DOI: 10.26599/FSHW.2026.9251173

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Structural characterization, physicochemical properties and antihypertensive efficacy evaluation of four novel ACE inhibitory peptides derived from *Flammulina velutipes*

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ABSTRACT: Four peptides with ACE inhibitory activity from the edible fungus *Flammulina velutipes* were prepared by proteolysis to develop high-activity functional peptides. Their secondary structures were analyzed by Fourier transform infrared (FTIR) spectroscopy and Circular dichroism (CD). The effects of pH, temperature, metal ions and gastrointestinal digestion on their ACE inhibitory activities were determined. Their antihypertensive effects were evaluated by an H_2O_2-induced oxidative stress injury, human umbilical vein endothelial cells (HUVECs) model. All four peptides had relatively stable secondary structures. The peptides with sequences FDGY and FHPGY had similar peak shapes but the FHPGY had the highest random coil content. The FAGGP peptide had a relatively flat peak shape and a high random coil content. The WADP peptide had the highest β-sheet content, the lowest random coil content, and the most stable secondary structure. The four peptides showed good resistance to pH and temperature changes, the presence of metal ions and gastrointestinal digestion. FHPGY had good ACE inhibitory activity in all tested pH and temperature ranges. Metal ions had the least effect on the ACE inhibitory activity of FHPGY. After gastrointestinal digestion, the ACE inhibitory activity of FHPGY was above 95%. At concentrations up to 3 mM, the four *F. velutipes*-derived ACE inhibitory peptides had no significant negative effect on HUVECs viability, while increasing intracellular NO and SOD levels and reducing ET-1 and MDA production. The four peptides all had strong antihypertensive effects. FHPGY increased the secretion of NO and reduced the ET-1 secretion of by 97.69% and 109.59%, respectively, of that of the positive control captopril, and appeared to have the potential to be developed into an effective ACE inhibitory peptide for medicinal use.

Keywords: *Flammulina velutipes*; ACE inhibitory peptide; Human umbilical vein endothelial cells; structural characterization; physicochemical properties; antihypertensive effect

1. Introduction

Hypertension is a chronic condition with a complex pathogenesis and is a major factor leading to various cardiovascular diseases and renal failure[1,2]. The renin-angiotensin system (RAS) and kallikrein-kinin system (KKS) are the primary regulators of blood pressure[3-5]. Angiotensin I-converting enzyme (ACE) is a key focus for medications that lower blood pressure[0]. Its mechanism of action is to catalyze the conversion of angiotensin I (Ang I) into the potent vasoconstrictor angiotensin II (Ang II).

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Received 1 December 2025
Received in revised from 15 January 2026
Accepted 29 January 2026

Therefore, inhibition of ACE can effectively reduce the production of Ang II, thereby achieving reduced blood[7,8].

Angiotensin I-converting enzyme inhibitor (ACEI) is bioactive compounds that can regulate blood pressure by inhibiting ACE activity[0]; they can be obtained by isolation from food grade microorganisms or by chemical synthesis. Synthetic drugs are extensively employed in the clinical treatment of hypertension and other diseases, but they may also cause adverse reactions, such as dry cough, dizziness, and angioneurotic edema[10,11]. Angiotensin-converting enzyme inhibitory peptides (ACEIPs) have attracted much attention in the development of functional foods because of their safety and ease of administration through the daily diet[12-14]. To date, a variety of antihypertensive peptides have been obtained from different sources, including soybean protein hydrolysates[0], small-aroma chicken[0], rice[0], Tenebrio Molitor protein[0], etc. These bioactive peptides inhibit the activity of ACE and the conversion of Ang I to Ang II, thereby effectively reducing blood pressure, and are therefore, a potential alternative to chemically synthesized drugs[7,19].

Flammulina velutipes is rich in nutrients such as protein, and a popular, commercially valuable medicinal and edible fungus[20-22]. ACEIPs have been isolated from a variety of edible fungi. For examples, Zeng et al.[0] and Cavenaghi et al.[0] isolated and identified peptides with ACE inhibitory activity from the hydrolysates of *Morchella esculenta* and *Pleurotus eryngii*, respectively. Song et al.[0] isolated and purified a novel ACEIP, APPLRP from *Grifola frondosa*, and verified its antihypertensive activity *in vivo* in an Ang II-induced hypertensive zebrafish model. However, although many bioactive peptides have been isolated from various edible fungi, none have been developed into a functional peptide product. Research on new ACEIPs derived from enzymic hydrolysates of edible fungi, such as *F. velutipes* is also limited. There is a lack of research on the *in vitro* stability of ACEIPs and verification of their antihypertensive effects at the cellular level. This limits their development into food-borne functional peptide products, resulting in the potential value of *F. velutipes* protein not being fully developed and utilized.

Our laboratory previously isolated and identified four new ACEIPs (FAGGP, FHPGY, FDGY and WADP) from an enzymic hydrolysate of *F. velutipes* protein[0], and elucidated their molecular mechanism of high blood pressure reduction, by *in silico* molecular docking. However, information on the secondary structure, *in vitro* stability and antihypertensive efficacy of these peptides is still largely unknown. Therefore, this study explored the effects of temperature, pH value and metal ions on the ACE inhibitory activity of the peptides. Their stability in the digestive tract was evaluated by simulating the gastrointestinal digestive environment *in vitro*, and their secondary structure was analyzed by Fourier transform infrared (FTIR) spectroscopy and Circular dichroism (CD). The effects of ACEIPs on the secretion of intracellular regulatory factors NO and ET-1, as well as changes of SOD activity and MDA content were determined using a cellular model of oxidative stress injury. Their protective effect against oxidative damage to human umbilical vein endothelial cells (HUVECs) and their hypotensive effects are discussed, to provide a

theoretical basis for the development and utilization of functional peptide-containing foods derived from *F. velutipes*.

2. Materials and Methods

2.1 Materials and reagents

F. velutipes ACEIPs (FAGGP, FHPGY, FDGY, WADP) were synthesized by Wuhan Dangang Biotechnology Co., Ltd. (Wuhan, China), at >98% purity. Human umbilical vein endothelial cells (HUVECs) and ECM complete medium were from Starfish Biotechnology Co., Ltd. (Suzhou, China). Angiotensin Converting Enzyme (ACE) was from Solarbio Science & Technology Co., Ltd., (Beijing, China). n-[3-(2-furylacryloyl)]-L-phenylalanyl-glycine-glycine (FAPGG), captopril, trypsin (4000 u/g), pepsin (30000 u/g) and penicillin-streptomycin mixed solution were from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). 0.25% trypsin EDTA and the CCK-8 assay kit were from Beijing Lanjieke Technology Co., Ltd. (Beijing, China).

2.2 Analysis of the secondary structure of ACEIPs from F. velutipes

2.2.1 Determination of FTIR

The secondary structure of ACEIPs from *F. velutipes* was determined by FTIR spectroscopy[0]. Potassium bromide (100 mg) was ground in an agate mortar with freeze-dried synthetic peptide powder (1 mg), pressed into 1-2 mm sheets and immediately scanned 32 times in a Fourier transform infrared spectrometer. The scanning wavenumber range was 4000–400 cm^{-1}, and the resolution was 4 cm^{-1}. The spectrum was analyzed by PeakFit V4.12 software, and the relative content of each secondary structure was calculated from the integrated peak areas.

2.2.2 Determination of CD

Each ACEIP, as a solution (0.2 mg/mL) in a 1 mm optical path quartz cuvette, was scanned over a wavelength range of 190–260 nm, for circular dichroism determination. The secondary structures present in each were determined and their relative content calculated.

2.3 Determination of ACE inhibitory activity

ACE inhibitory activity was determined as described previously[0]. FAPGG solution (1 mmol/L), ACE solution (0.1 U/mL) and sample solution were prepared using HEPES buffer (pH 8.2, 80 mmol/L). The assays were performed in 96-well plates, according to Table 1. The absorbance value at the wavelength of 340 nm at 37 ◦C and the absorbance value after 30 min of reaction were measured. The ACE inhibition activity was calculated using equation (1):

$$\text{ACE inhibition activity } (\%) = (1 - \Delta A/\Delta B) \times 100 \quad (1)$$

In the formula: ΔA (ΔA = A_1-A_2) is the absorbance change value of the sample pore solution; ΔB (ΔB = B_1-B_2) was the absorbance change value of the control pore solution. A_1 and B_1 were the absorbance values measured at the wavelength of 340 nm. A_2 and B_2 were the absorbance values measured at the wavelength of 340 nm after reacting for 30 min at 37 °C.

Table 1. Determination of ACE inhibition rate

reagent	*A* sample（μL）	*B* control（μL）
ACE（0.1 U/mL）	10	10
FAPGG (1 mmol/L)	50	50
ACEI	40	0
HEPES buffer（80 mmol/L）	0	40

ACEI：*F. velutipes* ACEIPs

2.4 Stability of F. velutipes ACEIPs

2.4.1 Effect of pH on ACEIP structural stability

The secondary structural stability of ACEIPs was evaluated by determining changes in ACE inhibitory activity under different pH conditions. The pH of each ACEIP solution (1 mg/mL, HEPES assay buffer) was adjusted to 3, 5, 7, 9, or 11. After standing at room temperature for 1 h, the ACE inhibitory activity was measured (Section 2.3).

2.4.2 Effect of temperature on structural stability

Changes in ACE activity were determined at different temperatures. Each ACEIP solution (1 mg/mL) was placed in a water bath set to 4, 25, 37, 65, or 100 °C, for one hour. After cooling to room temperature, the ACE inhibitory activity was determined.

2.4.3 Effect of metal ions on structural stability

Changes in ACE activity were determined in the presence of various metal ions. Each ACEIP solution (1 mg/mL) was shaken and incubated for 1 hour at room temperature, with K^+, Cu^{2+}, Ca^{2+}, or Mg^{2+} ions, at concentrations of 2, 4, 6, 8, and 10 mmol/L. After incubation, the ACE inhibitory activity was measured.

2.4.4 Effects of simulated digestion in vitro on chemical and structural stability

Simulated gastric digestion: the pH of each ACEIP solution (1 mg/mL) was adjusted to 2.0, then 2% pepsin was added. After 1 h of reaction at 37 °C, the digestion was terminated by heating in a boiling water bath for 10 min, cooling to room temperature and centrifugation at 10280 × g for 10 min, then the supernatant was retained for determination of the ACE inhibitory activity.

Simulated intestinal digestion: the pH of each ACEIP solution (1 mg/mL) was adjusted to 8.0, then 2% trypsin was added. After 1 h of reaction at 37 °C, the digestion was terminated by heating in a boiling water bath for 10 min, cooling to room temperature and centrifugation at 10280 × g for 10 min, then the supernatant was retained for determination of the ACE inhibition activity.

Simulated gastric digestion + intestinal digestion: each ACEIP solution (1 mg/mL) was subjected to simulated gastric digestion, then to simulated intestinal digestion, both as described above. The supernatant was retained for determination of the ACE inhibition activity.

2.5 Determination of the antihypertensive effect of ACEIPs using the H_2O_2-induced oxidative stress injury cellular model

2.5.1 HUVECs toxicity determination

HUVECs were cultured in ECM complete medium in an incubator at 37 °C and 5% CO_2. During cell culture, the cell state was monitored, the medium removed every 2-3 days, the cells rinsed with PBS buffer three times and fresh medium added.

When the cell confluence reached 80%, the cells were digested with trypsin, washed and resuspended in ECM complete medium. The cells were inoculated into 96-well plates (5×10^3 cells/well, 100 μL) for culture. After 24 h, the medium was discarded, and each peptide (FAGGP, FHPGY, FDGY, WADP) at different concentrations (0, 0.125, 0.5, 1, 2 and 3 mM), in serum-free ECM medium (100 μL) was added to each well. The effect of ACEIPs on the viability of HUVECs was determined using CCK-8.

2.5.2 Establishment of oxidative stress injury cell model

HUVECs were seeded in 96-well plates (5×10^3 cells, 100 μL/well). After 24 h, the old medium was discarded, and H_2O_2 solution (100 μL, serum-free medium configuration) with a final concentration of 0, 100, 300, 500, 700, 900, 1000 and 1100 μM was added to each well instead of serum-free ECM medium. Five replicate wells were set up at each concentration, and the cells were cultured for 24 h or 48 h. The old medium was removed, and the effect of H_2O_2 on the viability of HUVECs was evaluated using a CCK-8 assay kit.

2.5.3 Determination of nitric oxide (NO) content

HUVECs were inoculated into 6-well plates (2×10^5 cells, 2.5 mL/well). After culture in ECM complete medium for 24 h, the culture medium was discarded. The test conditions were as follows:

Blank control group: HUVECs cultured in serum-free ECM.

Model group: HUVECs cultured in 900 μM H_2O_2 (H_2O_2 solution instead of serum-free medium).

Positive control group: After HUVECs were cultured in 900 μM H_2O_2, 3 mM captopril solution was added for further culture.

Experimental group: HUVECs were cultured in 900 μM H_2O_2, and different concentrations (1, 2 and 3 mM) of each peptide (FAGGP, FHPGY, FDGY, WADP) were added for further culture.

After incubation, the supernatant was collected from each well, then the NO content of the supernatant was determined using an NO assay kit (Shanghai Preferred Biotechnology Co., Ltd., Shanghai, China). All the assay kits used on these samples were used according to the manufacturer's instructions.

2.5.4 Determination of Endothelin-1 (ET-1) content

After incubation as described in Section 2.5.3, the ET-1 content of the sample supernatant was determined using an ET-1 ELISA kit (Solarbio Science & Technology Co., Ltd., Beijing, China).

2.5.5 Determination of malondialdehyde (MDA) content

After incubation as described in Section 2.5.3, the content of MDA in the sample supernatant was determined using an MDA assay kit (Solarbio Science & Technology Co., Ltd., Beijing, China).

2.5.6 Determination of superoxide dismutase (SOD) content

After incubation as described in Section 2.5.3, the SOD content of the sample supernatant was determined using the SOD assay kit (Solarbio Science & Technology Co., Ltd., Beijing, China).

2.6 Data analysis

Each experiment was performed in triplicate and the results are expressed in the form of mean ± SD. One-way analysis of variance (ANOVA) was performed using Duncan's multiple comparison test and IBM SPSS Statistics 27. When $P < 0.05$, the difference was considered statistically significant and Origin 2021 was used for data processing.

3. Results and Discussion

3.1 Secondary structure analysis of ACEIPs from F. velutipes

3.1.1 FTIR analysis

Different protein functional groups selectively absorb different infrared wavelengths, resulting in different characteristic absorption peaks[29,30]. The peak at 1600–1700 cm^{-1} is the amide I band (Amide I), which is the most informative peak for analyzing the secondary structure of peptides[0]. The signal is mainly derived from the stretching vibration of the C=O group in the peptide bond. The relative content of secondary structural elements such as α-helix and β-sheet in a peptide can be accurately quantified by analyzing the spectral characteristics of the amide I band in the 1600–1700 cm^{-1} range[0].

The four peptides had absorption peaks in the characteristic peptide functional group regions (Fig. 1). The peaks around 3416 cm^{-1} are characteristic of the stretching vibration of hydroxyl-OH or amino-NH, which indicate the presence of hydroxyl or amino hydrogen bonds in the peptide molecule. The peak around 3083 cm^{-1} is characteristic of the C-H stretching vibration, indicating the presence of alkyl groups. The strong peak at 1674 cm^{-1} is characteristic of the carbonyl C=O stretching vibration and corresponds to the amide I band. The strong peak at 1513 cm^{-1} is characteristic of the N-H bending vibration of the peptide bond (Amide II). The peak around 1206 cm^{-1} is characteristic of the C-O stretching vibration, which arises from ester or hydroxyl groups in polypeptide molecules.

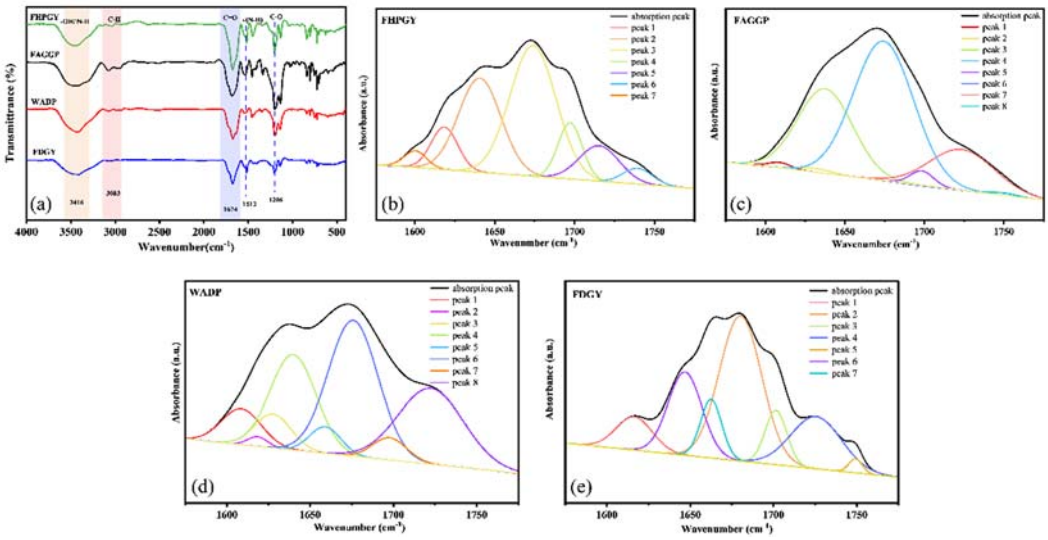

Fig. 1. Fourier transform infrared (FTIR) spectra of the ACEIPs. Complete FTIR spectra (a); expansion of the amide I region of FHPGY (b); FAGGP (c); WADP (d); FDGY (e).

WADP had the highest β-sheet content (50.97%), consistent with the most stable secondary structure. Its amide I band at 1674 cm^{-1} was sharp and split, consistent with the characteristics of β-sheet (Table 2). FDGY had the second highest β-sheet content (47.5%), consistent with the second highest stability. The β-sheet content of FAGGP was low, with a broad peak shape at 1674 cm^{-1}, and may have a higher α-helix content. FHPGY had the lowest β-sheet content (34.75%) and the highest random coil content (40.8%), indicated by the broadest peak at 1674 cm^{-1}, indicating a relatively unstable secondary structure[0].

Table 2. The secondary structure content distributions of the ACEIPs, calculated from the amide I band peak shape and area

peptide sequence	β-sheet/%	random coil/%	α-helix/%	β-turn/%
FDGY	47.5 ± 0.47^{b}	26.08 ± 0.27^{d}	18.27 ± 0.26^{bc}	8.05 ± 0.1^{b}
WADP	50.97 ± 0.1^{a}	26.73 ± 0.05^{c}	18.42 ± 0.04^{b}	3.85 ± 0.04^{d}
FAGGP	39.9 ± 0.32^{c}	31.29 ± 0.05^{b}	18.04 ± 0.05^{c}	10.52 ± 0.04^{a}
FHPGY	34.75 ± 0.03^{d}	40.8 ± 0.04^{a}	18.81 ± 0.03^{a}	5.62 ± 0.06^{c}

Different lowercase letters indicate significant differences ($P < 0.05$)

3.1.2 Circular dichroism (CD) analysis

Circular dichroism analysis can reveal the secondary structure characteristics of polypeptides, and reflect the conformation of polypeptides at the molecular level by the change of secondary structure content[0]. The secondary structure of the four peptides, as determined by circular dichroism, was dominated by β-sheet (39–48%) and the proportion of α-helix was generally low, indicating a stable secondary structure[0] (Table 3). WADP had the highest β-sheet content, the lowest random coil content and the most ordered and stable secondary structure, consistent with the findings from FTIR spectroscopy (Section 3.1.1). All four peptides had strong negative peaks at around 216 nm, characteristic of a relatively high β-sheet content (Fig. 2). WADP had the most intense negative peak around 216 nm and the positive peak around 222 nm was also the most intense, consistent with it having the highest β-sheet content. The peak shape of the peptide FAGGP was relatively flat, indicating a lower β-sheet content and a higher random coil content than WADP. The peak shapes of FDGY and FHPGY were similar, indicating similar secondary structures, with around 35% random coil.

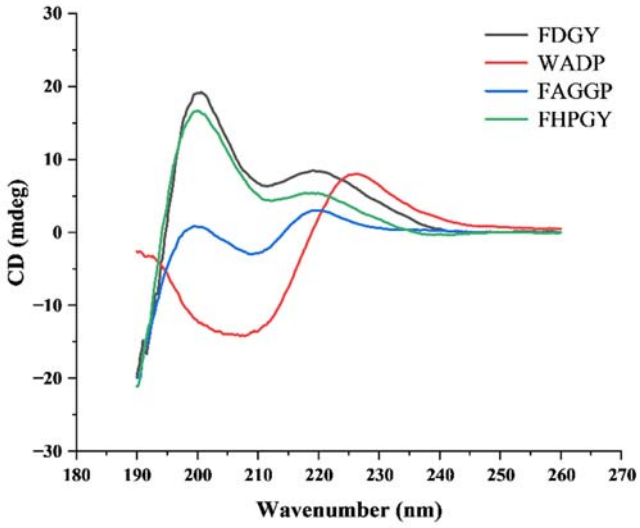

Fig. 2. Circular dichroism spectra of ACEIPs

Table 3. Circular dichroism determination of secondary structure content of ACEIPs

peptide sequence	β-sheet/%	random coil/%	α-helix/%	β-turn/%
FDGY	41.33 ± 0.05^{c}	35.33 ± 0.28^{b}	9.47 ± 0.05^{d}	13.87 ± 0.36^{d}
WADP	48.35 ± 0.27^{a}	21.98 ± 0.15^{d}	13.11 ± 0.36^{a}	16.48 ± 0.05^{a}
FAGGP	44.53 ± 0.14^{b}	29.48 ± 0.04^{c}	10.94 ± 0.04^{b}	15.05 ± 0.05^{b}
FHPGY	39.66 ± 0.03^{d}	35.88 ± 0.03^{a}	10.31 ± 0.03^{c}	14.16 ± 0.14^{c}

Different lowercase letters indicate significant differences ($P < 0.05$)

To sum up, this study used two techniques, FTIR and CD, to analyze the secondary structure of the four ACEIPs from two perspectives, namely, vibrational frequency and optical characteristics. The objective results of the two techniques characterized the peptide structure from different dimensions, which can reveal the secondary structural characteristics of ACEIPs more comprehensively [36,37]. It can be seen from the results that although there are some differences in the quantitative values obtained by the two techniques, such as the proportion of β-sheet and random coil in the secondary structure of the four ACEIPs, the trends of the detection results are consistent. This is mainly due to the different detection principles, the different states of the samples required for detection, and the different data calculation methods used by both.

3.2 Stability of F. velutipes ACEIPs

3.2.1 Effect of pH on ACE inhibitory activity of ACEIPs

The effect of pH on ACE inhibitory activity was determined (Fig. 3A). As the pH increased from 3 to 11, the ACE inhibitory activity of all four peptides initially increased, peaked, then decreased. Notably, the ACE inhibitory activity of FHPGY was >80% at all tested pHs, i.e., it was least affected by pH changes. The ACE inhibitory activity of FAGGP was >80% in weak acid, neutral and weak base conditions. The ACE inhibitory activity of WADP was >80% over the pH range of 5-7, whereas that of FDGY was barely above 80%, even under neutral conditions, so FDGY was most affected by pH. In summary, FHPGY had good ACE inhibitory activity over the widest pH range. FAGGP would be suitable for application and storage in weak acid, neutral and weak base food environments. WADP would be suitable for application and storage in weak acid and neutral environments, whereas FDGY would only be suitable for application in neutral environments.

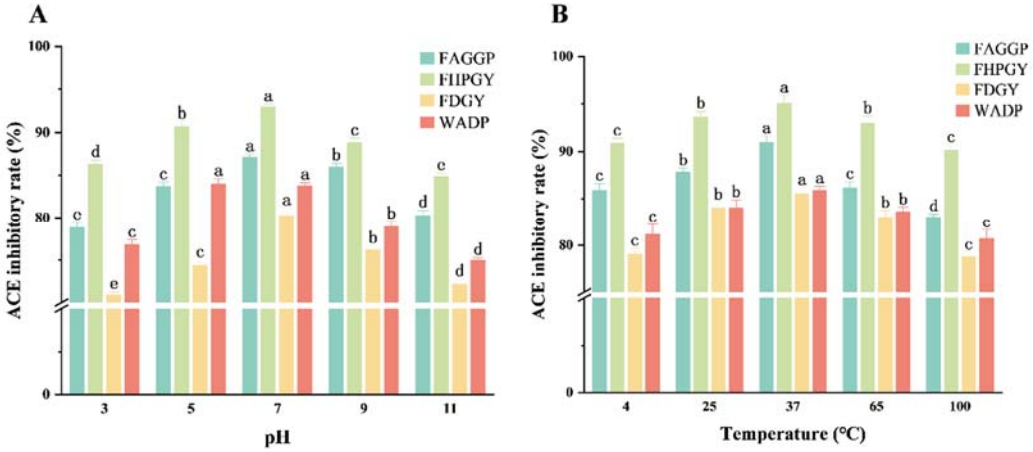

Fig. 3. Effect of pH and temperature on the ACE inhibitory activity of *F. velutipes* ACEIPs. A: Effect of pH on the ACE inhibitory activity of *F. velutipes* ACEIPs. B: Effect of temperature on the ACE inhibitory activity of *F. velutipes* ACEIPs. Note: The lowercase letters in this figure indicate significant activity differences between different pH or temperature treatments of the same peptide ($P < 0.05$). The same as below.

3.2.2 Effect of temperature on the ACE inhibitory activity of F. velutipes ACEIPs

The effects of temperature on the ACE inhibitory activity of *F. velutipes* ACEIPs was determined (Fig. 3B). Apart from FDGY, the activity of the peptides was >80% of control at all tested temperatures. The activity of FHPGY was >90%, at all tested temperatures, indicating that this peptide has high thermal stability and would be resistant to inactivation during heat treatment in food processing applications. The activity of FDGY at 100 °C was only 78.73 ± 0.54%, indicating that it may be ineffective in a high temperature environment. It appears that high temperature can disrupt the secondary structure of ACEIPs, thereby reducing their binding strength to ACE and consequently, their inhibitory activity. In general, however, temperature had a relatively small effect on the ACE inhibitory activity of the ACEIPs.

3.2.3 Effect of metal ions on the ACE inhibitory activity of F. velutipes ACEIPs

The effect of different metal ions on the ACE inhibitory activity of *F. velutipes* ACEIPs was determined (Fig. 4). The ACE inhibitory activity of all four peptides decreased with increasing metal ion concentration. Cu^{2+} influenced ACEIPs activity relatively strongly, especially with FAGGP; at a Cu^{2+} concentration of 250 μg/mL, ACE inhibition activity decreased by 21.12%, compared with 50 μg/mL. In addition, Ca^{2+} decreased the ACE inhibitory activity of FAGGP relatively strongly. In summary, Cu^{2+} and Ca^{2+} had a relatively strong effect on the ACE inhibition rate of the four peptides, followed by Mg^{2+}, with K^{+} having the least effect.

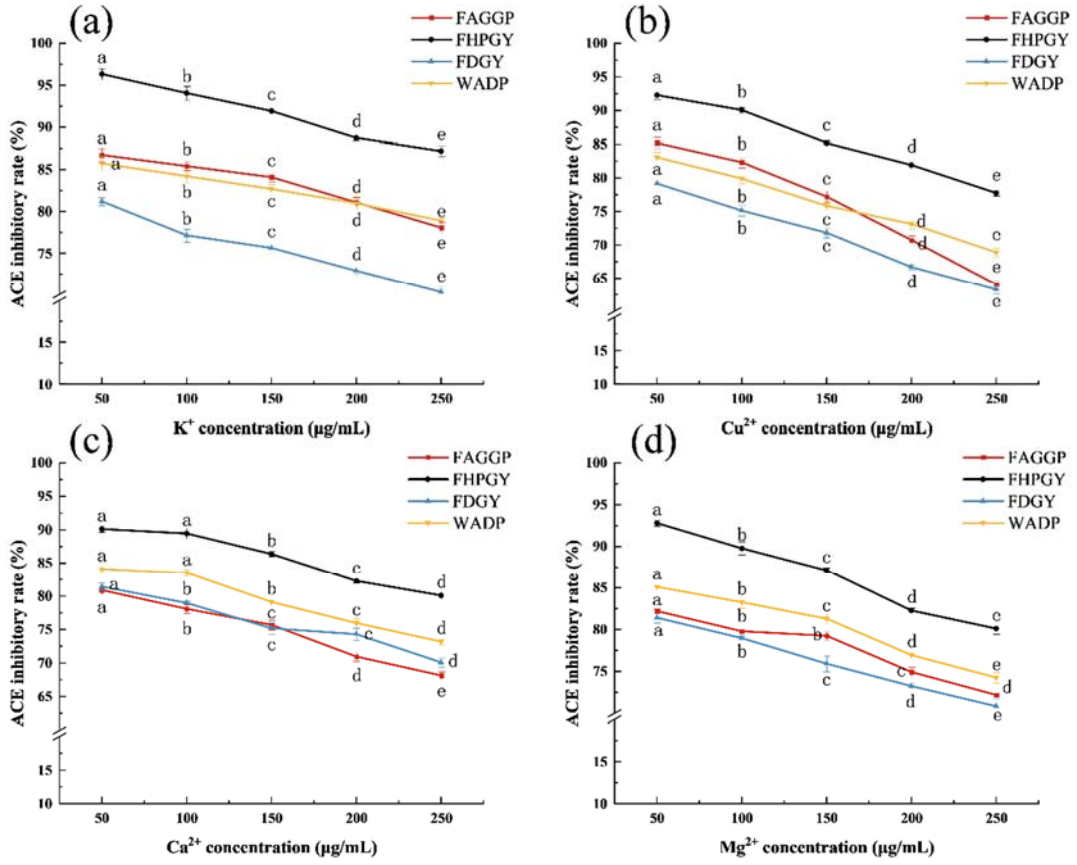

Fig. 4. Effect of metal ions on the ACE inhibitory activity of *F. velutipes* ACEIPs. K^{+} (a); Cu^{2+} (b); Ca^{2+} (c); Mg^{2+} (d). The lowercase letters in the figure indicate significant activity differences between different metal ions treatments of the same peptide ($P < 0.05$).

3.2.4 Effect of simulated digestion in vitro on the ACE inhibitory activity of F. velutipes ACEIPs

The antihypertensive effect of ACEIPs *in vivo* depends on their resistance to proteolysis by the digestive enzymes pepsin and trypsin and maintenance of their biological activity[0]. After 1 h of simulated gastric enzyme digestion, the ACE inhibitory activity of all four peptides increased significantly and was >90% (Fig. 5). The ACE inhibitory activity of FHPGY increased to 101.9 ± 0.60%. After 1 h of simulated intestinal digestion, the ACE inhibitory activity of WADP was the least affected, and the activity of all four peptides increased after both gastric and intestinal digestion. The four peptides can maintain good ACE inhibition under different gastrointestinal digestion conditions, which may be related to their short peptides[0].

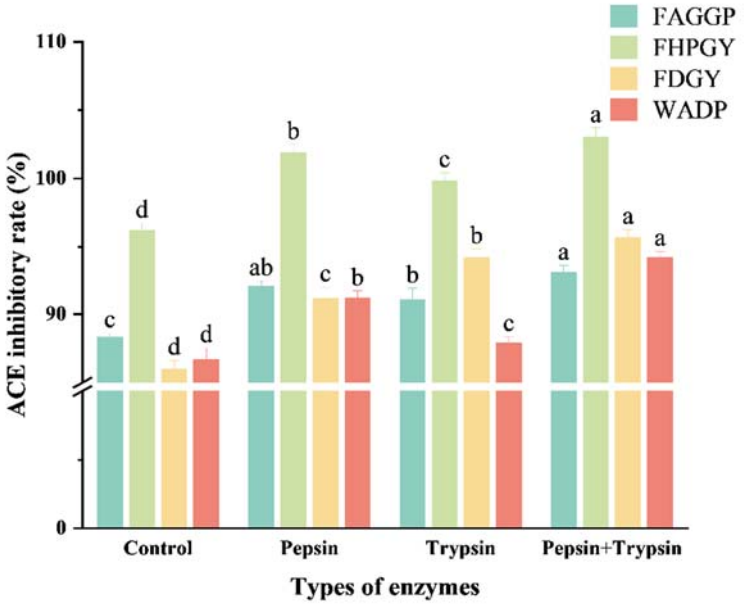

Fig. 5. Effect of simulated digestion *in vitro* on the stability of *F. velutipes* ACEIPs. The lowercase letters in the figure indicate significant activity differences between different simulated digestion *in vitro* treatments of the same peptide ($P < 0.05$).

In summary, FHPGY had good ACE inhibitory activity in all tested pH and temperature ranges. Metal ions had the least effect on its ACE inhibitory activity. After gastrointestinal digestion, the ACE inhibitory activity of FHPGY was still maintained above 95%. That is to say, FHPGY showed stronger resistance to pH, temperature, metal ions and gastrointestinal digestion than that of other three peptides.

3.3 Validation of antihypertensive effect of F. velutipes ACEIPs

3.3.1 Effect of different ACEIPs concentrations on HUVECs viability

Determination of the survival rate of ACEIP-treated HUVECs can be used evaluate any toxic effect of ACEIPs on the cells[0], as well as determining the appropriate concentration range for subsequent experiments. At an ACEIP concentration of 3 mM, HUVECs viability was >90%, indicating that these peptides have no significant cytotoxicity to HUVECs at this concentration (Fig. 6A). Therefore, subsequent antihypertensive effects were determined at ACEIP concentrations of 1, 2 and 3 mM.

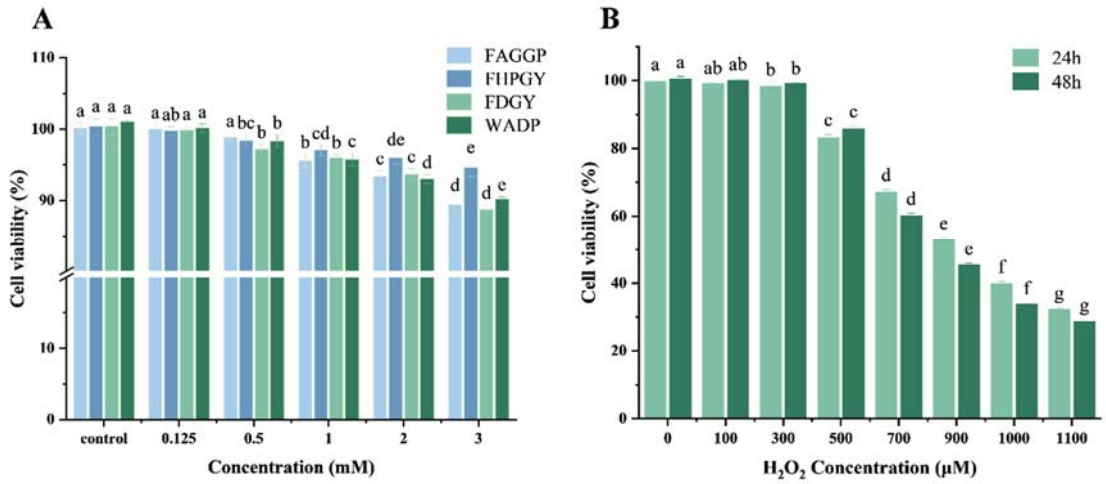

Fig. 6. The effect of different ACEIPs and H_2O_2 concentrations on HUVECs viability. A: The effect of ACEIPs on the viability of HUVECs. B: Effect of H_2O_2 treatment concentration on HUVECs viability. The lowercase letters in the figure indicate significant cell viability differences between different concentration treatment groups ($P < 0.05$).

3.3.2 Screening suitable conditions for H_2O_2-induced oxidative stress injury cell model

Measurement of HUVECs viability directly reflects its physiological state under oxidative stress, and it is often used as an important index to evaluate the sensitivity of cells to oxidative stress[0]. Over the range of 100–1100 μM H_2O_2, HUVECs viability decreased with increasing H_2O_2 concentration, after both 24 and 48 h treatment (Fig. 6B). At 900 μM H_2O_2, HUVECs viability decreased to 52.89% after 24 h and 45.49% after 48 h. Therefore, 900 μM H_2O_2 treatment for 24 h, resulting in cell viability close to 50%, was selected as the optimal condition for establishing the cell model.

3.3.3 Effect of F. velutipes ACEIPs on nitric oxide (NO) secretion by HUVECs

Nitric oxide (NO) has a variety of physiological functions in the human vascular system, such as regulating blood pressure, relaxing arteries and maintaining basic vascular tension, thereby helping to maintain normal blood flow[41,42]. However, if the activity of endothelial nitric oxide synthase (eNOS), which catalyzes the oxidation of arginine to NO is blocked, this results in insufficient NO production. This may cause vascular endothelial dysfunction, which in turn induces hypertension and other cardiovascular diseases[43-45]. Compared with the blank control, the amount of NO secreted by the cells in the H_2O_2 model group was significantly reduced, indicating that the endothelial cells were damaged by H_2O_2, resulting in inhibition of NO production and secretion (Fig. 7). However, treatment with *F. velutipes* ACEIPs increased the secretion of NO with increased ACEIP concentration in a dose dependent manner. At an ACEIP concentration of 3 mM, the NO secretion of HUVECs treated with FAGGP, FHPGY, FDGY, or WADP was 66.47, 97.69, 75.38 and 62.44%, respectively, of that of the positive control captopril. The NO secretion from HUVECs treated with FHPGY was essentially the same as that of the positive control captopril, i.e., FHPGY had a strong inhibitory effect on endothelial cell damage caused by H_2O_2. Similarly, the ACE inhibitory peptide STAP11 (LPRS) obtained from protein hydrolysate of skipjack tuna (*Katsuwonus pelamis*) processing by-products, which also showed the ability to promote NO levels in HUVECs[0].

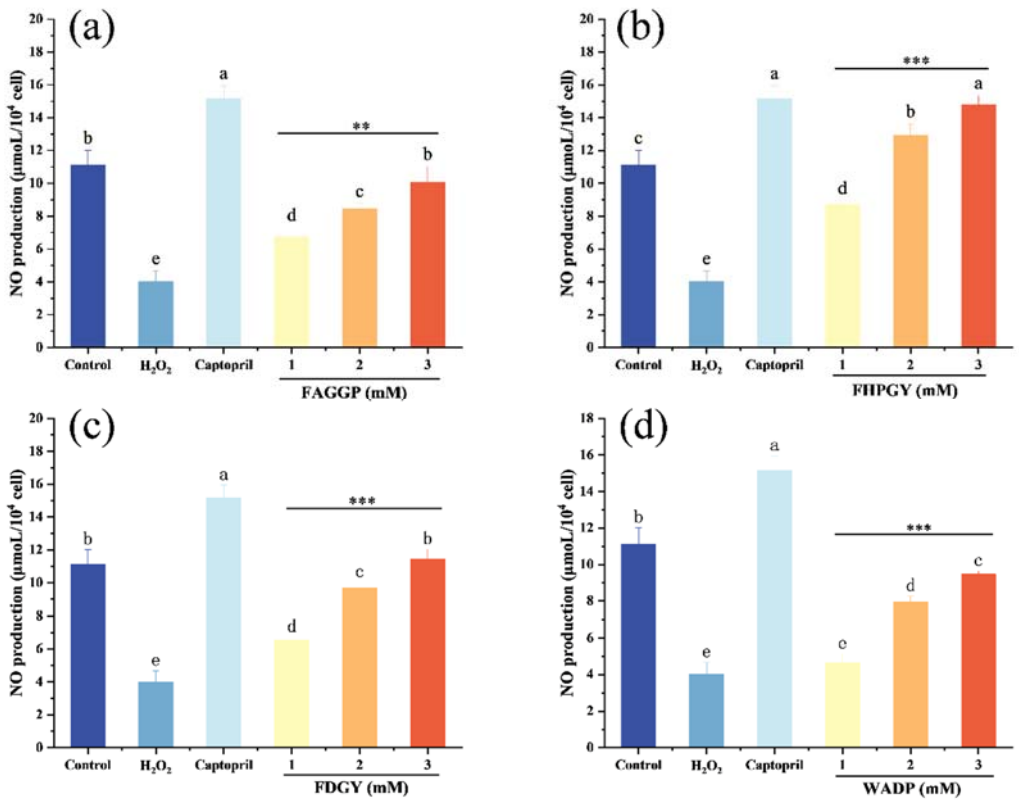

Fig. 7. Effect of *F. velutipes* ACEIPs concentration on the secretion of NO by HUVECs. FAGGP (a); FHPGY (b); FDGY (c); WADP (d). Different lowercase letters indicate significant differences in NO secretion between treatments ($P < 0.05$), and the same letter indicates that the difference is not significant. '**' means $P < 0.05$, '***' means $P < 0.001$.

3.3.4 Effect of F. velutipes ACEIPs on endothelin-1 (ET-1) secretion by HUVECs

ET-1 is a vasoconstriction factor secreted by endothelial cells, which induces vasoconstriction[0]. When endothelial cells are damaged, secretion of ET-1 increases, so ET-1 is an important indicator of endothelial dysfunction[0]. The inhibitory effect on ET-1 secretion gradually increased with ACEIP concentration in a dose dependent manner (Fig. 8). After treatment with FAGGP, FHPGY, FDGY and WADP at 3 mM, the ET-1 secretion by HUVECs was 133.51, 109.59, 169.09 and 186.54% of that of the positive control captopril, respectively. FAGGP and FHPGY reduced the secretion of ET-1 the most, indicating that they may be more effective than FDGY and WADP in regulating the secretion of ET-1 by HUVECs. These results are consisted with findings from other studies, such as the ACEIP from Wuyi rock tea residue, which also showed the ability to reduce ET-1 levels in HUVECs[0].

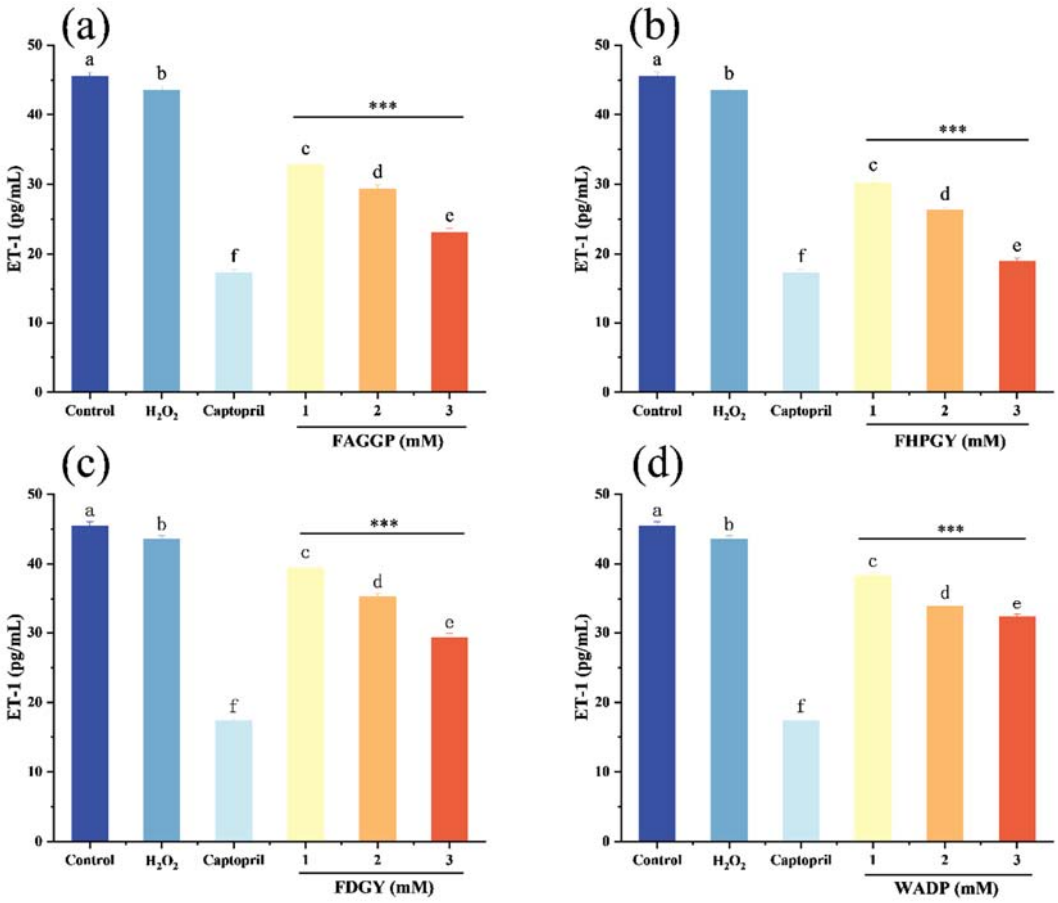

Fig. 8. Effect of *F. velutipes* ACEIPs concentration on the secretion of ET-1 by HUVECs. FAGGP (a); FHPGY (b); FDGY (c); WADP (d). Different lowercase letters indicate significant differences in ET-1 secretion between treatments ($P < 0.05$)'***' means $P < 0.001$.

3.3.5 Effect of F. velutipes ACEIPs on intracellular malondialdehyde (MDA) content

Under conditions of oxidative stress, polyunsaturated fatty acids in cell membranes are prone to lipid peroxidation and produce various lipid peroxides and their decomposition products, one of which is MDA, and which can induce cell structural damage. The extent of lipid peroxidation is usually evaluated by measuring the MDA content in the cells, a good indicator of the extent of oxidative damage[0]. Compared with the blank control, the MDA content of the H_2O_2 model cells was significantly higher, indicating that the cells were subjected to oxidative damage and increased lipid peroxidation (Fig. 9). However, treatment with an increased dose of ACEIPs decreased the HUVECs MDA content in a dose dependent manner, with FHPGY resulting in the greatest decrease. ACEIPs from *F. velutipes* significantly reduced MDA levels in injured HUVECs, which was consistent with the results reported in previous works[0].

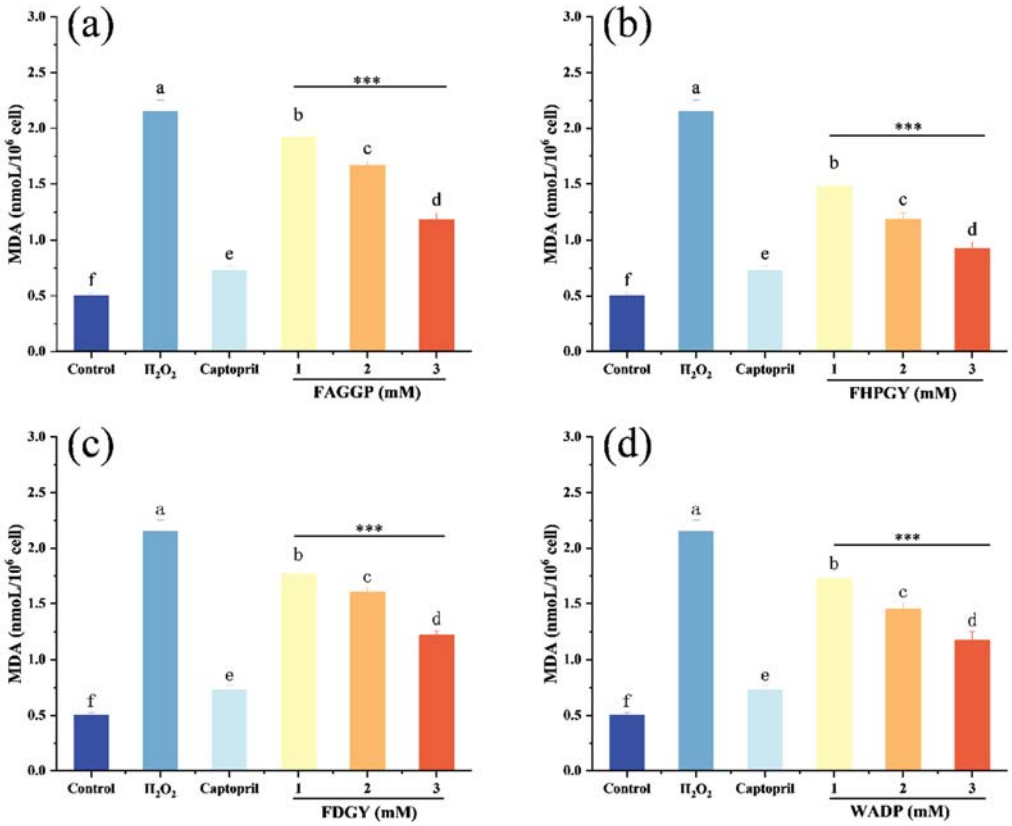

Fig. 9. Effect of *F. velutipes* ACEIPs on MDA content of H_2O_2-induced injured HUVECs. FAGGP (a); FHPGY (b); FDGY (c); WADP (d). Different lowercase letters in the figure indicate significant differences in MDA content between different treatments ($P < 0.05$). '***' means $P < 0.001$.

3.3.6 Effect of F. velutipes ACEIPs on intracellular superoxide dismutase (SOD) activity

The activity of SOD in patients with hypertension is significantly lower than that in healthy people. SOD can effectively scavenge superoxide anion free radicals, which not only helps to maintain the biological activity of NO, but is also an essential part of the oxidative stress response and maintenance of cellular homeostasis[52,53]. Compared with the blank control, the SOD activity in the H_2O_2 model group was significantly lower (Fig. 10). However, treatment with an increased dose of ACEIPs increased the HUVECs SOD activity in a dose dependent manner. Treatment with FHPGY and WADP at 3 mM increased the SOD activity of HUVECs to 94.90 and 88.22% of the positive control captopril, respectively. It appears that the four *F. velutipes* ACEIPs stimulate SOD production and probably other aspects of the oxidative stress response in HUVECs. The results of this study are consistent with previous research results. ACEIPs were isolated from the protein hydrolysate of blue mussel (*Mytilus edulis*) could provide protection to HUVECs against H_2O_2 damage by increasing SOD^0.

Overall, the NO and ET-1 secretion levels of HUVECs treated with FHPGY were basically equivalent to those of the positive control captopril, and FHPGY had a significant inhibitory effect on the MDA produced by HUVECs and a better protective effect on SOD activity.

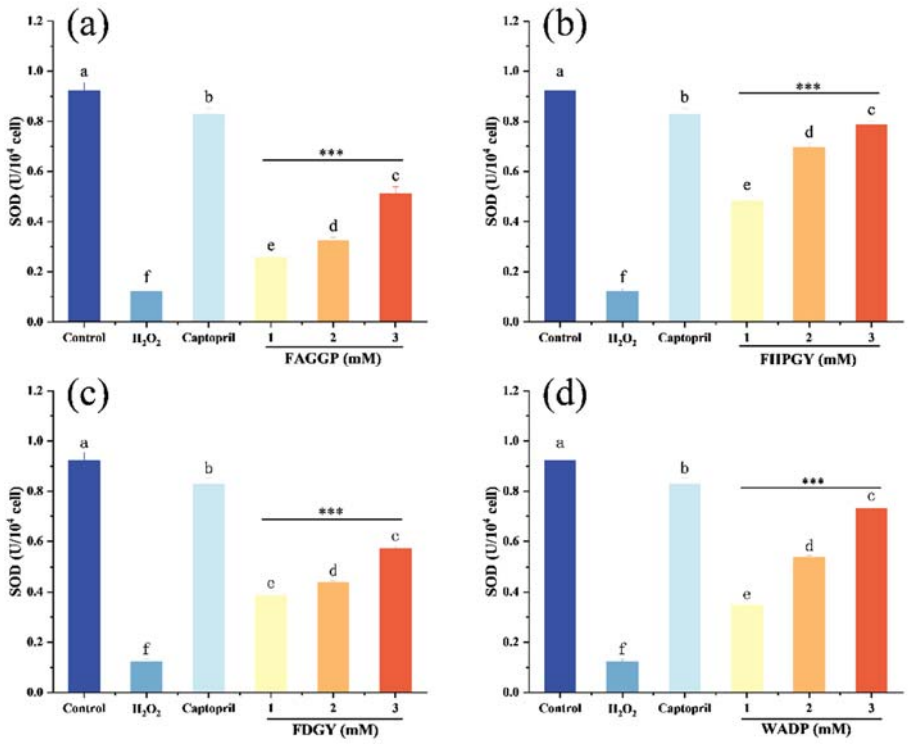

Fig. 10. Effect of *F. velutipes* ACEIPs on SOD activity in H_2O_2-induced injured HUVECs. FAGGP (a); FHPGY (b); FDGY (c); WADP (d). Different lowercase letters in the figure indicate significant differences in SOD activity between different treatments ($P < 0.05$). '***' means $P < 0.001$.

4. Conclusions

In this study, four ACEIPs derived from *F. velutipes* protein hydrolysates were investigated by structural characterization, analysis of physicochemical properties and determination of antihypertensive efficacy. The secondary structures of all four peptides was dominated by β-sheet and the peptide WADP had the highest β-sheet content and the greatest secondary structural stability. All four peptides retained good ACE inhibitory activity under various conditions of pH, temperature, the presence of metal ions, and after simulated human gastrointestinal digestion. The H_2O_2-induced HUVECs oxidative stress injury model showed that all four ACEIPs induced NO secretion, increased SOD activity, and inhibited the production of ET-1 and MDA. The peptide FHPGY was the most effective stimulator of NO secretion by HUVECs and the most effective suppressor of ET-1 secretion. Overall, FHPGY had the strongest antihypertensive effect and has potential for development into an effective ACE inhibitory peptide for medicinal use. However, its in vivo antihypertensive efficacy still needs to be tested in the future, so as to provide more reliable evidence for its industrial production.

Declaration of competing interest

There is no conflict of interests to declare.

Acknowledgement

This work was supported by the Science and Technology Major Special Project of Qingyang City (QY-STK-2024A-073).

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