

Full Length Research Paper

Studies on bioactive peptide from Chinese soft-shelled turtle (*Pelodiscus sinensis*) with functionalities of ACE inhibition and antioxidation

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This paper dealt with a novel anti-hypertensive collagen peptide from Chinese soft-shelled turtle (*Pelodiscus sinensis*), which was an efficient inhibitor of angiotensin converting enzyme (ACE, EC 3.4.15.1). ACE plays an important physiological role in the regulation of blood pressure by virtue of the renin angiotensin system. In traditional Chinese medicine, Chinese soft-shelled turtle possesses many health-function properties. This study investigated the effects of Chinese soft-shelled turtle hydrolysate (CTH) on ACE inhibition. The CTH showed limited inhibition effect on ACE with an IC_{50} value at 280 ± 8 $\mu\text{g/ml}$, while its graded fraction of molecular weight less than 5000 Da (CTH5K) obtained through membrane ultra filtration exhibited better inhibitory activity ($IC_{50} = 190 \pm 5$ $\mu\text{g/ml}$). The antioxidant activity of CTH was also determined by radical scavenging ability. The CTH5K showed higher antioxidant activity. These results indicate that CTH5K possesses potent antihypertensive and antioxidant activity, and provides a bioactive peptide material with potential applications as ingredients in functional foods.

Key words: Chinese soft-shelled turtle, collagen peptide, angiotensin converting enzyme inhibitor, antihypertensive, antioxidant.

INTRODUCTION

Hypertension is well known as one of the major risk factors of cardiovascular diseases (CVD). The renin-angiotensin system plays a key role in maintaining blood pressure homeostasis as well as fluid and salt balance in mammals (Turner and Hooper, 2002). Angiotensin converting enzyme (ACE) plays an important role in regulating blood pressure in the renin-angiotensin system because it catalyses the conversion of angiotensin I to angiotensin II (Hernández-Ledesma et al 2003). High levels of ACE can lead to increased vasoconstriction and high blood pressure with its accompanying pathological symptoms. Due to the multifunctional property of ACE enzyme, inhibition of ACE

may have further effects on different regulatory systems involved in immuno-defense and nervous system activities (Erdmann et al., 2008). Therefore, inhibition of ACE is considered to be an important therapeutic approach for controlling hypertension.

Bioactive peptides are short sequences fragments that have a positive impact on body functions. The bioactive peptides can be absorbed in the intestine and enter the blood stream directly, which ensures their bioavailability *in vivo* and physiological effect at the target site (Erdmann et al., 2008). Bioactive peptides can range from 2 to 20 amino acid residues and their activity is based on their amino acid composition and sequence (Rutherford-Markwick and Moughan, 2005; Korhonen, 2009). Protein hydrolysates are diverse in their functional and bioactive properties.

One approach to improving and upgrading the functional and nutritional properties of proteins is enzymatic hydrolysis. Bioactive peptides with ACE inhibitory abilities are generally derived from egg white hydrolysate

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Abbreviations: ACE, Angiotensin converting enzyme; CTH, Chinese soft-shelled turtle hydrolysate.

(Manso et al., 2008), whey proteins (Vermeirssen et al., 2003), soy proteins (Wu and Ding, 2002), Amaranth seed protein (Fritz et al., 2011), by-products proteins such as hydrolysates of sardinelle (*Sardinella aurita*) (Bougatef et al., 2010) and so on. Most food protein-derived peptides with ACE inhibitory abilities have relatively low molecular weight. However, ACE-inhibiting peptides with six, seven or 11 amino acids have also been reported (Haque and Chand, 2008; Otte et al., 2007). Since the discovery of ACE inhibitors in snake venom of *Bothrops jararaca* (Ferreira, 1965), many studies have attempted to synthesize ACE inhibitors such as captopril, enalapril which are currently used as clinical antihypertensive drugs. However, synthetic ACE inhibitors have certain side effects such as cough, taste disturbances and skin rashes (Li et al., 2004). Therefore, it is necessary to search for safe, innovative, and economical ACE inhibitors as alternatives to synthetic drugs to prevent and treat hypertension.

In recent years, ACE-inhibiting peptides from food sources are promising natural bio-functional alternatives to the synthetic drugs. Many ACE-inhibiting peptides have been discovered in enzymatic hydrolysates of different food-source proteins, and they were to be applied in the prevention of hypertension and in the initial treatment of mildly hypertensive individuals (Vermeirssen et al., 2003; Vercruyssen et al., 2005; Guang and Phillips, 2009). Therefore, searching for natural ACE inhibitors as alternatives to synthetic ones is of greater interest for safe and economical use as pharmaceuticals. Moreover, it is possible to obtain serial enzymatic digestions in a system by using a multi-step recycling membrane reactor combined with ultrafiltration membrane system to separate marine-derived bioactive peptides (Kim et al., 2001).

Recently, some protein hydrolysates have been reported to exhibit antioxidant activity (Turkoglu et al., 2007; Bougatef et al., 2010; Zhang et al., 2011). Thiansilakul et al. (2007) found that round scad muscle hydrolysates produced with flavourzyme exhibited a higher 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radical scavenging activity and reducing power. Pownall et al. (2011) found yellow pea seed protein-derived peptides through enzymatic hydrolysis and isolated by ultra filtration with < 3 kDa sizes have the ability of scavenging $O_2\cdot$ and H_2O_2 . The antihypertensive effects of collagen hydrolysates prepared from various sources and the isolation of several ACE-inhibitory peptides from those collagen hydrolysates have been reported in previous works (Kim et al., 2001; Saiga et al., 2008; Ichimura et al., 2009; Lee et al., 2011). In recent years, there are other findings showing that 'low molecular weight collagen hydrolysate' exerts a beneficial effect on osteoporosis by increasing the organic substance content of bone in rats (Watanabe-Kamiyama et al., 2010). Chinese soft-shelled turtle (*Pelodiscus sinensis*) contains abundant collagen and

has multiple functions in Chinese traditional medicine. Feng et al. (1996) demonstrated that long-term oral administration of Chinese soft-shelled powder reduces the development of hypertension in SHR rats and oral administration of Chinese soft-shelled turtle powder attenuates fatigue and accelerates recovery from stress in mice.

So, we obtain ACE-inhibiting peptide fractions by ultra filtration with neutral protease from Chinese soft-shelled turtle hydrolysates (CTH) which is rich in collagen. Bioactive peptides as ACE inhibitor also possess anti-oxidative property and shows cardio-vascular protection effects (Beermann et al., 2009).

ACE inhibitor plays an important role in regulating blood pressure while oxidative stress is implicated in the pathogenesis of hypertension and stroke (Münzel and Keaney, 2001; Fiordaliso et al., 2006). Therefore, anti-oxidant therapy provides another choice to hypertension patient.

MATERIALS AND METHODS

Chinese soft-shelled turtle was provided by Zhejiang Zhongde Agriculture Group Co. Ltd. Neutral protease AS1398 (EC 3.4.24.28) was produced by Genencor of Wuxi, China as well as DPPH, 2,2'-azinobis (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS), ACE (EC 3.4.15.1) from rabbit lung was purchased from Sigma-Aldrich. Hippuryl-L-histidyl-L-leucine (HHL) and captopril were also purchased from Sigma-Aldrich. Distilled deionized water was used for the preparation of all solutions. All other chemicals were of analytical reagent grade.

Ultra filtration methods for CTH treatment

Chinese soft-shelled turtle were collected from Zhejiang Zhongde agriculture group Co. Ltd in October, 2009 (Hangzhou, Zhejiang province). The Chinese soft-shelled turtle scales were washed twice in 1.5 mol/L NaCl solution to remove unnecessary proteins on the surface. The demineralised scales were washed twice with distilled water and then dried. The Chinese soft-shelled turtles were powdered using a crushing mill and a standard sample sieve with particle diameter of about 0.42 mm and then the powder was dispersed in water with a concentration of 50 g/L before hydrolysis. The neutral protease AS1398 was used for hydrolysis of the protein at a ratio of 1:25 (w:w) enzyme: protein, respectively, at pH 7.0 and 50°C. Ultra filtration fractions of CTH were prepared using membrane followed by MWCO 10 kDa and MWCO 5 kDa. Each fraction was collected, and the active fractions were concentrated and lyophilized and stored at 4°C for purification.

Amino acid composition analysis

CTH were dissolved in 6 mol/L HCl solution at 110°C for 24 h and the hydrolysates were analysed by an amino acid analyser (HP1100, Agilent).

Molecular weight distribution determination

In order to determine the molecular weight distribution of CTH, we selected several standard peptides with different molecular weight

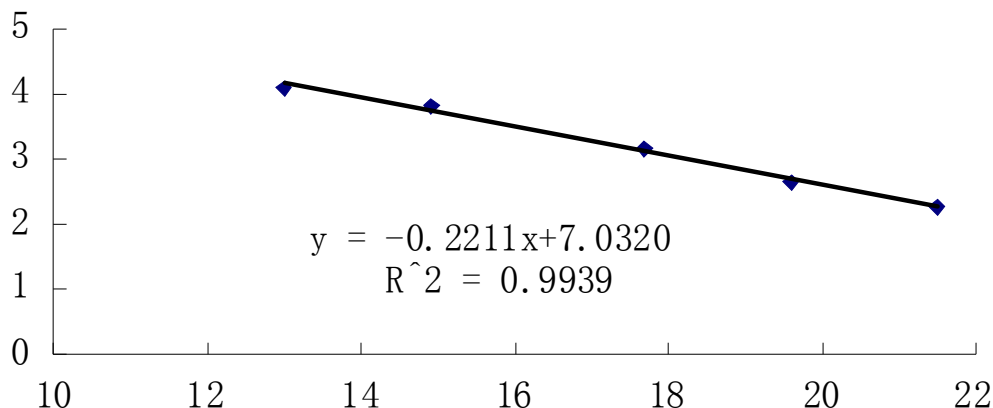


Figure 1. Peptide molecular weight standard curve. X is the retention time, while Y is the LogMW.

(MW) at 189, 451, 1450, 6500 and 12400 Da individually. We made a figure with LogMW plotted against retention time, by high-performance liquid chromatography. Peptide molecular weight standard curve was as followed (Figure 1). The sample was diluted with 0.45 μ m filter membrane. Chromatographic conditions: waters 2695 high performance liquid chromatography, waters 2996 diode array detector, TSK-GEL G2500PW \times L (7.8 \times 300 mm, Tosoh Corp., Tokyo Japan), Empower Pro data processing software. Acetonitrile: water: trifluoroacetic acid (45:55:0.1, v/v/v) as the mobile phase, flow rate: 0.5 ml/min, column temperature 30°C and the injection volume: 10 μ L.

Assay of ACE inhibitory activity *in vitro*

The antihypertensive activity of a peptide is determined based on its ACE inhibition rate or IC_{50} value. A higher ACE inhibition rate or lower IC_{50} value indicates stronger antihypertensive activity. ACE inhibition rate is determined based on the reduced percentage of peak area of hippuric acid using an adapted high performance liquid chromatography (HPLC) method. This method used was based on the assay developed by Wu et al. (2002) with some modifications. The substrate HHL was dissolved (5 mmol/L) in 0.1 mol/L sodium-borate buffer (pH 8.3) containing 0.3 mol/L NaCl. The assay was performed by mixing 50 μ L of substrate solution with 20 μ L of inhibitor solution (or borate buffer for control). After 10 min of incubation at 37°C, 20 μ L of ACE solution (100 mU/ml) were added and the sample was further incubated at 37°C for 30 min. The reaction was stopped by the addition of 85 μ L of 1 mol/L HCl and the solution was filtered through 0.45 μ m nylon syringe filter before reversed-phase HPLC analysis. The HPLC analysis was performed on C₁₈ column (150 \times 3.0 mm i.d.), particle size 5 μ m with a Varian chromatographic system and analytes were detected at the wavelength of 228 nm using Captopril as the reference drug. The blank group was also prepared using the previously mentioned procedure but the diluent was replaced by 0.1 mol/L sodium-borate buffer (pH 8.3).

The mobile phases consisted of solvent A composing of acetic acid/distilled water (0.5:99.5, v/v) and solvent B contained acetonitrile. HPLC analysis was performed using 5 μ L sample and 20% B in A at a flow rate of 1 ml/min, diode array detector (DAD) wavelength of 228 nm, detection temperature of 40°C and runtime of 40 min. The ACE inhibition rate (%) was calculated as follows:

$$R = (A - B) / A \times 100\%$$

Here, R is the ACE inhibition rate of peptide (%), A is the peak area

of hippuric acid in the blank and B is the peak area of hippuric acid in the diluent. IC_{50} value is defined as the concentration required to decrease the ACE activity by 50%. The determinations were plotted for each inhibitor concentration in triplicate.

Antioxidant activity of CTH assessment

Scavenging capacity on DPPH[•] radical

The antioxidant activities of the CTH were determined by measuring the capacity of bleaching a purple colored ethanol solution of DPPH as described by Turkoglu et al. (2007) with some modifications. Various concentrations samples (2 ml) in ethanol were added to 2 ml of 0.2 mmol/L DPPH in ethanol. After 30 min incubation at room temperature, the absorbance was measured against a blank control at 517 nm. Inhibition rate (%) on DPPH radical was calculated in the following formulation:

$$I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100\%$$

Here, A_{blank} is the absorbance of the control reaction (containing all reagents except the tested sample) and A_{sample} is the absorbance of the tested sample.

The sample concentration inhibition was calculated from the graph of inhibition percentage against sample concentration providing Trolox equivalent antioxidant capacity (TEAC), while Trolox was calculated as the reference. All determinations were performed at least in triplicate.

Scavenging capacity on ABTS^{•+} radical

ABTS^{•+} scavenging assay was carried out following a modified method of Thana et al. (2008). ABTS^{•+} radical cation (ABTS^{•+}) solution was produced by reacting ABTS^{•+} stock solution with 2.45 mmol/L potassium persulfate in the dark for 12 to 16 h and adjusting the absorbance to 0.70 ± 0.02 at 734 nm. For the photometric assay, 3 ml of the ABTS^{•+} solution and 100 μ L samples solution were mixed and measured immediately after 6 min at 734 nm (absorbance did not change significantly up to 10 min). Inhibition rate (%) on ABTS^{•+} radical and the sample concentration providing TEAC, while Trolox IC_{50} as the reference which was calculated as described in the DPPH assay. All determinations were performed at least in triplicate.

Table 1. CTH and CTH5K Amino acid composition analysis.

Amino acid composition	CTH (mg/g)	CTH5K (mg/g)
Aspartic acid	39.49	62.967
Threonine	18.613	28.272
Serine	20.632	35.762
Glutamine	62.273	109.235
Glycine	31.895	132.176
Alanine	26.384	67.436
Cysteine	4.942	5.614
Valine	21.496	26.701
Methionine	8.590	13.320
Isoleucine	18.458	22.662
Leucine	32.930	42.901
Tyrosine	13.245	14.199
Phenylalanine	19.383	24.014
Lysine	37.678	51.031
Histidine	10.281	16.675
Arginine	27.442	61.471
Proline	21.312	76.374

From the amino acid content, there is pure difference between CTH and CTH5K, especially hydrophobic amino acids such as Pro from 21.312 mg/g of CTH to 76.374 mg/g of CTH5K, Ala from 26.384 mg/g of CTH to 67.436 mg/g of CTH5K, Phe from 19.383 mg/g of CTH to 24.014 mg/g of CTH5K, Leu from 32.930 mg/g of CTH to 42.901 mg/g of CTH5K, Met from 8.590 mg/g of CTH to 13.320 mg/g of CTH5K, Val from 21.496 mg/g of CTH to 26.701 mg/g of CTH5K and Iso from 18.458 mg/g of CTH to 22.662 mg/g of CTH5K, which may contribute to ACE inhibitory activity and antioxidant activity.

RESULTS AND DISCUSSION

The amino acid composition of CTH

The amino acid composition is shown in Table 1. ACE-inhibiting peptide contained amino acids such as Pro from 21.312 mg/g of CTH to 76.374 mg/g of CTH5K, Ala from 26.384 mg/g of CTH to 67.436 mg/g of CTH5K, Phe from 19.383 mg/g of CTH to 24.014 mg/g of CTH5K, Leu from 32.930 mg/g of CTH to 42.901 mg/g of CTH5K, Met from 8.590 mg/g of CTH to 13.320 mg/g of CTH5K, Val from 21.496 mg/g of CTH to 26.701 mg/g of CTH5K, Iso from 18.458 mg/g of CTH to 22.662 mg/g of CTH5K, which may contribute to ACE inhibitory activity and antioxidant activity. Collagen is one of the unique proteins and they are rich in non-polar amino acids such as Gly, Ala, Val and Pro. The tripeptides with Trp, Tyr, Phe, Pro and a hydrophobic amino acid at the C-terminal were effective for ACE inhibitory activity due to interaction with three residues at the active site of ACE (Pihlanto-Leppälä et al., 2000). Biological activities of protein hydrolysates are related to amino acid composition and sequence, size and configuration of peptides. For example, the presence of certain amino acids such as His, Trp, Tyr, Phe, Met, Leu, Gly or Pro has been reported to enhance the scavenging activities of peptides (Park et al., 2001; Mendis et al., 2005; Li et al., 2007).

Although the structure–activity relationship of food derived ACE-inhibiting peptides has not yet been fully established, ACE preferred inhibitors containing non-polar amino acids has been established.

Molecular weight distribution

Generally, enzyme hydrolysates of protein are purified based on their molecular weight varied from ultra filtration to size exclusion chromatography or both (López-Fandiño et al., 2006; Quirós et al., 2007). Due to lower capacity and film blocking problem, process for purification is limited. Though hydrophobic amino acid content greatly affects ACE inhibition of these peptides, purification depends only on their molecular weight. Thus, these pre-purification methods limit the output of ACE-inhibiting peptides especially the method according to molecular size separation such as ultra filtration. After the separation membrane, we can see polypeptide molecular weight distribution: 5 to 2 KDa content is 24.94% in CTH5K and 2 to 1 KDa content is 28.46%, while below 1 KDa is 45% (Figure 2 and Table 2). According to previous report, molecular weight below 1 KDa showed potential power to be ACE inhibitor and antioxidant. So we evaluated the ACE inhibition and scavenging capacity of DPPH and ABTS⁺ radical.

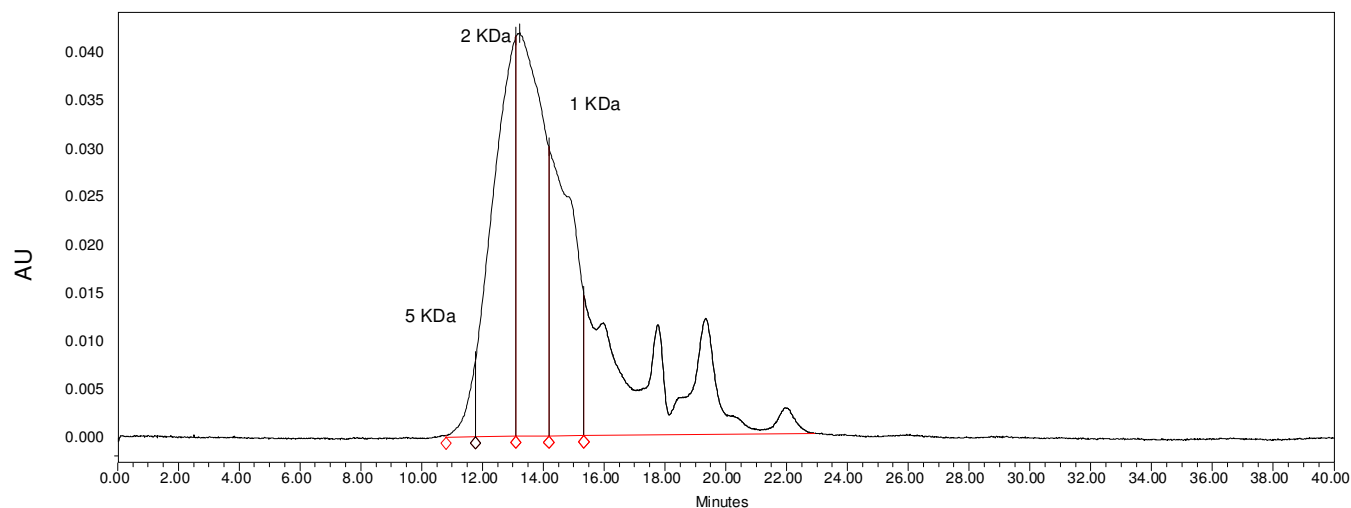


Figure 2. Molecular weight distribution of CTH5K. The molecular weight distributions showed in lines are as follow: 5, 2, 1 and 0.5 KDa.

Table 2. The CTH5K molecular weight distribution.

Molecular weight (KDa)	Retention time (min)	Peak area	Area (%)
>5	11.767	137162	1.61
5 - 2	13.100	2126610	24.94
2 - 1	13.220	2426501	28.46
1 - 0.5	14.183	1663331	19.51
<0.5	15.333	2173340	25.49

After the separation membrane, the polypeptide molecular weight distribution is clearly seen: 5 to 2 KDa content is 24.94% in CTH5K and 2 to 1 KDa content is 28.46%, while below 1 KDa is 45%.

Table 3. ACE inhibition and antioxidant activity of CTH and CTH5K.

Antioxidant	ACE inhibition IC ₅₀ value	IC ₅₀ of Scavenging DPPH (TEAC)	IC ₅₀ of Scavenging ABTS ^{•+} (TEAC)
CTH	280 ± 8 µg/ml	18.79 ± 1.3 mg/g	12.62 ± 0.8 mg/g
CTH 5K	190 ± 5 µg/ml	27.66 ± 1.7 mg/g	16.95 ± 0.7 mg/g

The IC₅₀ value of ACE inhibition obtained from CTH was 280 ± 8 µg/ml, while the mean value of CTH5K was 190 ± 5 µg/ml. The scavenging ability of CTH and CTH5K is lower than that of ascorbic acid; based on TEAC, the hierarchy of ABTS^{•+} radical scavenging activity was in agreement with that of DPPH radical scavenging capacity.

ACE inhibition IC₅₀ value of CTH and CTH5K

The ACE inhibition IC₅₀ value obtained from CTH was 280 ± 8 µg/ml, while the mean value of CTH5K was 190 ± 5 µg/ml (Table 3). Many studies have reported that ACE inhibition of hydrolysates depended on the material species and the purity level of the hydrolysate such as Alaska pollack frame protein hydrolysate between 1 to 3 kDa using an ultrafiltration membrane bioreactor system with higher ACE inhibition IC₅₀ value of 110 µg/ml, while further purification using consecutive chromatographic methods on SP-Sephadex C-25 column and HPLC on an octadecylsilane column was 14.7 µg/ml (Je

et al., 2004). The hydrolysate generated by the crude enzyme from Cuttlefish (*Sepia officinalis*) muscle proteins with high inhibition activity 87.11 ± 0.92% at 200 µg/ml, while fraction P₆ fractionated by size exclusion chromatography on a Sephadex G-25 with higher ACE inhibition. This fraction fractionated by reversed-phase (RP)-HPLC, and identified of Ala-His-Ser-Tyr, Gly-Asp-Ala-Pro, Ala-Gly-Ser-Pro and Asp-Phe-Gly displayed the highest ACE inhibition with an IC₅₀ of 11.6 µmol/L (Balti et al., 2010).

Pacific hake hydrolysates prepared with ultrafiltration (10 kDa molecular mass cutoff) showed an IC₅₀ value of 44 ± 7 µg of peptides, 2.5 times than the commercial product PeptACE peptides (IC₅₀ = 114 ± 8 µg/ml), which

may be a commercially competitive source of ACE-inhibitory peptides (Cinq-Mars and Li-Chan, 2007). CTH5K showed higher inhibition activity than CTH through ultra filtration and had relatively good stability even after several months of storage at -20°C.

Antioxidant activity

Scavenging capacity on DPPH radical

DPPH assay has been widely used to provide basic information on the antioxidant ability of extracts from plant, food material or single compounds because this method has shown to be rapid and simply available. DPPH is a stable free radical with a characteristic absorption which decreases significantly on exposure to proton radical scavengers (Singh et al., 2009). CTH5K showed better scavenging activities to reduce the stable radical DPPH to yellow-colored diphenyl picrylhydrazine in a concentration dependent manner than CTH. However, based upon the estimated TEAC, all fractions were relatively lower than ascorbic acid, a known antioxidant used as positive control. However, CTH and CTH5K have a mild scavenging activity on DPPH radical (Table 3).

Scavenging capacity on ABTS⁺ radical

ABTS⁺ is another synthetic radical and more versatile than DPPH because the ABTS⁺ model can assess the scavenging activity for both the polar and non-polar samples. What is more, the spectral interference is lessened in the absorption maximum and often evaluated by natural products. Therefore, it was considered necessary to further assess the CSSTH against the synthetic ABTS⁺ free radical. The CTH and CTH5K were also able to scavenge ABTS⁺ radicals. The scavenging ability of CTH and CTH5K is lower than that of ascorbic acid, based on TEAC, the hierarchy of ABTS⁺ radical scavenging activity was in agreement with that of DPPH radical scavenging capacity (Table 3).

Conclusions

CTH5K which is rich in collagen shows the property as ACE inhibitor and anti-oxidative peptides *in vitro*. The fractions exhibiting stronger activity will be further fractionated and screened for bioactivity using RP-HPLC. Peptides with stronger activity will be further sequenced and characterized by HPLC– mass spectrometry (MS). After the separation membrane, we can see below the molecular weight polypeptide 1 KDa in 45% of CTH5K. The membrane technology is suitable for industrial production of enzymatic hydrolysates. Industrial process

of CTH5K showed better repeatability which would be an attractive choice for commercial application as safe and effective hypertension inhibitor ingredients in functional foods.

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