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Antioxidant Activities Estimated by Different Measurments and Inhibitory Potential against Angiotensin Ⅰ- Converting Enzyme of Protein Hydrolysates from Tea (*Camellia sinensis* L.) Seed Cake

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Abstract In the present study, protein hydrolysates derived from tea (Camellia sinensis L.) seed cake were prepared by using five different enzymes (alkaline protease, papain, trypsin, flavourzyme and neutral protease). The antioxidant activities and inhibition on angiotensin I-converting enzyme (ACE) of the protein hydrolysates were evaluated in vitro. The results showed that hydrolysates derived from tea seed cake had remarkable antioxidant activities and inhibitory potential against ACE in vitro. Pearson correlation analysis indicated that the antioxidant capacity and ACE inhibitory activity of the hydrolysates might be mainly due to the kind of the enzyme used, not to the degree of hydrolysis (DH) of hydrolysates.

Keywords: *tea seed cake, antioxidant activity, ACE, protein hydrolysates*

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1. Introduction

Free radicals are not only closely associated with lipid peroxidation, but also involved in the development of a variety of diseases. It is recognized that excessive free radicals in human body that may induce damage to cell structures, DNA, lipids and proteins [1], leading to a variety of diseases such as cancers, cardiovascular disease, diabetes, neurological disorders and atherosclerosis [2]. In the past years, natural antioxidants have received considerable attention due to their ability to prevent human body against oxidative stress induced by imbalance between generation and removal of reactive oxygen species and retard the progress of many chronic diseases [3].

Bioactive peptides are regarded as specific protein fragments which are inactive in the parent protein sequence. They can exert several physiological functions after they are released by enzymatic hydrolysis. Previously, several enzyme-hydrolysed proteins from plant and animal sources, such as skin of sole and squid [4], soy [5], chickpea [6], milk porcine and beef muscle [7], were highlighted by their remarkable antioxidant capacity and potent inhibition on angiotensin I-converting enzyme (ACE), which is a key enzyme in the regulation of blood pressure [8], and could be developed as natural antioxidants and potential ACE inhibitors.

Tea (*Camellia sinensis* L.) seed cake is the main byproduct during the manufacture of tea seed oil, which has long been recognized as an edible oil of high quality. As a consequence of the increase in popularity of tea seed oil, the amount of tea seed cake produced has also increased. Till now, tea seed cake is not typically utilized except being used as cheap fertilizer. Recently, some attention has been put on the active components, such as phenols and glycosides in tea seed cake [9]. However, to the best of our knowledge, few studies have focused on protein hydrolyzates from tea seed cake as far.

Therefore, the aims of this study were to extract protein from tea seed cake and prepare protein hydrolyzates by different enzymes, and to evaluate their antioxidant activity and inhibitory capability on ACE *in vitro*.

2. Materials and Methods

2.1. Materials

Tea seed cake was purchased from the Zhejiang Taigu Agricultural Science and Technology Co., Ltd. (Zhejiang, China). Alkaline, flavourzyme, neutral were purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China). Papain, trypsin, angiotensin converting enzyme

(ACE) from rabbit lung, hipouril histidine leucine (HHL), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (Missouri, USA). Ninhydrin was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals were analytical grade and purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

2.2. Extraction of Protein from Tea Seed Cake

Tea seed cake protein was extracted from tea seed cake sing a method developed by Zhong et al. (2007) [5] with slight modification. Tea seed cake was mixed with n-hexane at a ratio of 1:5 (w/v), placed at 40°C for 2 h in a water bath. The precipitate was collected after centrifugation at 6000 × g for 6 min. The above operations were twice repeated. The degreased tea seed meal was mixed with ethanol at a ratio of 1:10 (w/v) and placed at 40°C for 2 h in a water bath. The precipitate, after standing, was collected after centrifugation at 6000 × g for 6 min. The above operations were twice repeated. The processed tea seed meal was placed on ventilators at 20°C for 24 h.

The comminuted tea seed cake was mixed with water at a ratio of 1:10 (w/v) at room temperature. The solution pH was adjusted to 8.5 with 1 M NaOH and stirred for 1 h, the mixture was centrifuged 15 min at 8000 × g for 15 min, then adjusted to pH 5.0 with 1 M HCl and stood for 1 h. The obtained precipitate was centrifuged (8000 × g for 15 min) and again dissolved with a small amount of distilled water. The supernatant, previously adjusted to pH 7.0, was lyophilized and stored at 20°C until use

2.3. Determination of Protein and Amino Acid Content

The protein content was determined by the Bradford method [10].

The *amino acid content* was determined by Nihydrin Colorimetry [11]. One ml of tea seed cake protein (1 mg/ml) was placed in a 25 ml-capacity volumetric flask, added 0.5 ml of phosphate buffer (pH 8.04) and 0.5 ml of 2% solution ninhydrin. The mixture were heated in boiling water for 15 min, then cooled and added to 25 ml, finally determined at 570 nm.

2.4. Protease Hydrolysis of Tea Seed Cake Protein

Five enzymes, Alkaline, Papain, Trypsin, Flavourzyme and Neutral were used to prepare hydrolysate of the extracted protein from tea seed cake. Various conditions based on the working temperature and pH [12] of the enzymes were used (Table 1). The protein was dissolved in buffer with specific pH at 1% (w/v) (10 mg/ml) and then was stood at 90°C for 10 min in a water bath. The protein was hydrolyzed by the individual enzyme at a concentration of 5000 u/g protein. The enzymatic hydrolysis was stood at different temperature for 4 h. The enzymatic reactions were terminated by boiling for 5 min. Each mixture was adjusted to pH 7.0 and centrifuged at 7000 × g for 10 min. The supernatant was freeze-dried and stored at -20°C.

2.5. Determination of the Degree of Hydrolysis (DH)

The degree of hydrolysis (DH) of tea seed cake hydrolysates was determined according to ninhydrin colormetry [13] with tiny modification. Supernatant (0.2 ml) of tea seed cake hydrolysates was placed into a 100-ml-capacity volumetric flask, distilled water was added up to 100 ml. 1 ml of sample, 1 ml of water, 3 ml of ninhydrin solution (0.4 g of ninhydrin, 5 ml of n-propanol, 10 ml of n-butyl alcohol, 20 ml of ethylene glycol and 3 ml of pH 4.5 acetic acid buffer), and 0.1 ml of 1% (w/w) ascorbic acid were mixed. The mixture was heated in boiling water for 15 min and then shook and cooled shook to room temperature by cold water.-Ethanol solution (60% v/v) was added to make sure that the total volume of the mixture was 5 ml. The absorbance of the mixture was read at 580 nm, and distilled water was used to calibrate. The degree of hydrolysis (DH) was calculated as follows:

$$\mathrm{DH}\ (\%) = (A_1 - A_2) / (A_3 - A_2) \times 100$$

A_1: supernatant of tea seed cake hydrolysates;
A_2: crude protein from tea seed cake;
A_3: tea seed cake protein hydrolyzed by 6 M HCl at 110°C for 24 h.

2.6. In Vitro Antioxidant Capacity

2.6.1. DPPH Radical Scavenging

The DPPH free radical scavenging activity of the tea seed cake protein hydrolysates was determined by the method of Mohsen et al. [14], with a slight modification. One milliliter of the tested samples at various concentrations (156.25–10000 μg/ml) was added to 3 ml of ethanolic DPPH solutions (0.1 mM). The reaction mixture was incubated for 30 min in the darkness at room temperature. The increasing of absorbance was measured at 517 nm. The DPPH scavenging effect was calculated as follows:

$$\text{DPPH scavenging effect}(\%) = (1 - A_{samp} / A_{cont}) \times 100$$

where A_{samp} and A_{cont} are defined as absorbance of the sample and the control, respectively.

2.6.2. ABTS Radical Scavenging

ABTS assay was carried out according to the method of Cai et al. (2004) [15]. The ABTS cation radical solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate and incubating in the dark at room temperature for 12 h. The ABTS cation radical solution was then diluted with water to obtain an absorbance of 0.70 ± 0.02 at 734 nm. ABTS cation radical solution (3 ml) was added to 0.1 ml of the test samples with various concentrations (156.25–10000 μg/ml) and vigorously mixed. The absorbance was measured at 734 nm after standing for 6 min. The ABTS scavenging effect was calculated as follows:

$$\text{ABTS scavenging effect}(\%) = \left(1 - A_{samp} / A_{cont}\right) \times 100$$

where A_{samp} and A_{cont} are defined as absorbance of the sample and the control, respectively.

2.6.3. Ferric Reducing Antioxidant Power (FRAP)

The ferric-reducing antioxidant power (FRAP) assay was performed according to a modified method of Benzie et al., (1999) [16]. Briefly, the working FRAP reagent was prepared by mixing 10 vol of 300 mM acetate buffer (pH 3.6) with 1 vol of TPTZ (10 mM) in HCl (40 mM) and with 1 vol of $FeCl_3$ (20 mM). Freshly prepared FRAP reagent was warmed at 37 °C, and a reagent blank reading was taken at 593 nm. Subsequently, 0.6 ml of test samples with various concentrations (156.25 –10000 µg/ml) was added to the FRAP reagent (4.5 ml). A second reading at 593 nm was performed after 8 min. The initial blank reading with the FRAP reagent alone was subtracted from the final reading of the FRAP reagent with the sample to determine the FRAP value of the sample. A standard curve was prepared using different concentrations (25 – 1500 µM) of $FeSO_4 \cdot 7H_2O$. The reducing ability of the samples were expressed as the equivalent to that of 1 µM $FeSO_4 \cdot 7H_2O$.

2.7. In Vitro ACE Inhibitory Activity

ACE inhibition was performed by the method of Nakamura et al., (1995) [17] with a slight modification. In brief, 20 µl of sample and 120 µl of hippuryl-L-histidyl-L-leucine (HHL, 5 mM in 0.1M sodium borate buffer containing NaCl 0.3 M, pH 8.3) were incubated at 37°C for 5 min. Then, 150 µl of ACE was added and the mixture was incubated for an hour at 37°C. Sample blanks (inactivating the enzyme at first) and a blank (buffer instead of sample) were also included. The enzymatic reaction was stopped by adding 150 µl of 1 M HCl. The hippuric acid formed by the action of the ACE on HHL, was extracted from the acidified solution into 1ml ethyl acetate by stirring for 15 s. The mixture was centrifuged at 4000× *g* for 10 min, and a 750 µl aliquot of each ethyl acetate layer was transferred to clean tubes and evaporated by heating at 120°C in an oven. The hippuric acid was redissolved in 3 ml of water, and the absorbance was measured at 228 nm after 0.5 min.

2.8. Statistical Analysis

All the experiments were carried out in triplicate. The results were expressed as means ± SD and evaluated by analysis of variance (ANOVA) followed by Turkey's studentized range test carried out on the SAS system for windows V9 (Version 9.1, SAS Institute Inc., Cary, NC, USA), and $p < 0.05$ was regarded as statistically significant. Pearson's correlation coefficients were determined by SPSS (version 16.0). EC_{50} values were calculated by curve fitting using OriginPro (version 8.0).

3. Results

3.1. Chemical Compositions

In this study, the yield of protein from tea seed cake was 2.62%. Meanwhile, the contents of protein and amino acid in the extract of tea seed cake were 53.37±6.62 and 20.52±0.75%, respectively.

3.2. Degree of Hydrolysis (DH) of Enzymatic Hydrolysates

The enzymatic hydrolysis was prepared by varied condition according to the five proteases (Table 1). As shown in Figure 1, the highest DH was 25.71%, which was found in papain hydrolysate, followed by that of neutral (12.11%). The hydrolysis capacity of alkaline and flavourzyme to tea seed protein seemed to be similar, where, the DH of the hydrolysates by them were 10.68% and 10.87%, respectively.

Table 1. The optimum hydrolysis condition of enzymes

Protease type	pH	Temperature(°C)
Alkaline	8.0	55
Papain	6.5	55
Trypsin	8.0	37
Flavourzyme	7.0	50
Neutral	7.0	50

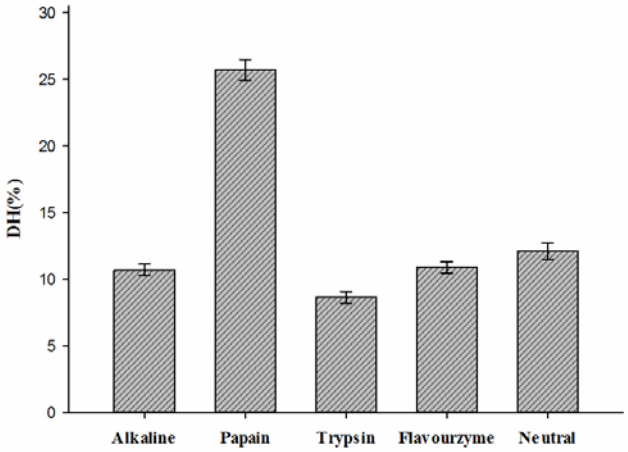

Figure 1. The DH of five hydrolysis of tea seed cake protein by five enzymes. Alkaline, papain, trypsin, flavourzyme and neutral are the tea seed cake protein hydrolysates hydrolyzed by alkaline, papain, trypsin and flavourzyme and neutral and respectively

3.3. Antioxidant Properties

3.3.1. DPPH Radical Scavenging Activity

DPPH is one kind of relatively stable free radicals with unpaired electron and DPPH free radical assay were used to test the free radical scavenging activity of the hydrolysates. As shown in Figure 2, all hydrolysates showed a concentration-dependent effect on scavenging ability DHHP radical. In the tested concentration range, the order of scavenging ability was alkaline >

flavourzyme > neutral > papain > trypsin with EC_{50} (mg/ml) of 1.65, 2.17, 2.23, 2.74, 3.82, respectively (Table 2).

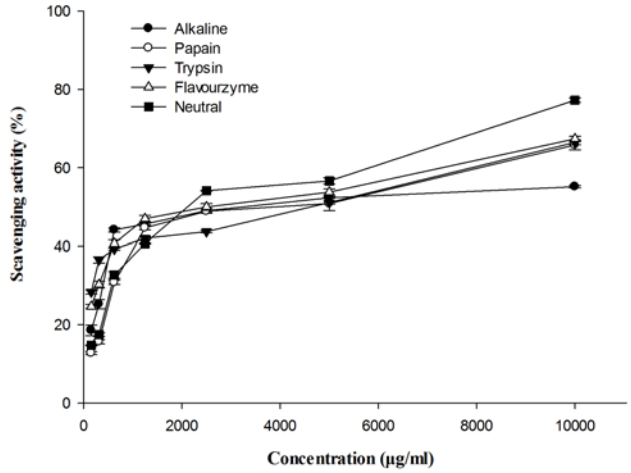

Figure 2. DPPH radical scavenging activity of tea seed cake protein hydrolysis. Alkaline, papain, trypsin, flavourzyme, and neutral are the tea seed cake protein hydrolysates hydrolyzed by alkaline, papain, trypsin, flavourzyme and neutral, respectively

3.3.2. ABTS Radical Scavenging Activity

The ABTS radical scavenging activity of five protein hydrolysates was shown in Figure 3. It can be observed that all these hydrolysates showed a concentration-dependent scavenging activity on ABTS radical. The scavenging ability of the hydrolysates on ABTS radical was in the following order: flavourzyme> trypsin >alkaline > neutral > papain with EC_{50} (mg/ml) of 1.89, 2.23, 2.14, 2.37, 3.39, respectively (Table 2).

Table 2. Effective concentrations of the antioxidant and ACE inhibitory activities of the tea seed cake protein hydrolysis

	DPPH (EC_{50}, mg/ml)	ABTS (EC_{50}, mg/ml)	FRAP (μmol/L) (5000 μg/ml)	ACE (EC_{30}, mg/ml)
Alkaline	1.65	2.14	199.82	0.65
Papain	2.74	3.39	144.65	0.65
Trypsin	3.82	2.23	165.82	0.71
Flavourzyme	2.17	1.89	216.38	1.15
Neutral	2.23	2.37	243.14	0.17

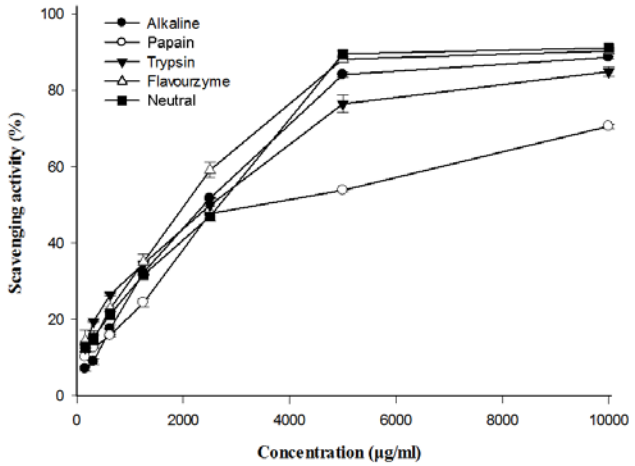

Figure 3. ABTS radical scavenging activity of tea seed cake protein hydrolysis. Alkaline, papain, trypsin, flavourzyme, and neutral are the tea seed cake protein hydrolysates hydrolyzed by alkaline, papain, trypsin, flavourzyme and neutral, respectively

3.3.3. Reducing Activity

The results of FRAP assay were sown in Figure 4. The ferric-reducing power of these protein hydrolysates exhibited a significant concentration-dependent effect. The order of reducing activity of these five hydrolysates was as follows neutral > flavourzyme > alkaline > trypsin > papain at a concentration of 5000 μg/ml.

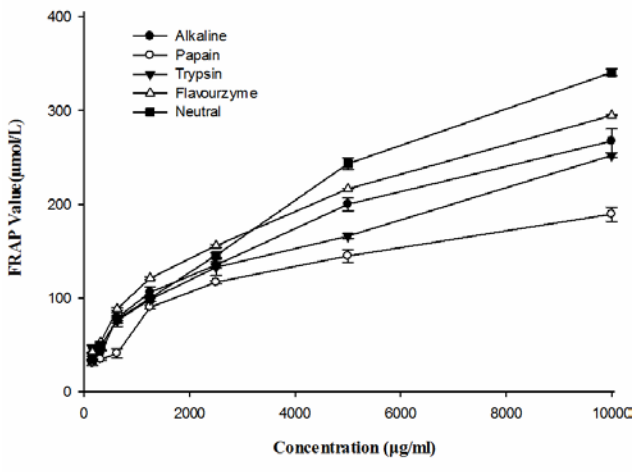

Figure 4. Ferric-reducing antioxidant power (FRAP) ability of tea seed cake protein hydrolysis. Alkaline, papain, trypsin, flavourzyme and neutral are the tea seed cake protein hydrolysates hydrolyzed by alkaline, papain, trypsin, flavourzyme and neutral, respectively

3.4. ACE Inhibitory Activity

The inhibitory activity on ACE of five hydrolysates was presented in Figure 5. As shown, the ACE inhibitory activity of neutral hydrolysates increased from 24.00% to 58.16% with the concentration from 125 μg/ml to 4000 μg/ml. The order of reducing activity of these five hydrolysates was as follows neutral > alkaline= papain> trypsin > flavourzyme with EC_{30} (mg/ml) of 0.17, 0.65, 0.65, 0.71, 1.15, respectively (Table 2).

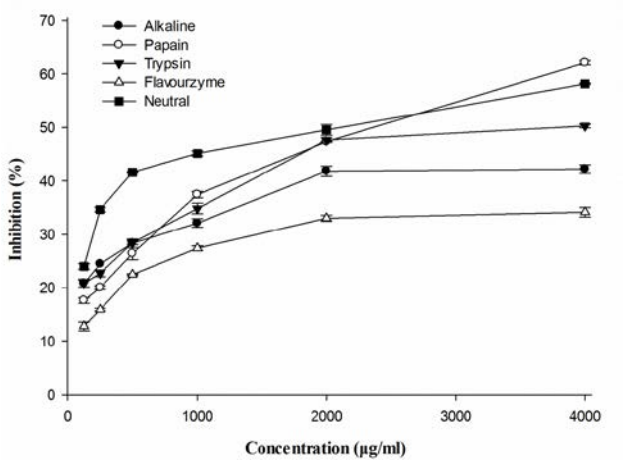

Figure 5. ACE inhibitory activities of tea seed cake protein hydrolysis. Alkaline, papain, trypsin, flavourzyme and neutral are the tea seed cake protein hydrolysates hydrolyzed by alkaline, papain, trypsin, flavourzyme and neutral, respectively

4. Discussion

Protein is regarded as a source of energy and essential amino acids, which plays an important role in the growth, metabolism and maintenance of physiological functions [18]. Recent studies focused on the bioactive peptides, exhibited various physiological functions, derived from animal and plant protein [19], and indicated that such protein hydrolysates could be safe and effective agents with some advantages in comparison to protein. Specially, the utilization of the wastes or by-products for such agents has been recently attracting lots of attention. In this study, protein hydrolysates were prepared from tea seed cake, the main by-product during tea (*Camellia sinensis* L.) seed oil production. China is the world's largest tea producing and exporting country and also a big tea consumer. Over 1000 million kg of tea fruit are produced annually in China [20]. Many studies have shown that tea seed oil has several health benefits for human beings as the tea seed oil contains more than 84% unsaturated fatty acid, such as oleic acid (62.5% by weight), linoleic acid (18.1% by weight) and linolenic acid [21]. Increasing amounts of tea seed cake have been produced as the byproducts during the manufacture of tea seed oil, which was recognized by the Ministry of Health, China, as a new food resource in 2009. The extraction rate of tea seed oil is about 14-15% and after expelling the rest, except tea seed peel, is mainly tea seed cake so there is large amount of tea seed cake in this industry. In addition, the extraction method showed above of tea seed cake protein hydrolysates is simple, feasible and non-toxic. The article suggests that utilizing this readily available and low cost resource can brings both economic and environmental interest.

A vast number of evidence has implicated that oxidative stress was associated with a wide range of diseases [22]. Lots of studies have shown the peptides have inhibitions on lipid peroxidation [23,24], scavengers of free radicals [25,26] and chelators of transition metalions [26,27] (In the present study, DPPH, ABTS and FRAP assays were used to evaluate the antioxidant activity of the protein hydrolysates from tea seed cake, respectively. DPPH free radical is capable of accepting an electron or a hydrogen radical to become a stable diamagnetic molecule [28], and it has been widely used to test the free radical scavenging ability of various natural products [29,30], ABTS cation radicals are more reactive than DPPH radicals and unlike the reactions with DPPH radicals, which involve H atom transfer, the reactions with ABTS cation radicals involve an electron transfer process [31]. Moreover, The FRAP assay is based on a redox reaction where a ferric (Fe^{3+}) complex is reduced to the blue ferrous (Fe^{2+}) form by oxidation of a reductant (antioxidant) [16]. FRAP measures is alternatively considered as the ability of a natural antioxidant to donate electrons [32]. By calculating EC_{50} (Table 2), it was found that protein hydrolysates had inconsistent performance in varied systems, which may attributed to the different mechanisms as discussed above, and to the composition and the sequence of amino acid of hydrolysates as well. In literature, Tyr, Trp, Met, Lys, Cys, and His are examples of amino acids that have antioxidant activity [33]. However, the exact mechanism underlying the antioxidant activity of peptides has not been fully understood yet.

Hypertension is one of the top five most common diseases in the world and over 25% of the adult populations nearly one billion in 2000 were affected by hypertension [34]. ACE plays an important physiological role in the regulation of blood pressure by virtue of the rennin angiotensin system. ACE also inactivates the vasodilative peptide bradykinin in the kallikrein-kinin system [35]. Additionally, ACE functions as a stimulant for the release of aldosterone in the adrenal cortex [36]. Therefore Inhibition of ACE is considered to be a widely used and effective approach in the treatment of hypertension. ACE inhibitors have been developed as antihypertensive medicine, but such synthetic chemical inhibitors can cause serious side effects [37]. The search for natural alternatives has resulted in the identification of numerous ACE inhibitory peptides derived from various sources. In our study, all these protein hydrolysates had ACE inhibitory activity, and the EC_{30} values for ACE inhibition of all hydrolysates varied between 0.17 and 1.15 mg/ml (Table 2). Meanwhile, ACE inhibitory activity of hydrolysates derived from oyster, scallop, codfish skin, and codfish bone has been already reported, and the IC_{50} of most of them was higher than 10 mg/ml [38]. Saiga *et al.*, (2003) [27] demonstrated that a disparity in activity of two similar hydrolysates (regarding their amino acid compositions) could be related to the structure and length of the peptides in the hydrolysates.

Meanwhile, oxidative stress was suggested to play a very important role in the mechanism of essential hypertension [39]. Oxidative stress was defined as the sustained increase in the levels of reactive oxygen species (ROS), and it has been reported that antioxidant peptides keep cells safe from damage by ROS through the induction of genes [40]. According to Table 3, it was observed that DH of the hydrolysates had significant *($p < 0.05$)* positive correlation with ABTS radical scavenging capacity, and was not statistically significant *($p > 0.05$)* with DPPH radical scavenging, FRAP reducing activity and ACE inhibitory activity. These results may imply that the different antioxidant capacity and ACE inhibitory activity of the hydrolysates was mainly due to the kind of the enzyme used, not to the DH of hydrolysates. Antioxidant activity of protein hydrolysates is affected by amount and composition of free amino acid and peptides [24], the type of protease, degree of hydrolysis [41] as well as size [24], structure, amino acid composition and amino acid sequences of peptides in hydrolysates [42]. Therefore, purification and analysis of the amino acid sequence of the hydrolysates should be carried out in the future.

Table 3. Pearson correlation analysis

	DH	DPPH	ABTS	FRAP	ACE
DH	1.000	0.008	0.944*	-0.563	-0.094
DPPH		1.000	0.217	-0.580	0.032
ABTS			1.000	-0.643	-0.311
FRAP				1.000	-0.257
ACE					1.000

*$p < 0.05$

5. Conclusion

In conclusion, the protein hydrolysates derived from tea seed cake possessed remarkable antioxidant activities and

inhibitory potential against ACE *in vitro*, and may have a potential to be used as functional agents, such as antioxidants and ACE inhibitors. Meanwhile, tea seed cake, as worthless by-product, could be developed as cheap, renewable and readily resource for preparation of bioactive peptides. In addition, separation and purification of these protein hydrolysates are required in further researches, and animal models should be employed to validate the bioactivities of them as well.

Acknowledgements

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