly bind with the antioxidant enzymes, SOD, CAT, GSR, GPx1, and GPx4, through hydrogen bonds or hydrophobic interactions. As shown in Fig. 5C, amino-acid residues of SOD form hydrogen bonds with FVEG at Arg 143, Val 148, Cys 227 and Ile 229. Additionally, the Lys 9, Leu 137 and Gln 15 residues of SOD form hydrogen bonds with FYDQ. FVEG and FYDQ may have the potential to stabilize and enhance the catalytic activity of SOD. Binding of FVEG and FYDQ peptides with CAT is shown in Fig. 5D. Arg 66, Arg 363 and His 364 residues of CAT form hydrogen bonds with FVEG. The Pro 70 and Pro 391 residues of CAT form π-interactions with the Phe residue of FVEG. Moreover, Phe and Tyr residues of FYDQ form π-interactions with Arg 122, Val 126 and Phe 200 residues of CAT.

Interactions of FVEG and FYDQ peptides with GSR are shown in Fig. 6A. Hydrophobic amino acids such as Asp 227, Leu 337, and Ile 367 of GSR form hydrogen bonds with FVEG, while Tyr 197, Ala 336, and Ile 367 form five hydrogen bonds with FYDQ. Phe residues of FVEG form π-interactions with Tyr 197 and Arg 291, whereas Ile 367 show hydrophobic interactions with the Tyr residue of FYDQ. Since both the FVEG and FYDQ peptides also exhibit a binding position with Tyr 197, they may have the potential to enhance GSR activity. Interactions of antioxidant enzymes, GPx1 and GPx4, are shown in Fig. 6B and C. Both peptides tend to bind with GPx1 with lower binding energies than GPx4. FVEG and FYDQ form hydrogen bonds and show π-interactions with the hydrophobic amino-acid residues of GPx1 and GPx4 (Fig. 6B and C).

The results from molecular docking suggest that both tetrapeptides could bind with SOD, CAT, GSR, and GPx. Consequently, they have the potential to protect or enhance the activity of antioxidant enzymes. The hydrophobic Phe, Tyr and Val amino-acid residues in FVEG and FYDQ peptides readily form hydrogen bonds and hydrophobic interactions with antioxidant protein targets, contributing to enhanced antioxidant effects. Moreover, the presence of hydrophobic amino acids in FVEG and FYDQ enhances peptide solubility in lipids and improves accessibility to hydrophobic radical species. Consequently, these peptides can potentially attenuate oxidative stress and enhance antioxidant activity. In summary, the results suggest that peptides from cricket protein hydrolysate may exhibit an antioxidant function by scavenging free radicals, enhancing the activity of antioxidant enzymes through modulation of the Keap1-Nrf2 pathway, and directly conjugating with free radicals. Further studies are necessary to assess the efficacy of synthetic novel peptides, such as FVEG and FYDQ, on antioxidant and ACE inhibition properties.

4. Discussion

Antioxidant activity is generally higher for peptides with hydrophobic and aromatic amino acids having molecular weights lower than 1 kDa [31]. [17] revealed 10 peptides derived from field cricket (*G. bimaculatus*) protein fractions with 3–13 amino acids and their associated antioxidant activities. The percentage of hydrophobic amino acids ranged from 50 % to 100 %, significantly elevating their antioxidant activity. Amino acids containing aromatic residues function as hydrogen donors to radicals lacking electrons. [32] reported two antioxidant peptides from sardinelle (*Sardinella aurita*) hydrolysate, containing tryptophan or tyrosine. This characteristic has the potential to enhance the capability of peptides for effective radical scavenging. The effectiveness of antioxidant peptides is generally related to the capability of hydrophobic and aromatic amino acids to donate protons, electrons, or directly scavenge lipid radicals [33]. Since tyrosine, tryptophan and methionine scavenge radicals better than other amino acids, these amino acid residues in peptide sequences may contribute to their antioxidant activity [34]. Moreover [17], revealed peptides with a molecular weight range of 359.23–721.37 Da derived from field crickets showed significantly higher antioxidant activities than peptides with molecular weights above 1.5 kDa. Altogether, the high antioxidant activity of fractions F44 to F46 might be due to their low molecular weight and hydrophobic amino acid content, which provide powerful free radical scavenging capabilities.

ACE inhibitory peptides are typically composed of 2–12 amino acids. Large peptides are unable to bind to the active sites of ACE [20]. [16] reported that a 5 amino-acid peptide from tropical banded crickets (*G. sigillatus*) exhibited high ACE inhibition. [35] also reported high ACE inhibition activity of peptides with a greater number of hydrophobic amino acids derived from leatherjacket (*Meuchenia* sp.) hydrolysates. Proline, lysine, or arginine at the C-terminal end of the peptides can enhance ACE inhibition potency. From this, it can be inferred that the ACE inhibition activity of peptides is influenced by their typical structure and amino acid composition.

ROS is an important indicator of oxidative stress in SH-SY5Y and HepG2 cells. The oxidation of DCFH by ROS results in conversion to DCF. This conversion is accompanied by a change in fluorescence, with DCF emitting green fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 530 nm [36]. The scavenging activity of peptides depends on their amino acid composition, size, and the type of free radical being scavenged. Antioxidant peptides with hydrophobic amino acid residues from housefly pupae protein hydrolysate exhibited a neuroprotective effect against H_2O_2 induced oxidative damage in PC12 cells [37]. Peptides from marine pearl oysters, RL, RGL, and PR, showed strong antioxidant activity to protect HepG2 cells from oxidative damage induced by H_2O_2 and scavenging accumulated ROS [38].

A previous study reported that a peptide derived from fish protein enhanced the activation of multiple antioxidant genes, including *SOD* and *CAT*, in mice liver cells. This led to a reduction in oxidative stress [39]. [40] revealed that peptides derived from casein showed antioxidant activity by upregulating mRNA expression of antioxidant enzymes including SOD, CAT and GPx, as well as Nrf2. Similarly, six novel antioxidant peptides derived from Chinese baijiu exhibited ROS reduction by activating the Nrf2/ARE signaling pathway as well as antioxidant enzyme activities, including SOD, CAT, and GPx [41]. ROS reduction by peptide pretreatment is correlated with upregulation of the *Nrf2*, *SOD* and *CAT* genes. According to these results, cricket peptides have the potential to upregulate *Nrf2* in SH-SY5Y and HepG2 cells, leading to increased transcript levels of antioxidant enzymes. It can be inferred that

cricket peptides hold promise as potential antioxidant agents.

Antioxidant enzymes have the capability to decrease the buildup of oxygen free radicals within the body, thereby safeguarding cells against oxidative damage [42]. [43] revealed that peptides from pearl shell meat hydrolysate exhibited antioxidant activity, with enhanced antioxidant enzyme activity for SOD and CAT in AAPH-damaged HepG2 cells. A well-regulated enzymatic system within the mitochondrial matrix functions related to activity of the SOD present in the intermembrane space and cytosol of mitochondria convert superoxide anion ($O_2^{\bullet-}$) into H_2O_2 [44]. After that, H_2O_2 is eliminated in mitochondria and the cytosol through detoxification by GPx, which utilizes glutathione (GSH) as a substrate. Additionally, in peroxisomes, CAT converts H_2O_2 into H_2O. Finally, oxidized glutathione (GSSG) is reduced by glutathione reductase (GR) to restore intracellular GSH [45]. Furthermore, these peptides also enhance the expression of genes related to antioxidant enzymes, resulting in elevated cellular antioxidant enzyme levels to combat ROS. Then, recovering antioxidant enzyme activity from oxidative stress could potentially protect against overall oxidative damage in cells.

Numerous antioxidant and ACE inhibitory peptides have been reported and identified with sequences ranging from 2 to 12 amino acids. Smaller peptides have a better chance of effectively binding with the ACE active site, consequently leading to an increased ACE inhibitory activity [21]. The peptide, KVEGDLK, derived from crickets contains leucine and lysine, and exhibits inhibitory activity against ACE [46]. Three peptides, YKPRP, PHGAP, and VGPPQ from crickets, exhibit lower binding energies when interacting with ACE [19]. Additionally, antioxidant activity was identified as a property of bioactive peptides. [40] found that the novel peptides, SPSSS, SGTAV, and NSVAA, identified from pearl shell meat hydrolysate, exhibited high antioxidant activities.

The primary interacting residues at the ACE active site were categorized into three pockets including an S1 pocket (Ala 354, Glu 384, and Tyr 523), S2 pocket (Gln 281, His 353, Lys 511, His 513, and Tyr 520) and S'1 pocket (Glu 162) [47]. Notably, hydrogen bonding interactions are likely the strongest among these. An optimal ACE inhibitory peptide with high activity should be composed of hydrophobic and aromatic amino acids. The ratio of hydrophilic to hydrophobic amino acids in the peptide sequence plays a crucial role in ACE inhibitory activity. Hydrophilic amino-acid residues can potentially impede access of the peptide to the active site of ACE [48]. Moreover, peptides containing aromatic residues such as Trp, Phe, Tyr, or Pro and branched aliphatic amino acids are preferred for inhibition of ACE activity [49]. Furthermore, Asp and Gln within the FYDQ sequence can potentially enhance ACE inhibitory activity by forming a complex through chelation with zinc in its active site [50]. This binding mode illustrates that both peptides can be accommodated within the subdomain of ACE.

The molecular docking of Keap1 and tetrapeptides from cricket proteins showed a high number of hydrogen bonds and π-interactions. [51] found that PAGY isolated from Amur sturgeon skin gelatin showed scavenging activities against free radicals. A tetrapeptide, AKRA, was identified from sesame flavor-type Baijiu and showed preventive effects against oxidative stress in HepG2 cells [41]. Binding of the active compound to the active site of Keap1 can prevent the nuclear translocation of Nrf2, leading to initiation of an antioxidant mechanism [30]. In this study, FVEG and FVDQ were found to interact with Keap1 protein molecules with low binding energies, suggesting their potential to enhance antioxidant enzyme activity and reduce oxidative stress.

The binding of FVEG and FYDQ with antioxidant enzymes, SOD, CAT, GSR, GPx1, and GPx4, showed high hydrogen bonding and hydrophobic interactions. [52] reported that the Arg 143 residue in the active site of SOD attracts and stabilizes the anionic superoxide substrate for a catalytic reaction. Since peptide FVEG formed a hydrogen bond with the Arg 143 residue of SOD, it might contribute to enhancing SOD activity. Finding peptide FYDQ with the SOD catalytic site involves the Lys 9 residue, as it has been reported as a binding site for phytochemical active substances from *Heliotropium indicum* Linn [53]. [54] reported that the hydrophilic residue Ser 198 guides H_2O_2 molecules to CAT active sites (His 75 and Asn 148) for reduction to water. Although FVEG and FYDQ do not directly interact with these amino acids within the active site of CAT, they still form interactions in the surrounding area through hydrogen bonds and π-bonds, contributing to a molecular recognition mechanism with hydrogen peroxide [55]. Since FVEG and FYDQ do not interact directly with the active site of CAT, the activity of CAT can be increased by the influence of the Nrf2-Keap1 mechanism.

The Tyr 197 residue, located in the NADPH binding site of GSR, plays a crucial role in catalytic activity by acting as an entrance for NADPH and stabilizing the complex [56]. [57] reported that syringic acid and syringaldehyde, active substances from plants, formed a complex with GPx through hydrogen bonds. Moreover, binding of our FVEG and FYDQ peptides with the Lys 131 and Thr 152 residues of GPx catalytic sites via hydrogen bonds, respectively, is consistent with the binding sites of botulin to the catalytic sites of GPx as previously reported [54]. FYDQ also showed a binding site with not only the Asn 151 residue but also the His 93 residue at the catalytic sites of GPx, as previously reported [55]. This result indicates that the FVEG and FYDQ tetrapeptides can potentially enhance GPx activity.

5. Conclusions

The present study demonstrates that fractionation of cricket protein hydrolysate by size exclusion chromatography provides low molecular weight novel bioactive peptides with essential functions for health. These peptide fractions have strong antioxidant capacities to scavenge accumulated ROS and upregulate antioxidant related gene expression. Not only high antioxidant activities but also ACE inhibitory properties were observed through chemical and cellular assays from peptides in the fraction F45B. The mechanisms of the two most effective antioxidant and ACE inhibition novel tetrapeptides, FVEG and FYDQ, were investigated using molecular docking. Although further studies are needed to elucidate other functions of novel bioactive peptides with improved bioavailability through physiological digestion and uptake conditions, our results provide strong support for the use of cricket protein as a source of novel bioactive peptides for health products.

Data availability statement

The data that support this study are available from the corresponding author, [Natteewan Udomsil], upon reasonable request.

CRediT authorship contribution statement

Ratasark Summart: Writing – original draft. **Sumeth Imsoonthornruksa:** Formal analysis, Data curation. **Jirawat Yongsa-watdigul:** Supervision, Funding acquisition. **Mariena Ketudat-Cairns:** Supervision, Investigation. **Natteewan Udomsil:** Writing – review & editing, Validation, Investigation, Conceptualization.

Declaration of competing interest

The authors declared that they have no conflicts of interest in this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the submitted manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35156.

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