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Isolation, purification and bioactivity of ACE inhibitory peptides from peach kernel protein enzymatic hydrolysate

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Abstract

Peaches mainly produced in China are a source of food-derived proteins, but much of it is wasted. To make full use of the peaches and peaches related resources, we extracted proteins, including albumin, globulin, gliadin, and glutelin, from defatted Tibet wild peach kernels using Osborne method to study their angiotensin I-converting enzyme (ACE) inhibiting activity, which is an active ingredient for hypertension treatment. The different enzymatic products of these extracts were further separated by ultrafiltration membranes and reversed phase high-performance liquid chromatography (RP-HPLC). The component K2 originated from globulin hydrolysate by alcalase treatment showed the highest ACE inhibiting activity (IC_{50} 0.78 mg/mL). Through further analyzing the Mass spectrometry results of K2 and searching the BIOEP database, the specific dipeptide AH (consisted of Ala and His) has been identified as the ACE inhibitory peptide, providing a potential for using Tibet peach kernel-derived peptides as an ingredient in functional food to control hypertension.

Keywords: peach kernel protein enzymatic hydrolysate; ACE inhibitory peptides; isolation and purification; bioactivity.

Practical Application: Hypertension is one of the common chronic diseases that seriously harm human health, and food-derived ACE inhibitory peptides are functional food resources that have been proven to inhibit hypertension. In this study, Tibet wild peach kernel proteins were hydrolyzed by protease to obtain enzymatic hydrolysates with different ACE inhibitory activity. The different protein enzymatic hydrolysates were further separated by ultrafiltration membranes and reversed phase high-performance liquid chromatography (RP-HPLC). The component with the highest ACE inhibitory activity was identified by liquid-mass technology (HPLC-MS). the specific dipeptide AH (consisted of Ala and His) has been identified as the ACE inhibitory peptide, providing a potential for using Tibet peach kernel-derived peptides as an ingredient in functional food to control hypertension.

1 Introduction

Hypertension is a common chronic disease that threatens the health of the elderly. Angiotensin I-converting enzyme (ACE), which can hydrolyze bradykinin leading to vasoconstriction, is one of causes of hypertension (Salampessy et al., 2017; Zheng et al., 2019; Tan et al., 2019). To relieve the hypertension, developing some ACE inhibitors are useful. The chemically synthesized ACE inhibitors, which are usually used to treat hypertension, have side-effects, such as skin rashes and cough, on human body (Gupta et al., 2018; Wang et al., 2020). To overcome these issues, the bioactive ACE inhibitory peptides from food-derived proteins have received extensive attention from researchers and have been introduced to control hypertension (Ahn et al., 2014; Wang et al., 2018; Cicero et al., 2017; Teh et al., 2016).

Peaches as a source of food-derived proteins are mainly produced in China. However, most of peaches are discarded as wastes in peach processing industry (Koprivica et al., 2018; Ishimoto et al., 2020; Dias et al., 2020; Florêncio et al., 2020). Particularly, the annually yield of peach is about 5 million kilograms in Tibet of China. Only a fraction of the peaches is consumed by locals directly, and the most are thrown away. To make full use of peach and peach-related resources, it is meaningful to develop other utilization value of peaches (Hao et al., 2019; Cassiem & de Kock, 2019). In traditional Chinese medicine, peach kernel was used to treat hypertension (Wang et al., 2013). Our previous study presented that a component isolated from Tibet peach kernels by alkaline proteinase hydrolyzation has a good ACE inhibiting activity (Yang et al., 2019). Considering that the targeting sites of different enzymes to the same protein are different, the hydrolysates from a protein treated by different enzymes are not same (Wang et al., 2021; Bougatef et al., 2008). It is necessary to provide a comprehensive study to reveal the ACE inhibiting activity of peach kernel enzymatic hydrolysates treated by different enzymes for better using the Tibet peach resources.

In this study, we extracted albumin, globulin, gliadin, and glutelin from defatted Tibet wild peach kernels by Osborne method. The different enzymatic hydrolysates of these extracts were further separated by ultrafiltration membranes and reversed phase high-performance liquid chromatography (RP-HPLC). The component K2 originated from globulin hydrolysate by

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alcalase treatment showed the highest ACE inhibiting activity (IC_{50} 0.78 mg/mL). The dipeptide AH (consisted of Ala and His) has been identified as the key ACE inhibitory peptide through further analyzing the Mass spectrometry results of K2 and searching the BIOEP database, providing a potential for using Tibet peach kernel peptides as an ingredient in functional food for hypertension treatment.

2 Experimental

2.1 Materials and instruments

Tibet wild peach kernels were provided by professor Zhengchang Zhong from the School of Food Science, Tibet Agriculture and Animal Husbandry College. Flavourzyme (20 U/mg), neutrase (50 U/mg), and alcalase (200 U/mg) were purchased from Shanghai Ryon Biological Technology Co., Ltd. (Shanghai, China). ACE (from rabbit lung), captopril, and N-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). Tris-HCl solution was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Petroleum ether with analytical pure was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetonitrile, trifluoroacetic acid and methanol with chromatographically pure were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

Freeze-dried samples were obtained using a FD5-4 free dryer (Gold-Sim, USA). Absorbance was measured by a UV2600 spectrophotometer (Shimadzu, Japan). RP-HPLC results were obtained from a LC-30A HPLC system (Shimadzu, Japan). LC-MS results were collected by a MSQ Plus /U3000 LC-MS system (Thermo Fisher Scientific, China).

2.2 Extraction of different protein fractions

Peach kernels sample was mixed with petroleum ether with the ratio 1:6 (weight of sample/volume of petroleum ether) and stirred at 25 °C for 12 h. After evaporating the petroleum ether in the mixture, the degreased peach kernels were collected and dried at 45 °C in a blast drying oven. Different fractions of proteins (albumin, globulin, gliadin, and glutelin) in the degreased peach kernels were extracted according to the Osboren classification procedure reported by Van de Vondel et al. (2020) and Tan et al. (2011) with some modifications. The yield was calculated by mass ratio (extracted protein/degreased peach kernel). The purity of protein fractions of each extract was determined using the Kjeldal method.

2.3 Preparation of protein hydrolysates

A solution containing 4% (w/w) albumin, 4% (w/w) globulin, and 4% (w/w) glutelin solution was stirred separately. The pH of each solution was adjusted with 0.5 M NaOH according to the manufacturer's recommendation. Subsequently, flavourzyme, neutrase, and alcalase were separately added into above solutions and incubated at 50 °C in a water bath for 2, 4, 6, 8, and 10 h, respectively. To maintain the pH constant, NaOH was continually added into these solutions. The hydrolysis reaction was stopped by heating the solutions at 95 °C for 20 min. After adjusting the pH of these solutions to 4, the hydrolysates were centrifuged at 5000 rpm under 4 °C for 20 min. The peach kernel peptides in the supernatant were lyophilized and stored at 4 °C for further use.

2.4 Determination of ACE inhibiting activity

Each hydrolysate was tested for ACE inhibitory activity using polypeptide FAPGG as the substrate for ACE according to the method of Jiang et al (Jiang et al., 2007) with slight modification. The assay was performed by mixing 20 μL of 1 mM FAPGG, 20 μL of ACE (0.2 U/mL), and 80 μL of 1.2 mg/mL sample solution or 80 μL of 50 mM Tirs-HCl as the blank. The mixture was incubated at 37 °C for 40 min and then detected by UV-Vis spectrophotometer at 290 nm. ACE inhibiting efficiency was calculated by the following Equation 1.

$$\text{ACE inhibition efficiency } (\%) = \left(1 - \frac{A1 - A2}{A3 - A4}\right) \times 100\% \quad (1)$$

Where A1 is the absorbance of the enzyme-substrate-inhibitor mixture before incubation; A2 is the absorbance of the enzyme-substrate-inhibitor mixture after incubation; A3 is the absorbance of the enzyme-substrate-Tris-HCl buffer mixture before incubation; A4 is the absorbance of the enzyme-substrate-Tris-HCl buffer mixture after incubation.

The half inhibition rate (IC_{50}) value of ACE inhibiting peptides repents the concentration of the sample when the ACE inhibition efficiency reaches 50%.

2.5 Isolation and purification of ACE inhibiting peptides

The hydrolysates that showed strong ACE inhibition were ultrafiltered using ultrafiltration membranes with molecular weight cut-offs of 10, 5, and 1 kDa. The obtained four components ((<1, 1 - 5, 5 - 10, and >10 kDa) from each hydrolysate were lyophilized for evaluating the ACE inhibiting efficiency

The component with the highest ACE inhibiting efficiency was selected for further purification using RP-HPLC with shim-pack GIST C18 column (4.6 mm 150 mm, 5 μm). The peptide solution was filtered with a microfilter and applied to the column. Solvent A was acetonitrile solution with 0.1% (v/v) of trifluoroacetic acid (TFA) and solvent B was deionized water with 0.1% (v/v) of TFA. The purification was eluted with a linear gradient of solvent A by increasing the solvent B (0-10 min, 5-25% A, 10-25 min, 25-40% A, 25-3 0 min, and 40-100% A). The absorbance of the eluent was monitored at 280 nm. The temperature, injection volume of the peptide solution, and flow rate of mobile phase were maintained at 25 °C, 10 μL and 0.5 mL/min, respectively. The fractions were collected by a semipreparative RP-HPLC with Unitary C18 (20 mm 250 mm, 5 μm,100 A) column at flow rate of 5 mL/min. The injection volume was set at 2 mL. Finally, peaks were collected and lyophilized for further use.

2.6 Peptide identification by LC-MS

The fraction purified by RP-HPLC that exhibited strongest ACE inhibiting efficiency was furtherly identified by LC-MS with Acclaim TM 120 C18 column. The ion source was ESI and full scan of mass spectrometry was performed.

2.7 Statistical analysis

SPSS statistics 22 was used for one-way ANOVA based on Duncan's multiple comparison method.

3 Results and discussion

3.1 The yield and purity of four proteins extracted from defatted peach kernel

Figure 1 shows that the yield and purity of four proteins including albumin, globulin, gliadin, and glutelin are significantly different. Albumin has the highest yield (40.3%), while the gliadin has the lowest yield (0.13%). The yield of globulin and glutelin are 3.47% and 3.7%, respectively. These results suggest that the main proteins in peach kernel are correlated to other plant kernel. As reported by Deng et al. (2020), the main proteins extracted from melon seed kernels were albumin and glutelin with yield of 27.3% and 69.53%, respectively. The purity of albumin, globulin, gliadin, and glutelin are 87.57%, 84.77%, 81.07, and 79.69%, respectively (Figure 1).

3.2 Evaluation for ACE inhibiting efficiency of enzymatic hydrolysates

The ACE inhibiting activity test of gliadin enzymatic hydrolysate was not carried out due to the low yield of gliadin (0.13%). Flavourzyme, alcalase, and neutrase were separately used to treat the other proteins including albumin, globulin, and glutelin. As shown in Figure 2, ACE inhibiting efficiency of these enzymatic hydrolysates are increased with the increasing of the enzyme treatment time and then gradually decrease as the enzyme treatment time extended. The neutrase treatment to albumin (AN4) and glutelin (CN6) has the best ACE inhibiting effect with 45% and 60% inhibiting efficiency, respectively. The highest ACE inhibiting efficiency of enzymatic hydrolysate by alcalase is 58% for globulin at 6 h (BA6). These results are like those of Qu et al. (2010), in which the ACE inhibiting peptides were prepared from nori by alcalase, indicating that the prolonged enzyme treatment time furtherly cause the covalent bond breakage and decomposition of peptides (Yang et al., 2017).

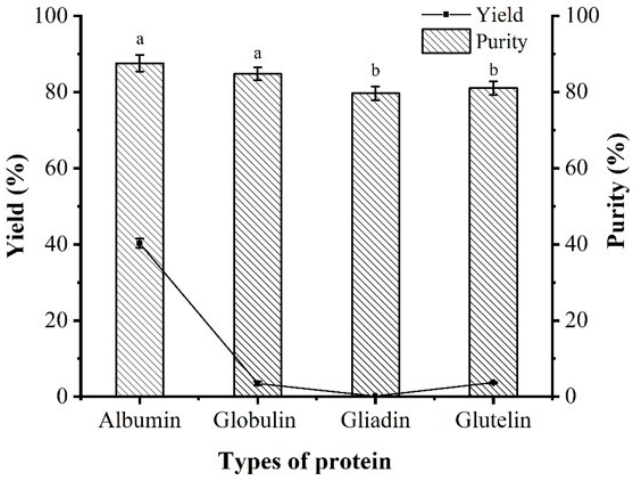

Figure 1. The yield and purity of four proteins extracted from defatted peach kernel powder. Different letters denoted significant differences in the purity of the various proteins ($P < 0.05$).

The hydrolysates from a protein treated by different enzymes are not same because that the targeting sites of different enzymes to the same protein are different. As shown in Figure 2, the ACE inhibiting efficiency of albumin enzymatic hydrolysate treated by neutrase is significantly higher than that of flavourzyme and alcalase treatment, ranging from 24% to 45%. The ACE inhibiting efficiency of glutelin shows the similar trend as albumin with a range of 4% - 60%. The globulin enzymatic hydrolysate has the strongest ACE inhibiting effect treated by alcalase.

To further evaluate the ACE inhibiting effect of these protein enzymatic hydrolysates, IC_{50} was introduced to compare ACE inhibiting rates of AN4, BA6, and CN6 using the captopril as a positive control. Figure 3 shows that the IC_{50} values of AN4, BA6, and CN6 are 1.65 mg/mL, 1.11 mg/mL, and 1.15 mg/mL, respectively. The different inhibiting efficiency of ACE for these hydrolysates may be attributed to the water-soluble degree of each protein, in which the hydrophobic proteins are more likely to coordinate and bind to zinc ions at the active center of ACE (Auwal et al., 2019; Sagardia et al., 2013; Yu et al., 2018; Durak et al., 2013). AN4 originated from water-soluble protein has the lower ACE inhibiting rate than BA6 and CN6, which are obtained from water-insoluble proteins (Javed et al., 2021). These results are correlated to the work of Salampessy et al. (2017) that the IC_{50} of water-insoluble protein hydrolysate was significantly higher than the water-soluble protein hydrolysate.

3.3 Evaluation for ACE inhibiting efficiency of enzymatic hydrolysate based on molecular weight

The previous results showed that hydrolysates with highest ACE inhibiting efficiency treated by each enzyme are AN4, BA6, and CN6. To further identify the specific component for ACE inhibiting, these hydrolysates were separated by ultrafiltration membranes based on different molecular weight (<1, 1-5, 5-10, and >10 kDa). The ACE inhibiting efficiency of each component are shown in Figure 4 with the unseparated enzymatic hydrolysate as the control. For each hydrolysate, the lower molecular weight, the higher the ACE inhibiting rate is. The components below 1 kDa have the best ACE inhibiting effects, with inhibiting efficiency of 67% for AN4, 76% for BA6, and 71% for CN6. These results are consistent with previous studies. As reported by Pan et al. (2012), the isolated components of trypsin treated protein products by ultrafiltration membranes (<6, 6-10, and >10 kDa) had the similar trends as our work. Ma et al. (2019) found that the IC_{50} of isolated components for alcalase treated Ginkgo protein by 3 - 5 kDa ultrafiltration membrane was lower than that of isolated by 5 - 10 kDa ultrafiltration membrane. Figure 5 shows that the IC_{50} values of the isolated components

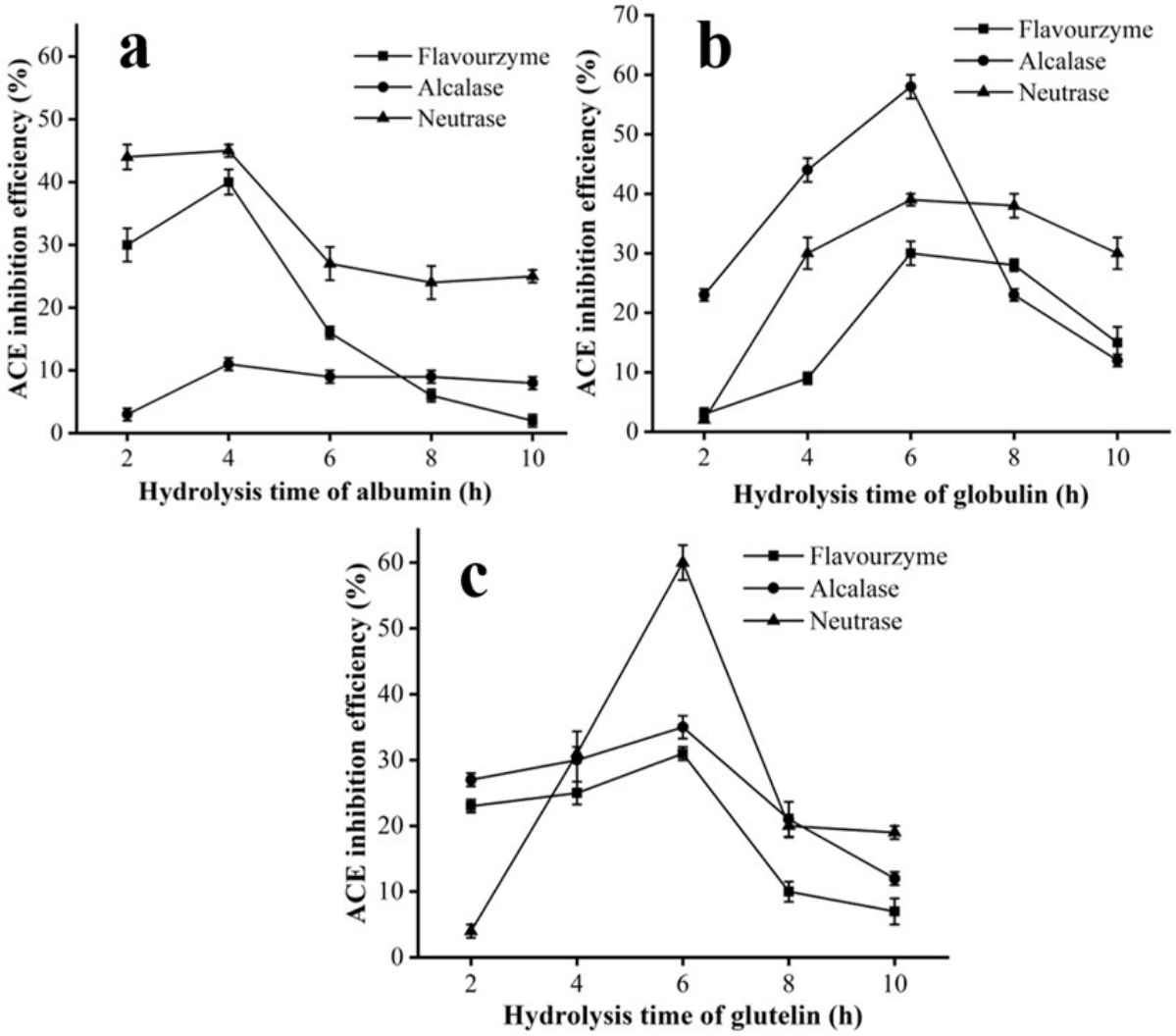

Figure 2. Evaluation for ACE inhibition efficiency of albumin hydrolysate (a), globulin hydrolysate (b) and glutelin hydrolysate (c).

below 1 kDa for each protein. The IC_{50} of components (< 1 kDa) from BA6 was significantly lower than the components (< 1 kDa) from AN4 and CN4, indicating that the ACE inhibiting efficiency of BA6 is greatly superior to AN4 and CN6, that is consistent with the conclusion in Figure 3.

3.4 Evaluation of ACE inhibiting efficiency for the component below 1 kDa from BA6 isolated by RP-HPLC

To find the key component for ACE inhibiting, BA6 (below 1 kDa) with highest ACE inhibiting efficiency was further separated by RP-HPLC using C18 as the non-polar stationary phase and acetonitrile as the polar mobile phase. Figure 6a shows that the separation chromatograms for each component from BA6 below 1 kDa. The characteristic peaks are defined as K1, K2, and K3. Among these components, the K2 with an IC_{50} of 0.78 mg/mL is significantly lower than the BA6 below 1 kDa (0.89 mg/mL) without a RP-HPLC separation, indicating that K2 contains the key component to inhibit the ACE.

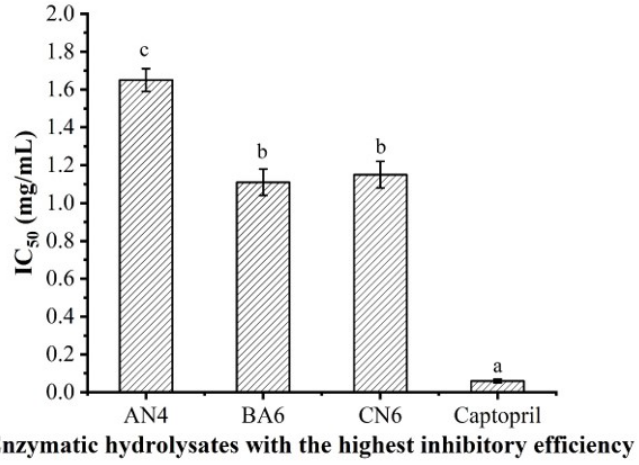

Figure 3. Evaluation of ACE inhibiting efficiency ofAN4, BA6, CN6. Different letters indicates that there were significant differences in ACE inhibiting efficiency between AN4, BA6, CN6 and Captopril ($P < 0.05$).

Table 1. Prediction for amino acids composition of major peptide chains in the K2.

Peptide chain	Mass-to-charge ratio	Peptide chain length	Amino acid composition prediction of peptide chain
P1	227.15	Dipeptide	AH
P2	268.2	Dipeptide	SY, CF, IH
P3	342.13	Dipeptide	HW
		Tripeptide	GCY, GEH, APR, ADH, SVH, PVQ, PIN
P4	363.09	Tripeptide	GTW, GMR, ASW, AHH, STR, PTF, VNM, VDM
P5	430.83	Tripeptide	HHH, HLY, HIY, FHE, FHK, FHQ
		Tetrapeptide	CCCC
P6	498.93	Tripeptide	MYW
		Tetrapeptide	GPYY, GEFF, ASHW, ACFY, ADFF, SVMY, STEY
		Pentapeptide	GASCY, GASQH, GATNH, ASPII

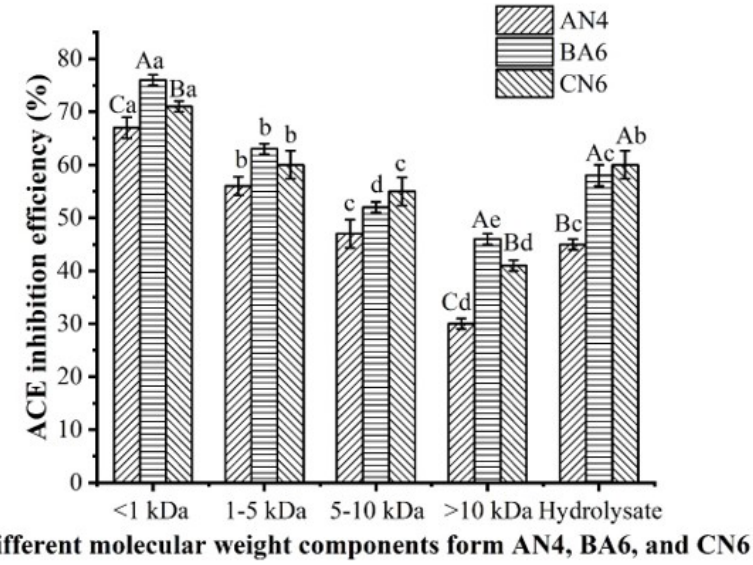

Figure 4. Evaluation of ACE inhibiting efficiency for the component isolated from AN4, BA6 and CN6 by ultrafiltration membranes (<1, 1-5, 5-10, >10 kDa). Different capital letters indicates that the ACE inhibition rate of different hydrolysates with the same relative molecular weight range was significantly different ($P < 0.05$). Different lowercase letters indicates that there were significant differences in ACE inhibiting efficiency between different relative molecular weight ranges of the same hydrolysates ($P < 0.05$).

To investigate the amino acids composition, K2 was analyzed by first-level mass spectrometry. As shown in Figure 7, components of K2 are mainly composed of peptides with a relative molecular weight range of 200 - 500 Da, which is consistent with the reported results that the ACE inhibitory peptides have a molecular weight range of 200 - 800 Da. According to the molecular mass, the amino acids composition of K2 was predicated and shown in Table 1. The K2 is mainly composed of 2 - 5 peptides with a relative molecular weight range of 200-500 Da, which is consistent with the results of most studies showing that ACE inhibitory peptides have a molecular weight range of 200-800 Da (Kumagai et al., 2020; Daskaya-Dikmen et al., 2017). Among the peptides from P1 to P6, the most abundant ingredient P1 with an m/z of 227.15 matches the dipeptide AH consisted of Ala and His (Figure 7), indicating that AH plays an important role in ACE inhibiting. Through searching the BIOEP database, we found that the AH does in the database as an ACE inhibitory peptide.

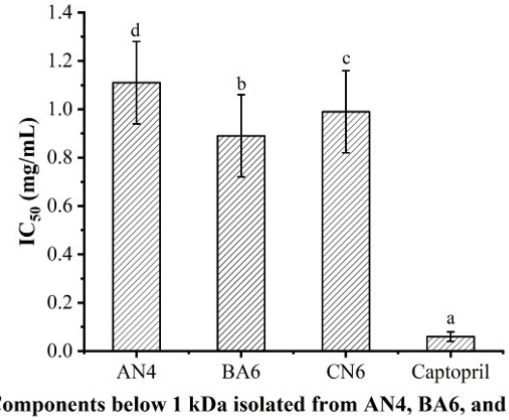

Figure 5. Evaluation of ACE inhibiting efficiency for component below 1 kDa isolated from AN4, BA6, CN6. Different letters indicates that there were significant differences in ACE inhibiting efficiency between component below 1 kDa isolated from AN4, component below 1 kDa isolated from BA6, component below 1 kDa isolated from CN6, Captopril ($P < 0.05$).

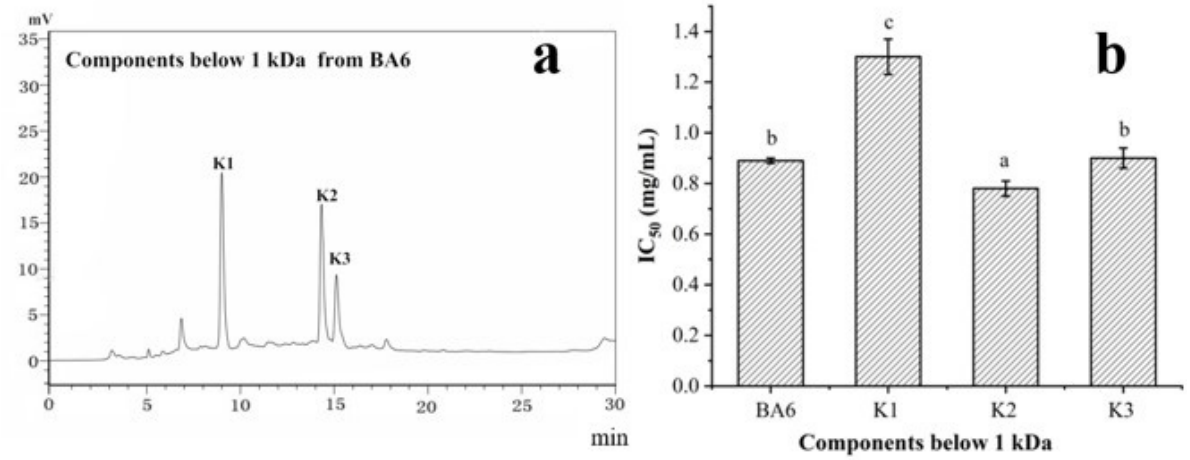

Figure 6. RP-HPLC chromatogram of components below 1 kDa from BA6 (a) and ACE inhibiting efficiency evaluation of these RP-HPLC separated components (b). Different letters indicates that there were significant differences in ACE inhibiting efficiency between BA6 and the RP-HPLC separated components noted K1, K2, K3 ($P < 0.05$).

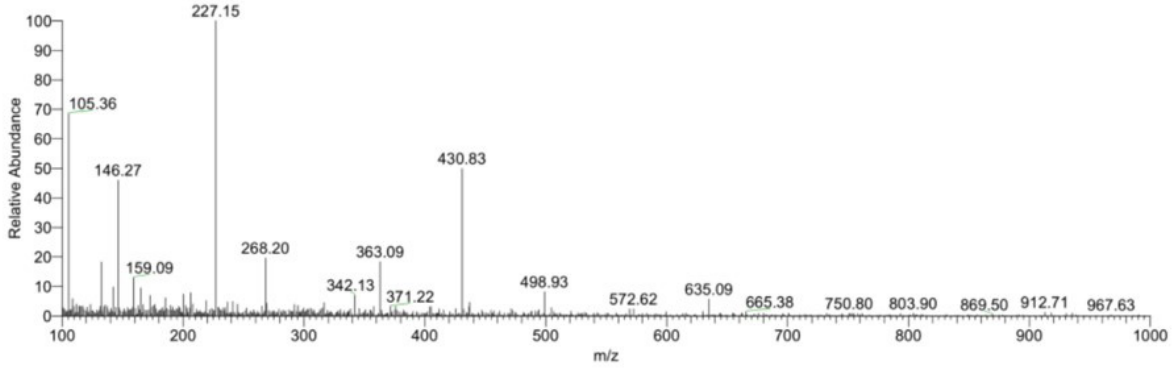

Figure 7. Mass spectrogram of K2.

4 Conclusions

In this study, albumin, gliadin, glutelin, and gliadin were separated from defatted Tibet wild peach kernels and treated by enzymes for investigating the ACE inhibiting effect. The component K2, which isolated from globulin hydrolysate by alcalase treatment using ultrafiltration membrane (<1 kDa) and RP-HPLC, exhibited the highest ACE inhibiting efficiency with an IC_{50} of 0.78 mg/mL. The first-level mass spectrometry results showed that the K2 is mainly composed of 2-5 peptides with a relative molecular mass ranged from 200 to 500 Da. The contributor in the K2 (m/z 227.15) for ACE inhibiting activity is the dipeptide (P1) composed of Ala and His according to the molecular mass, which is consistent with the searching result of BIOEP database.

Conflict of interest

The author declares that there is no conflict of interest in this article. All the authors agreed to the submission of the work.

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