Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.61622

Preparation of angiotensin I-converting enzyme (ACE) inhibitory peptides from Tie Guanyin tea residue protein using two-step enzymatic hydrolysis

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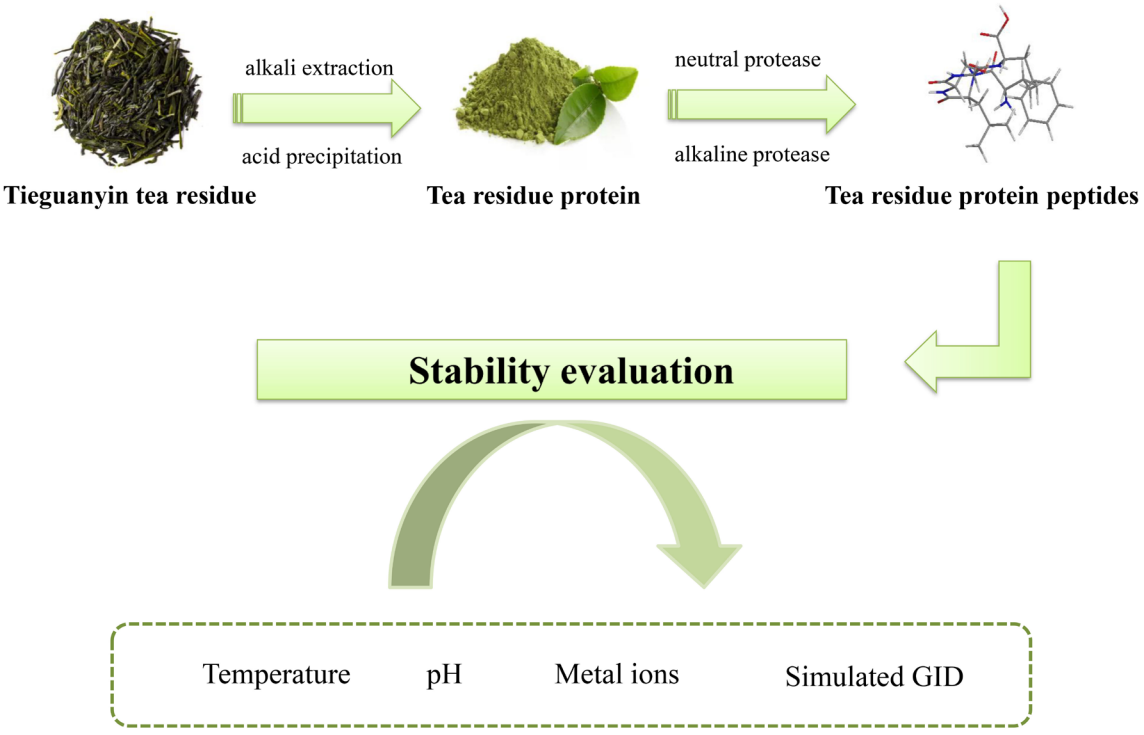
Abstract

In this study, we attempted to prepare novel angiotensin I-converting enzyme inhibitory (ACEI) peptides from Tie Guanyin tea residue protein (TTRP). Different proteases were used to hydrolyze TTRP to prepare ACEI peptides, neutral protease and alkaline protease hydrolysates exhibited higher ACEI activity and was chosen in further hydrolysis treatment. Response surface methodology (RSM) was used to optimize the enzymatic condition, and the results indicated that the optimal conditions of enzymatic hydrolysis were: 4% (w/v) of TTRP in water was hydrolyzed with 3500 U/g neutral protease at 50 °C and pH 6.5 for 2.5 h in the first step and then with 5000 U/g alkaline protease at 50 °C and pH 10.0 for 3 h in the second step. Under the optimum condition, the ACE inhibitory rate of the final products was 88.45% ± 0.45% at the concentration of 1.0 mg/mL. Moreover, ACEI peptides derived from TTRP showed good stability under different temperatures, pHs, metal ions and gastrointestinal digestion. These results indicated that the TTRP has potential to be used for preparing ACEI peptides.

Keywords: Tie Guanyin tea residue protein; two-step enzymatic hydrolysis; ACEI peptides; response surface methodology (RSM); stability.

Practical Application: It is practicable to use dual-enzyme hydrolysis in preparation of high-value ACE-inhibitory peptides. Tie Guanyin tea residue protein hydrolysates showed strong ACEI activity and stability, and could be explored as a potential source of ACEI peptides and applied in the research as well as the development of functional food.

Graphical Abstract



Received 15 Mar., 2022
Accepted 28 June, 2022
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1 Introduction

Tea is one of the most popular functional beverages globally and rich source of polyphenols (Wang et al., 2022). Tea leaves contain multiple secondary metabolites, such as polyphenols, caffeine, and amino acids, which are important functional food and beneficial to human health (Liu et al., 2022b). The bioactive chemicals in Tie Guanyin and their functions have been widely investigated (Zhang et al., 2018). Recently, researchers discovered that tea polyphenols supplementation improved the glucose metabolism and reversed the sick changes of intestinal flora (Liu et al., 2022a). In addition, the formation of tea aroma profiles of has been well studied (Guo et al., 2021). However, tea residue is considered as a by-product and waste in tea processing. It contains various amino acids and can be used as a potential high-quality source of bioactive protein hydrolysates (Chen et al., 2016). At present, the reuse methods of tea residue mainly focus on making animal feed, adsorbing heavy metal ions and inoculating cultivated fungi, while the research and development of other components and their derivatives in tea residue are still immature (Zhang et al., 2021). Tie Guanyin, a kind of semi-fermented oolong tea, is famous for the unique and elegant floral aroma, and ripe fruity flavor (Yao et al., 2021). Research indicated that Tie Guanyin extracts could alleviate Alzheimer's disease (AD) in a mouse model (Tu et al., 2022). Phenolics obtained from Tie Guanyin exhibited strong antioxidant capacity and inhibition of the growth of 4T1 murine breast cancer cells (Wang et al., 2019). Tie Guanyin tea residue has not been well studied. Therefore, it is very necessary to carry out further research on Tie Guanyin tea.

Nowadays, under the influence of dietary habits, work and rest rules, mental pressure and other factors, high blood pressure is becoming more and more common. Research shows that hypertension affects about 25% of the adult population in the world (Zhao et al., 2022). Hypertension is a kind of cardiovascular disease syndrome which is often accompanied by other diseases, such as atherosclerosis, myocardial infarction and stroke (Gallo et al., 2022). Angiotensin I-converting enzyme (ACE) regulates the balance between Renin-angiotensin system (RAS) and the Kallikrein-kinin system (KKS) which maintain normal blood pressure (Zhang et al., 2022). ACE inhibitory peptide can bind to the active center of ACE, inhibiting the activity of ACE and lowering blood pressure. ACE inhibitory peptide of natural origin is more effective than traditional antihypertensive drugs, without toxic side effects, and has no effect on normal blood pressure, which is important for the treatment of hypertension and human health (Su et al., 2021).

Although various ACEI peptides have been isolated from different food proteins, most of these researches mainly have focused on the separation and identification of the peptides and the relationship between the structure and function. However, little is known about the influence of the processing methods and storage conditions such as enzymatic hydrolysis, refining, concentration, inactivation of protease, drying and sterilization on the ACE inhibitory activity of peptides (Wang et al., 2021). Therefore, it's worth noting that these bioactive peptides must undergo gastrointestinal digestion (GID) and then reach their target sites in active form to exert biological effects *in vivo* (Ma & Xiong, 2009). Hence, before applying these bioactive peptides as nutraceuticals or functional food ingredients, their stability towards potential food processing conditions needs to be considered.

This paper focused on the further decomposition and preparation of tea residue protein, in order to obtain high activity tea residue protein derivatives. Meanwhile, only a few literatures mention the enzymatic preparation of tea residue peptides and verification of the antioxidant activity of the products, but there are few studies on tea residue ACE inhibitory peptides. Moreover, at present, single protease is mostly used to prepare ACEI inhibitory peptide, for example, Wu et al. (2016) hydrolyzed sweet sorghum grain protein with alkaline protease to produce ACEI peptides with high ACE inhibitory activity, while there is no report on the preparation of ACEI peptide by two-step enzymatic hydrolysis of tea residue protein. In this study, two-step enzymatic hydrolysis was used to prepare ACEI peptides from Tie Guanyin tea residue protein. Response surface method was applied to optimize the enzymatic conditions of double enzymes, so as to improve the ACE inhibitory ability of tea residue protein hydrolysate, which provided a research basis for further improving the development and utilization efficiency and economic value of tea residue.

2 Materials and methods

2.1 Materials and chemical regents

Tie Guanyin tea residue was provided by Fujian Anxi Huaxiang Tea Factory Co., Ltd (Quanzhou Fujian, China). ACE (from rabbit lung) and N-(3-(2-furyl) acryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Papain, neutral protease, trypsin, alkaline protease, flavor protease, acid protease and pepsin were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The chemicals used in this study were of analytical grade and supplied by Sinopharm Chemical Reagent Co., Ltd. unless stated otherwise.

2.2 Preparation of enzymatic hydrolysates and screening of the protease

Tea residue was crushed in a grinder and sieved through an 80 mesh. The powder (100 g) was then mixed with 3 L of 0.12 M NaOH and extracted at 60 °C for 2.5 h. Next, the mixture was centrifuged at 8000 rpm for 20 min at 4 °C and the supernatant was collected. The pH of the supernatant was adjusted to 3.5 and left undisturbed for 1 h at 4 °C. It was then centrifuged to obtain precipitate which was collected and redissolved with distilled water, while adjusted pH to 7.0 with 1M NaOH. The extract (Tie Guanyin tea residue protein, TTRP) was filtered and lyophilized as raw material for enzymatic hydrolysis.

The protein content of TTRP was 45.88% as determined by Kjeldahl method (Miner et al., 1995). TTRP was dissolved in ultrapure water at a protein concentration of 3% (w/v) and digested by seven different enzymes (papain, neutral protease, trypsin, alkaline protease, flavor protease, acid protease and pepsin) for 2 h under their optimal conditions (shown in Table S1), respectively. Afterward, the hydrolysate was heated in a boiling

water bath for 10 min to inactivate the enzyme. The precipitate was removed by centrifuged at 8000 rpm for 20 min at 4 °C and the hydrolysates were subjected to ACEI assay afterwards.

2.3 Double enzymatic hydrolysis

Two of seven enzymes with highly ACEI active hydrolysates were selected for double enzyme optimization. The double enzymatic hydrolysis was carried out as follows: TTRP (4%, w/v) was hydrolyzed by neutral protease, then heated to inactive, and further hydrolyzed by alkaline protease. The hydrolysates designated as Tie Guanyin tea residue protein peptides (TTRPPs) were lyophilized for further use.

2.4 Response surface design of double enzymatic stepwise hydrolysis

Single-factor experimental design

The variable factors in the single factor test included enzyme addition, substrate concentration, temperature, pH, and enzymatic hydrolysis time. When the other factors are deterministic and one of the factors is a variable, the effect of the variable on the test can be determined.

In the first step of enzymatic hydrolysis, the effect of enzymatic hydrolysis time on the ACE inhibition rate and degree of hydrolysis (DH) of Tie Guanyin tea residue protein hydrolysates (TTRPH) were investigated at the substrate concentration of 3% (w/v), pH 7.0, enzyme addition of 5000 U/g, and enzymatic hydrolysis time of 2.0 h, 2.5 h, 3.0 h, 3.5 h, 4.0 h. The effect of enzyme addition was investigated when the substrate concentration was set to 3% (w/v), the pH was 7.0, the enzymatic hydrolysis time was 2.5 h, and the enzyme addition was set to 2000 U/g, 2500 U/g, 3000 U/g, 3500 U/g, 4000 U/g. The effect of pH was investigated when the enzyme addition was set at 3000 U/g, the substrate concentration was 3% (w/v), the enzymatic hydrolysis time was 2.5 h, and the pH was set at 6.5, 7.0, 7.5, 8.0 and 8.5. The effect of substrate concentration was investigated when enzyme addition was set at 3000 U/g, the enzymatic hydrolysis time of 2.5 h, the pH was 7.0, and the substrate concentration was set at 1%, 2%, 3%, 4%, and 5%.

After first step of enzymatic hydrolysis, the optimal enzymatic hydrolysis conditions of alkaline protease were explored. The effect of enzyme addition on the ACE inhibition rate of TTRPH was investigated when the temperature was set to 50 °C, the pH was 10.0, the enzymatic hydrolysis time was 3 h, and the enzyme addition was set to 4000 U/g, 5000 U/g, 6000 U/g, 7000 U/g, 8000 U/g . The effect of enzymatic hydrolysis time was investigated at the pH of 10.0, the temperature of 50 °C, enzyme addition of 6000 U/g, and enzymatic hydrolysis time of 2.0 h, 2.5 h, 3.0 h, 3.5 h, 4.0 h.

RSM experimental design

Based on the results of single factor test, response surface analysis was used to optimize the hydrolysis conditions of neutral protease and alkaline protease. Box-Behnken design (BBD) was used to predict the effects of three independent variables in neutral protease hydrolysis, i.e., enzyme addition (A), pH value (B), and substrate concentration (C), on ACE inhibitory activity (Y). Central composite design (CCD) was then used to predict the effects of two independent variables in alkaline protease hydrolysis, i.e., enzyme addition (A) and hydrolysis time (B), on ACE inhibitory activity (Y). The variables and their levels are shown in Table 1 and Table 2 and a total of 17 and 13 runs with different conditions were designed and implemented, respectively, including 3 repetitions.

2.5 Determination of the Degree of Hydrolysis (DH)

The DH of the TTRPH was measured according to the method of Yang (Wen-bo, 2014) with some modification. Diluted samples (2 mL each) were pipetted into 10 mL test tubes and 1 mL of ninhydrin reagent was added, followed by mixing and incubation at boiling water for 15 min (to exclude light). At the end of the incubation, the solutions were cooled to room temperature, and 5 mL of 40% ethanol solution was added, mixed and incubated for 15 min. Finally, distilled water was used as a blank control and the absorbance was measured at 570 nm. Glycine was used to generate a standard curve. The DH values were calculated using Equation 1:

$$DH(\%) = \frac{h}{h_{tot}} \times 100\% \quad (1)$$

Where h is the number of peptide bonds broken and h_{tot} is the total number of bonds per unit weight with h_{tot} equaling 7.12 mmol/g of protein for tea (Nannan, 2021).

2.6 Assay of ACE-inhibitory activity

ACEI activity of TTRPPs was determined in accordance with Murray et al. (2004) with several modifications. The reaction system was comprised of 50 μL FAPGG (dissolved in 100 mM Tris–HCl buffer containing 0.3 M NaCl, pH 8.3), 10 μL ACE (0.1 U/mL) and 40 μL sample dissolved in the same buffer. The absorbance value A_1 before the reaction was measured by microplate reader at 340 nm, and then the absorbance value A_2 was measured after incubation at 37 °C for 30 min, and the change of absorbance was calculated as ΔA ($\Delta A = A_1 - A_2$), the ACE inhibitory rate (%) was calculated from Equation 2:

Table 1. Box-Behnken design factors and levels of neutral protease.

Level	Enzyme addition: A/(U/g)	pH: B	Substrate concentration: C/(%)
-1	2500	6.5	2
0	3000	7.0	3
1	3500	7.5	4

Table 2. Central composite design factors and levels of alkaline protease.

Factor	Code	Level				
		-1.414	-1	0	1	1.414
Addition: A/(U/g)	X1	4585.79	5000	6000	7000	7414.21
Time: B/(h)	X2	1.79	2	2.5	3	3.21

$$ACE\ inhibitory\ rate\ (\%) = \left(1 - \frac{\Delta A_a}{\Delta A_b}\right) \times 100 \quad (2)$$

Where ΔA_a is the change of absorbance within 30 min when the peptides were added; ΔA_b is the change of absorbance within 30 min when buffer solution was added.

2.7 Stability against heat, pH, metal ions and simulated GID treatments

Thermal, pH and metal ions stability

Thermal stability of TTRPPs was determined according to Yarnpakdee et al. (2015) with minor modifications. TTRPPs solution (2 mg/mL) was placed in a temperature controlled water bath at different temperatures (20, 40, 60, 80 and 100 °C) for 2 h. Thereafter, the solutions were centrifuged at 8000 rpm for 20 min at 4 °C to evaluate the ACE inhibitory activities of supernatant. Effects of pH treatments were assessed according to Zhu et al. (2014) with minor modifications. TTRPPs solution (2 mg/mL) adjusted to pH 2.0, 4.0, 6.0, 8.0 and 10.0 were incubated at 37 °C for 2 h. Following adjustment back to pH 7.0 using with 1 M HCl or 1 M NaOH and centrifuge at 8000 rpm for 20 min at 4 °C to evaluate the ACE inhibitory activities of supernatant. Effects of metal ions on the stability of TTRPPs were evaluated according to Lai et al. (2020a) with slight modifications. The lyophilized TTRPPs powder was dissolved in NaCl, $MgCl_2$, KCl, $CaCl_2$, and $CuCl_2$ at a concentration of 1 mM to make a final concentration of 2 mg/mL. After incubation for 24 h at room temperature, the solution was centrifuged at 8000 rpm for 20 min at 4 °C and the pH was adjusted to 7.0 for measurement of the ACE inhibitory rate.

Simulated GID stability

A pepsin–trypsin *in vitro* system simulating gastro-intestinal digestion was carried out essentially according to the method of Zhu et al. (2008). TTRPPs solution (2 mg/mL) was first hydrolyzed with pepsin (enzyme dosage of 3% w/v) at pH 2.0 and 37 °C for 3 h followed by hydrolysis with trypsin (enzyme dosage of 3% w/v) at pH 7.0 and 37 °C for 3 h. The reaction was deactivated by heating at 95 °C for 10 min. In addition, the method of trypsin digestion experiment (3% of protein hydrolysate, pH 6.8, 37 °C for 3 h) is the same as that of pepsin digestion test. The ACE inhibitory activity of peptides hydrolyzed with pepsin, trypsin and pepsin-trypsin were determined.

2.8 Statistical analysis

Experimental data were analyzed using IBM SPSS 26.0 Software (SPSS, Inc., Chicago, IL, USA). Data were analyzed by one-way ANOVA. GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze and to plot graphs. The results were presented as the mean ± standard deviation (SD) derived from three replications. The differences between the average values were determined using Duncan's multiple range test ($P < 0.05$).

3 Results and discussion

3.1 Hydrolysis of tea residue protein with proteolytic enzymes

To generate tea residue-derived peptides with ACE inhibitory capacity, TTRP was hydrolyzed with seven different proteases under their respective optimum conditions. After 2 h of hydrolysis, neutral protease hydrolysates showed the highest ACE inhibition rate of 47.70% ± 3.67% at concentration of 0.5 mg/mL (Figure 1), followed by alkaline protease with ACE inhibition rate of 35.61% ± 3.53%. The results indicated that neutral protease was more effective at hydrolyzing TTRP compared to the other six proteolytic enzymes. The hydrolysates produced from various enzymes exhibited different levels of inhibition against ACE, which can be attributed to the characters of the various peptides. Studies have shown that the identity of the amino acid at the C-terminal of the peptide has a major influence upon its potency against ACE (Nchienzia et al., 2010). Peptides containing tyrosine, phenylalanine and tryptophan located at the C-terminal were found to be most effective (Ke-Han et al., 2015). This would explain the effectiveness of alkaline protease, which prefers to cleave peptide bonds with hydrophobic amino acids, in producing hydrolysates with higher ACE inhibitory potency. Therefore, neutral protease and alkaline protease were selected as enzymes for the two-step enzymatic preparation of ACE inhibitory peptides.

3.2 Optimization of hydrolysis conditions of neutral protease

Single factor test of neutral protease

The effects of hydrolysis time on DH and ACE inhibition activity of TTRPH were determined (Figure 2A). The DH and ACE inhibitory rate increased linearly during 2.0-2.5 h, and then reached a plateau during 2.5-4.0 h. The increase of DH and ACE inhibitory activity with the increase of time is not very obvious,

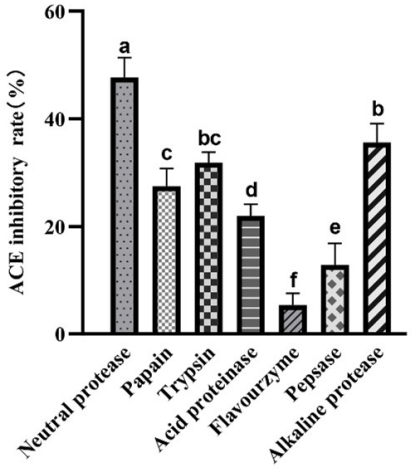

Figure 1. Average ACE inhibitory rate achieved by seven proteolytic enzymes after 2 h of hydrolysis. Data points are presented as the mean and standard deviation of three replications. Different lowercase letters indicate significant differences ($P<0.05$)

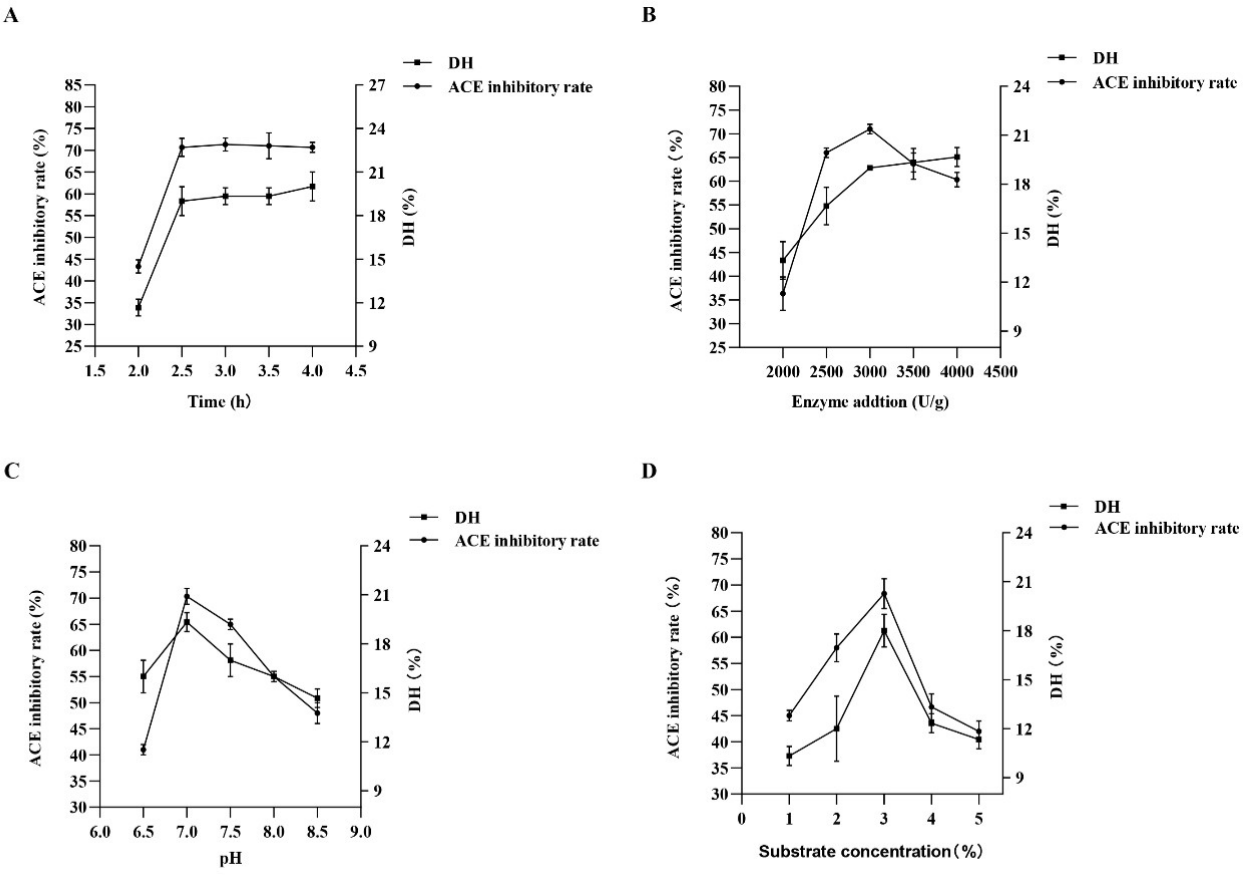

Figure 2. Effects of hydrolysis factors on DH and ACE-inhibitory activity of neutral protease. (A) Time; (B) Enzyme addition; (C) pH; and (D) Substrate concentration.

and the extension of the enzymatic digestion time will increase the energy consumption and production cost, and it is more reasonable to choose a shorter level of enzymatic digestion time at the same level. Therefore, 2.5 h was ensured as the optimum hydrolysis time.

As shown in Figure 2B, the DH continuously increased with the enzyme addition (2000-4000 U/g). However, the ACE inhibitory activity increased with the enzyme addition (2000-3000 U/g) and then decreased with the further increment of enzyme addition. The lowering ACE inhibitory activity at higher enzyme addition may be due to further hydrolysis of the ACE inhibitory peptide into more fragmented small peptides without ACE inhibitory activity (Gao et al., 2019). This is also consistent with earlier findings on the hydrolysis of whey protein that the loss of ACEI activity could be due to greater hydrolysis of the protein when more proteases were added (Guo et al., 2009). As a result, enzyme addition of 3000 U/g was selected.

The effect of pH (6.5-8.5) on DH and ACE inhibitory rate was determined (Figure 2C). The DH and ACE inhibitory rate increased when initial pH was 6.5-7.0 and peaked at initial pH of 7.0, and decrease afterwards. The results may be due to the fact that the structure of the neutral enzyme is altered so that it is no longer complementary to its specific substrate in the acidic or alkaline environment, resulting in a gradually decrease or even loss of enzyme activity. Hence, pH of 7.0 was chosen.

The DH and ACE inhibitory activity increased with the increment of substrate concentration and reached maximum at 3% (Figure 2D). The further increase of substrate concentration caused the decrease of the value of DH and ACE inhibitory rate. The results could be due to that the viscosity of the liquid also becomes larger as the concentration of the substrate increases, which will lead to poor mobility of the substrate and reduced contact opportunities with the enzyme, ultimately leading to a decrease in the efficiency of enzymatic hydrolysis (Ryan et al., 2008). In this study, the optimal substrate concentration was 3%.

RSM of neutral protease

Single factor test was required to select the significant enzymatic factors and their ranges to enhance the success rate of RSM. Based on the results in single factor tests, BBD model-designed experiments were conducted to visualize the effects of independent factors (enzyme addition, pH and substrate concentration) on ACE inhibitory activity (Y) (Table 3).

As shown in Table 4, the RSM models for ACE inhibitory activity (Y) had been successfully constructed based on the low p value of models ($P < 0.01$), insignificant lack of fit ($P > 0.05$),

and high R^2 values. Response surface and contour plots were used to describe the relationship between the dependent and independent variables (Figure 3). In generally, the steeper the slope, the more significant the effect of the factor; the gentler the slope, the less the effect of the factor. On the other hand, the contour plots reflect the significance of the interaction of the two variables. As shown in Figure 3, the slopes are steeper on the pH (B) and substrate concentration (C) axes and gentler on the enzyme addition (A) axis, indicating that the effects of pH and substrate concentration on the ACE inhibition rate are more significant, while the effect of enzyme addition on the ACE inhibition rate is less. The obtained quadratic polynomial regression equation was shown in Equation 3.

Table 3. RSM responses of the neutral protease.

Run	Enzyme addition (A, U/g)	pH (B)	Substrate concentration (C, %)	ACE inhibitory rate (%)
1	2500	6.50	3	60.22
2	3500	6.50	3	72.25
3	2500	7.50	3	56.49
4	3500	7.50	3	51.54
5	2500	7.00	2	56.45
6	3500	7.00	2	60.44
7	2500	7.00	4	69.28
8	3500	7.00	4	73.14
9	3000	6.50	2	53.27
10	3000	7.50	2	56.18
11	3000	6.50	4	68.25
12	3000	7.50	4	54.21
13	3000	7.00	3	64.12
14	3000	7.00	3	64.32
15	3000	7.00	3	66.08
16	3000	7.00	3	61.18
17	3000	7.00	3	64.04

$$Y = 63.95 + 1.87A - 4.45B + 4.82C - 4.25AB - 0.032AC - 4.24BC + 1.51A^2 - 5.34B^2 - 0.63C^2 \quad (3)$$

According to mathematic models of RSM, the predicted optimal hydrolysis factors were as follows: enzyme addition 3500 U/g, pH 6.5, substrate concentration 4%. Under these conditions, the highest peptide yield was 79.11% ± 1.31% that corresponding ACE inhibitory activity of 79.07% at the concentration of 1.0 mg/mL. This experimental ACE-inhibitory activity was consistent with the predicted data at 95% confidence interval, which confirmed that the RSM model was robust and suitable for predicting experimental data.

3.3 Optimization of hydrolysis conditions of alkaline protease

Single factor test of alkaline protease

As shown in Figure 4A, the increase of enzyme addition resulted in an increase in DH and ACE inhibitory activity in the second step enzymatic hydrolysis (< 6000 U/g). The DH increased as the further alkaline protease addition, but ACE inhibitory activity decreased. The results could be due to greater hydrolysis of the protein when more proteases were added. On the contrary, excessive enzyme addition did not yield significant increase in DH and resulted in decrease in ACE inhibitory activity. It may be attributed to either enzyme being saturated or enzyme inhibition by the derived peptides. Considering the cost, the optimum enzyme addition was 6000 U/g.

As shown in Figure 4B, the hydrolytic curve showed gradually increased from 2.0 to 4.5 h. The ACE inhibitory activity increased from 2.0 to 2.5 h, however decreased steadily from 2.5 to 4.0 h. Further treatment may result in the hydrolysis of the ACE inhibitory peptides into amino acids and thus destroy the ACE inhibitory activity (Cui et al., 2014). Thus, considering the production cost and efficiency, 2.5 h was selected as the

Table 4. Statistic analysis for mathematical models of ACE inhibitory activity from RSM design of the neutral protease.

Source	Sum of squares	df	Mean square	F value	p-value	
Model	644.95	9	71.66	9.26	0.0039	**
A-A	27.86	1	27.86	3.60	0.0996	
B-B	158.15	1	158.15	20.43	0.0027	**
C-C	185.67	1	185.67	23.98	0.0018	**
AB	72.08	1	72.08	9.31	0.0186	*
AC	4.225E-003	1	4.225E-003	5.457E-004	0.9820	
BC	71.83	1	71.83	9.28	0.0187	*
A^2	9.64	1	9.64	1.25	0.3012	
B^2	119.91	1	119.91	15.49	0.0056	**
C^2	1.69	1	1.69	0.22	0.6543	
Residual	54.20	7	7.74			
Lack of Fit	41.81	3	13.94	4.50	0.0901	Not significant
Pure Error	12.38	4	3.10			
Cor Total	699.15	16				
R^2	0.9225					
Adj. R^2	0.8228					

**P < 0.01, representing highly significant. *P < 0.05, representing significant. P > 0.05, representing not significant.

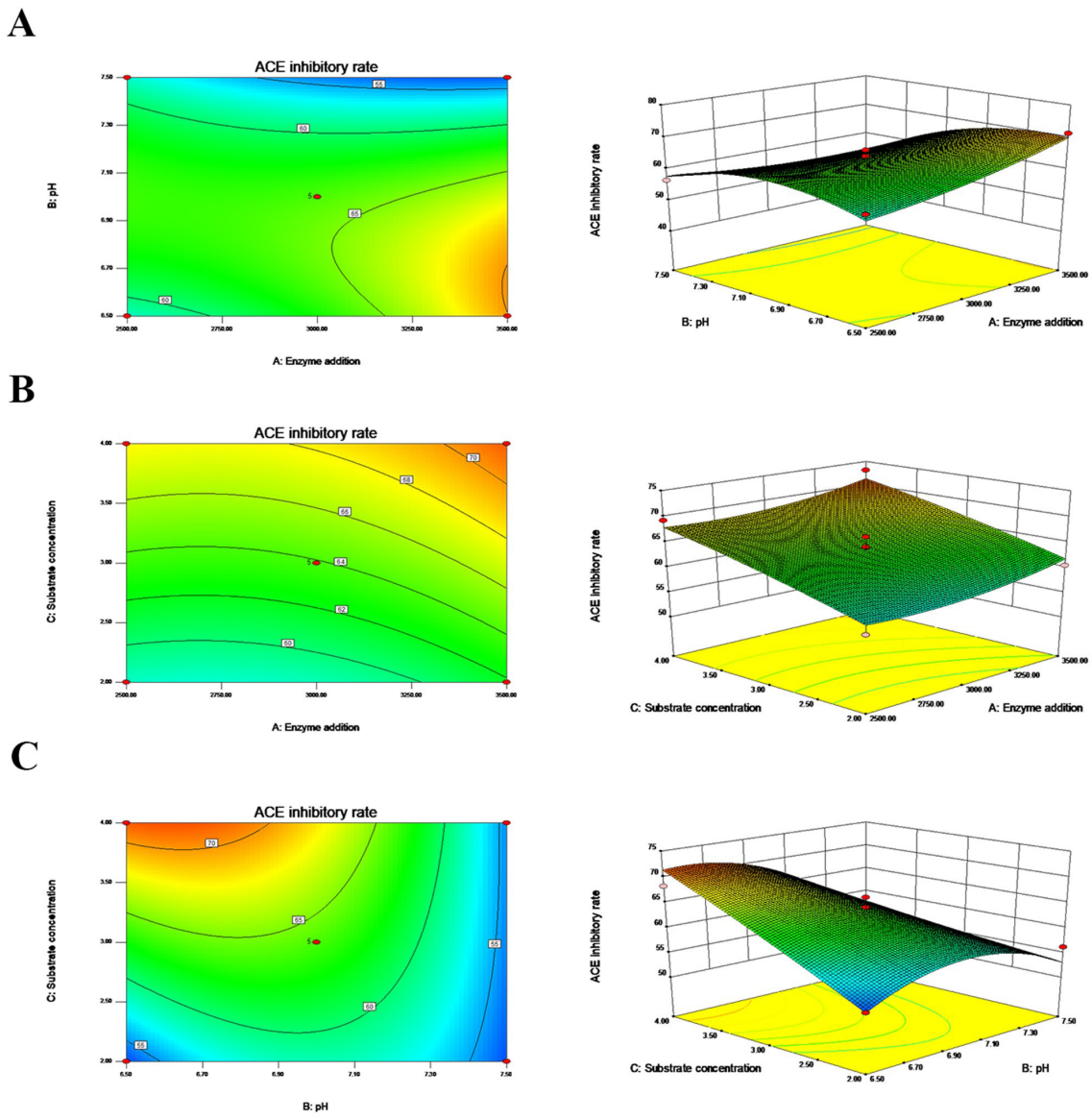

Figure 3. Contour plots for ACE inhibitory activity under independent variables of neutral protease. (A) Distribution of ACE inhibitory activity under enzyme addition and pH (substrate concentration fixed at 3%); (B) Distribution of ACE inhibitory activity under enzyme addition and substrate concentration (pH fixed at 7.0); (C) Distribution of ACE inhibitory activity under pH and substrate concentration (enzyme addition fix at 3000 U/g).

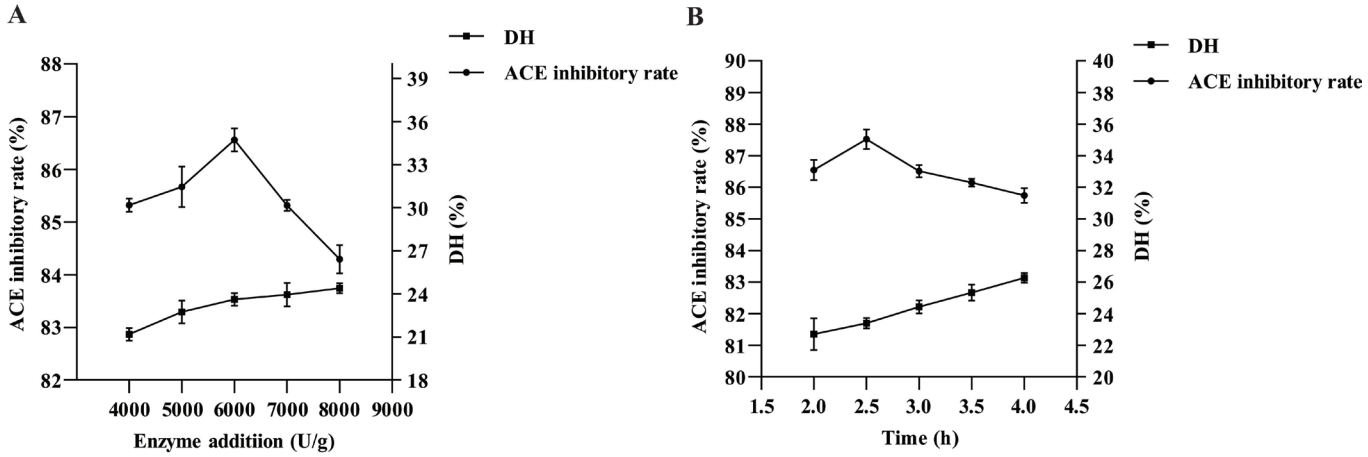

Figure 4. Effects of enzyme addition (A) and time (B) of alkaline protease on DH and ACE-inhibitory activity.

central value of the time factor of the RSM. This conclusion was consistent with Lu et al. (2021), who found that with the extension of time, the reaction becomes more and more sufficient, and the ACE inhibition rate increases gradually, but the long-term enzymatic hydrolysis will hydrolyze the polypeptide into amino acids without ACE inhibition activity.

RMS of alkaline protease

A three-level-two-factor central composited design (CCD) approach-based RSM analysis was used to statistically specify the effect of independent variables (enzyme addition and time) on the ACE inhibitory activity and the results were shown in Figure 5 and Table 5. The analysis of variance was displayed in Table 6. The enzyme addition and hydrolysis time had a significant effect ($P < 0.05$) on the ACE inhibitory activity. The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.9650, indicating that 96.50% of the variability in the response on ACE inhibitory rate can be explained by the model Equation 4.

Table 5. RSM design and the experiment results of the alkaline protease.

Run	Enzyme addition (A, U/g)	Time (B, h)	ACE inhibitory rate (%)
1	5000.00	2.00	86.36
2	6000.00	1.79	84.55
3	7414.21	2.50	85.76
4	6000.00	3.21	83.11
5	5000.00	3.00	87.2
6	6000.00	2.50	86.97
7	6000.00	2.50	87.88
8	6000.00	2.50	87.58
9	6000.00	2.50	87.71
10	7000.00	2.00	85.15
11	6000.00	2.50	87.42
12	4585.79	2.50	88.48
13	7000.00	3.00	81.82

$$Y = 87.51 - 1.30A - 0.57B - 1.04AB - 0.28A^2 - 1.93B^2 \quad (4)$$

The Adj.R^2 of ACE inhibitory rate was 0.9400 which was close to R^2, indicating the accuracy of the model, which can be used for the study and evaluation of ACE inhibition rate. The high R^2 indicates that the models are well adapted to the responses. The lack of fit was insignificant ($P > 0.05$) indicated that the responses are adequately described by the regression equation. The statistical analysis of data revealed that linear, quadratic and interaction coefficients were significant. Equation 4 showed the dependence of the ACE inhibitory activity on enzyme addition and hydrolysis time. The maximum ACE inhibitory activity obtained by using the above optimized concentrations of the variables was enzyme addition of 5000 U/g and hydrolysis time of 3 h. On this condition, the predicted ACE inhibitory activity was 88.56% at the concentration of 1 mg/mL. Experimental verification result showed that ACE inhibitory activity was 88.45% ± 0.45% under the optimal conditions. This confirmed that these conditions were optimal for ACE inhibitory peptides production.

3.4 Thermal, pH, metal ions and GID stability of TTRPPs

The pH and thermal stability of peptides are the main concerns in the production and processing of peptide and also the main evaluation indicators for simulating processing treatments of peptides (Singh & Vij, 2018). As shown in Figure 6A, thermal treatments did not significantly alter the ACE inhibitory activity of TTRPPs. TTRPPs can tolerate temperature up to 100 °C, which is interesting in preparing foods with these functional ingredients. In accordance with our results, Wu et al. studied the stability of soy protein derived peptides at different temperature (20-100 °C) and found that soy-protein-derived ACEI peptides had satisfactory heat stability (Wu & Ding, 2002). While, there was no significant different in ACE inhibitory activity of TTRPPs for pH treatments from pH 2.0 to pH 8.0, except pH 10.0 ($P < 0.01$, Figure 6B). The decrease of activity at pH 10.0 may be due to alkaline hydrolysis which affect the amount,

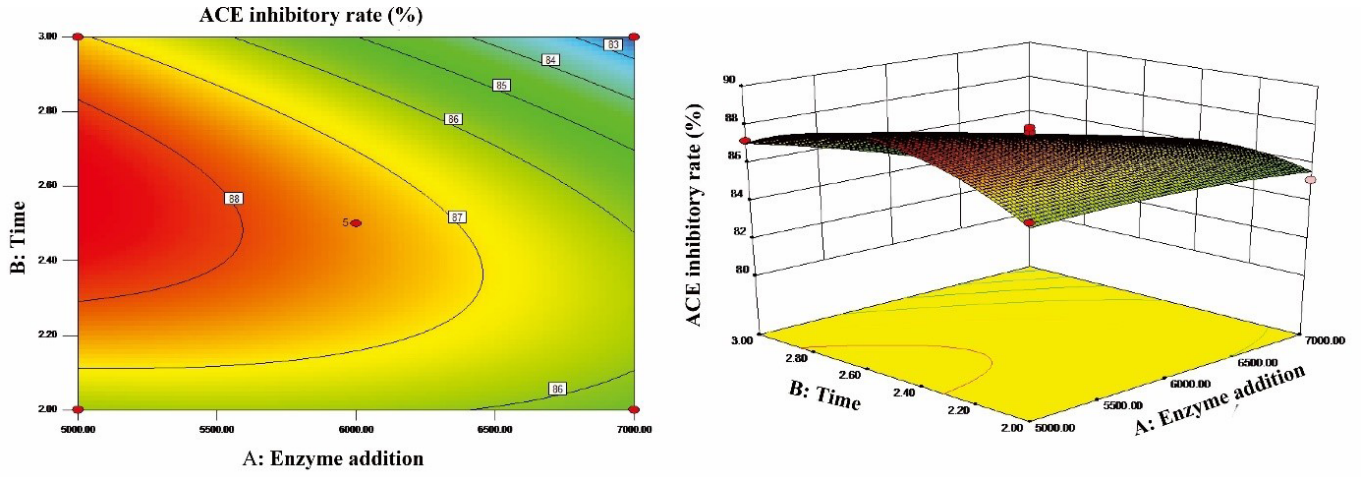

Figure 5. Contour plots for ACE inhibitory activity under independent variables of alkaline protease. Distribution of ACE inhibitory activity under enzyme addition and time.

Table 6. Statistic analysis for mathematical models of ACE inhibitory activity from RSM design of the alkaline protease.

Source	Sum of squares	df	Mean squares	F value	p-value	
Model	46.35	5	9.27	38.57	< 0.0001	**
A-A	13.62	1	13.62	56.65	0.0001	**
B-B	2.56	1	2.56	10.66	0.0138	*
AB	4.35	1	4.35	18.09	0.0038	**
A^2	0.55	1	0.55	2.30	0.1735	
B^2	25.82	1	25.82	107.43	< 0.0001	**
Residual	1.68	7	0.24			
Lack of Fit	1.20	3	0.40	3.33	0.1380	Not significant
Pure Error	0.48	4	0.12			
Cor Total	48.03	12				
R^2	0.9650					
Adj. R^2	0.9400					

**P < 0.01, representing highly significant. *P < 0.05, representing significant. P > 0.05, representing not significant.

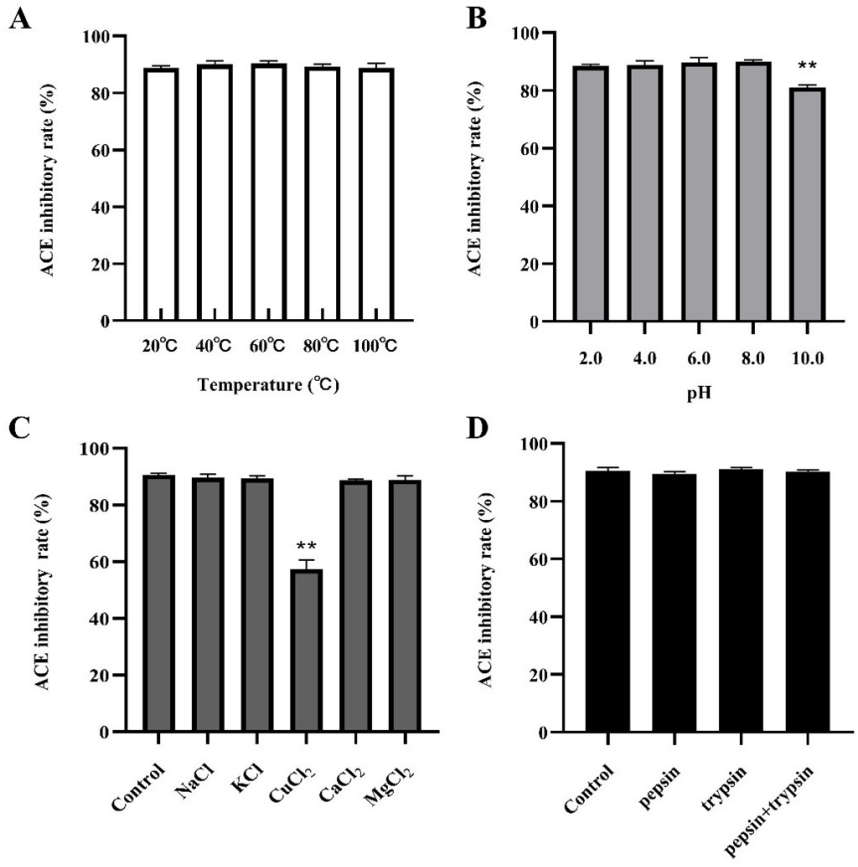

Figure 6. Stability analysis of tea residue proteolytic products. A is the ACE inhibitory rate of tea residue proteolytic products under different temperature, B is different pH, C is different metal ions (NaCl, $MgCl_2$, KCl, $CaCl_2$, and $CuCl_2$), D is the ACE inhibitory rate of tea residue proteolytic products under *in vitro* digestive environment of the stomach and intestines. Columns bearing with '**' represent P < 0.01 statistically significant difference.

size, structure, amino acid composition and hydrophobicity of peptides (Singh & Vij, 2018). Above results about the thermal and pH stability of TTRPPs implied that these peptides of tea residue may be applied during food processing with minimum concerns of loss of activity.

Metal ions are major component of daily food and important for maintaining body heath and cardiac function (Li et al., 2010). As seen in Figure 6C, the ACE inhibitory activity of TTRPPs did not change significantly in the presence of different metal ions, except Cu^{2+}. The reason may be attributed that Cu^{2+} can

alter the aggregation pathway of polypeptides as well as the secondary spatial structure of the aggregates (Lu et al., 2018). In the food industry, Ca^{2+} is abundant in dairy products, meat products, and legumes (Rohrmann & Van Hemelrijck, 2015). Some studies found that the ACE inhibitory activity decreased with increasing Ca^{2+} concentration, which meant peptides should not be consumed with these foods, avoiding their antagonism with Ca^{2+} and affecting their activity (Lai et al., 2020b). While, in our study, the presence of Ca^{2+} did not alter the activity of TTRPPs. Summerly, the above experimental results indicated TTRPPs had stable hypotensive activity in the existence of metal ions and can be consumed with such foods without affecting their activity.

It is necessary for an ACE inhibitory peptide to retain its activity in the gastrointestinal (GI) tract so that it can be absorbed intact into the bloodstream, and exert their hypotensive effect (Bhaskar et al., 2019). However, there are several results found that some food-protein derived ACE inhibitors with strong bioactivities *in vitro* failed to show hypotensive activity after oral administration *in vivo*. It may be attributed to that they are hydrolyzed into peptides with reduced activity or free amino acids in the gastrointestinal tract (Tsai et al., 2008). Conversely, several studies find that some peptides showed an increase in antihypertensive activity upon digestion (Salampessy et al., 2015). Thus, the stability of ACE inhibitory peptides towards *in vivo* GID was evaluated by incubating TTRPPs with proteases (pepsin, trypsin, pepsin and trypsin) present in the GI tract *in vitro*. As shown in Figure 6D, the ACE inhibitory activity of TTRPPs digested by proteases was not significantly different from the control group. This suggests the possible therapeutic use of TTRPPs as antihypertensive agents *in vivo*.

4 Conclusion

In this study, seven different enzymes were used to generate tea residue protein ACE inhibitory peptides. Among all treatments, neutral protease induced TTRPH possessed greatest ACE inhibitory activity and alkaline protease followed. ACE inhibitory peptides were prepared from tea residue proteins by optimization of two-step enzymatic hydrolysis. The optimal conditions for producing peptide with high ACE-inhibitory activity were neutral protease addition 3500 U/g, pH 6.5, substrate concentration of 4%, hydrolysis time of 2.5 h, hydrolysis temperature of 50 °C in the first step of enzymatic hydrolysis and alkaline protease addition 5000 U/g, hydrolysis time of 3 h at 50 °C and pH 10.0 in the second step of enzymatic hydrolysis. Under the optimal conditions, the ACE-inhibitory activity was 88.45% ± 0.45% at the concentration of 1.0 mg/mL. The ACE inhibitory activity of the two-step enzymatic hydrolysis makes it potentially commercially attractive in the future as "health enhancing ingredient" and as a functional food in the food industry.

The results of stability indicated that thermal treatments, different pH conditions, different kinds of metal ions did not alter the ACE inhibitory activity of the TTRPPs. Moreover, the TTRPPs maintained its ACE inhibitory activity after simulated gastrointestinal digestion. Collectively, these peptides hydrolyzed from tea residue protein have potential applications as functional additives in food or nutraceuticals. However, further clinical allergenicity test for TTRPPs is necessary. At the same time, further characterization of the composition of the protein hydrolysate, elucidation of the relationship between peptide structure and activity, and its potential application as "functional food" awaits future study.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Shufang Ye: Investigation, Data curation, Visualization, Formal analysis, Writing - original draft. Jinyan Luo: Investigation, Data curation, Formal analysis, Methodology, Formal analysis. Jiarong Lin: Data curation. Chun Meng: Supervision. Jing Hong: Supervision, Project administration, Conceptualization, Writing - review & editing.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. Optimum conditions of enzymatic hydrolysis for various enzymes.

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