CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals	Company
Alcalase® CLEA	Sigma-Aldrich, USA
Ammonium thiocyanate	Ajax Finechem, AUS
Angiotensin converting enzyme from rabbit lung, ACE	Sigma-Aldrich, USA
2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ABTS	Sigma-Aldrich, USA
Bromelain from pineapple stem	Sigma-Aldrich, USA
Ethanol (absolute)	Qrëc, New Zealand
Ethyl acetate	J.T. Baker®, USA
Ethylenediaminetetraacetic acid, EDTA	Scharlau, Spain
Ferric chloride	POCH, USA
Ferrous chloride	Sigma-Aldrich, USA
Ferrous sulphate	RDH, USA
Hippuryl-L-histidyl-L-leucine hydrate, HHL	Sigma-Aldrich, USA
Hydrochloric acid (37%), conc. HCl	Merck, Germany

Chemicals	**Company**
6-hydroxy-2,5,7,8-tetramethylchromane-2-caboxylic acid, Trolox	Sigma-Aldrich, USA
Linoleic acid	Sigma-Aldrich, USA
Papain lyophilized	Sigma-Aldrich, USA
Potassium persulfate	BDH Porlabo, UK
Protease from *Aspergillus oryzea*, Flavourzyme	Sigma-Aldrich, USA
Protease from *Bacillus amyloliquifaciens*, Neutrase	Sigma-Aldrich, USA
3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p′*-disulfonic acid monosodium salt hydrate, Ferrozine	Sigma-Aldrich, USA
Sephadex® G-50 medium	Sigma-Aldrich, USA
SP Sephadex®	Sigma-Aldrich, USA
Sodium acetate trihydrate	Carlo Erba, Italy
Sodium chloride	Merck, Germany
di-Sodium hydrogen phosphate dihydrate	Qrëc, New Zealand
Sodium hydroxide	RCI Lab Scan, Thailand
Sodium phosphate monobasic dihydrate	Sigma-Aldrich, USA
Sodium sulfite anhydrous	Carlo Erba, Italy
2,4,6-tri(2-pyridyl)-s-triazine, TPTZ	Fluka Chemika, Switzerland
2,4,6-trinitrobenzenesulfonic acid, TNBS	Sigma-Aldrich, USA
Trisma base	Sigma-Aldrich, USA
Trypsin type I from bovine pancreas	Sigma-Aldrich, USA

2.1.2 Instruments/Apparatus

Instruments/Apparatus	**Company**
Amicon® Ultra-15 centrifugal filter devices	Merck millipore, USA
Incubator, WB M15	Falc, Italy
Lambda25 UV/VIS spectrometer	Perkin Elmer, USA
Lyophilizer LY-3-TT	Schneiger Electric, France
MS1 minishaker	IKA, Malaysia
Power supply for electrophoresis, EC250-90	Thermo Fisher Scientific, AUS
pH meter, 713	Metrohm, Switzerland
Spectrophotometer model Genesys 20	Thermo Fisher Scientific, AUS
Vertical electrophoresis system, miniVE	Amersham Biosciences, USA
Waterbath, WNE 14	Memnert, Germany

2.1.3 Nile Tilapia Skin Materials

Nile tilapia (*Oreochromis niloticus*) skins (Figure 2.1) were obtained from a farm under Charoen Pokphand Group in Doi Tao district, Chiang Mai, Thailand. The skins were cleaned up and stored at -18 °C until used.

Figure 2.1 Nile tilapia skin

2.1.4 Peptides

Peptide 1 (GPEGPAGAR) and peptide 2 (GETGPAGPAGAAGPAGPR) were synthesized by China Peptides Co. Ltd. (Shanghai, China).

2.2 Gelatin Extraction

The frozen tilapia skins were thawed, washed with tap water and then cut into small pieces (1×1 cm) by knife. The gelatin extraction method was followed from Songchotikunpan et al. (2008) with slight modification. First, the skins were drenched with 0.8 M NaOH for 2 hrs to remove noncollagenous proteins. The skin:solution ratio was 1:7 (w/v) and the solution was changed when it became dark. Then, tap water was used to wash these treated skins for approximately 1 hr and followed by soaked in 0.5% (v/v) HCl, with the same ratio of skin:solution mentioned previously, for another hour to swell the skin collagen for easier gelatin extraction. The acid treated skins were washed again with tap water until a neutral pH of the wash water was obtained. The distilled water was added to the washed fish skins with the skin:water ratio of 1:2 (w/v) and heated up to 60 °C for 1.5 hrs to extract the gelatin. The resulting gelatin solution was filtered through double layer cheesecloth to remove non gelatin debris. After that, the solution was lyophilized and kept at room temperature for further hydrolysis (Figure 2.2).

Figure 2.2 Lyophilized gelatin from Nile tilapia skin

2.3 Preparation of Gelatin Hydrolysate

Six protease enzymes, including bromelain, papain, trysin, alcalase, flavourzyme and neutrase, were used to hydrolyze the Nile tilapia skin gelatin. The hydrolysis conditions for each enzyme were: bromelain (pH 7.0, 55 °C), papain (pH 6.0, 37 °C), trypsin (pH 8.0, 37 °C), alcalase (pH 7.0, 50 °C), flavourzyme (pH 7.0, 50 °C) and neutrase (pH 8.0, 50 °C) (Alemán et al. 2011a; Li-Chan et al. 2012; Song et al. 2008; Zhao et al. 2009;). The lyophilized gelatin was dissolved in 0.1 M sodium phosphate buffer with optimum pH for hydrolysis. The enzyme:substrate ratio was 1:100 (w/w). The gelatin and enzyme solutions were pre-incubated for 10 min at their optimal temperature before mixing. The hydrolysis was performed for 4 hrs. The hydrolysate mixture was collected every 30 min for 2 hr and every hour for next 2 hr to determine their degree of hydrolysis (DH). These mixtures were boiled for 10 min to deactivate the enzymes and subsequently centrifuged at 4,000×*g* for 15 min. After 4 hrs of hydrolysis, the supernatant was collected, lyophilized and stored at 4 °C until used.

2.4 Determination of Degree of Hydrolysis

The amount of α-amino acid was evaluated from previously described method by Benjakul and Morrissey (1997) to determine the DH. Aliquot of each sample (125 µL) was added to 2.0 mL of 0.2125 M phosphate buffer solution pH 8.2 and 1.0 mL of 0.01% TNBS solution. The mixtures were incubated at 50 °C in the dark for 30 min, followed by adding 2.0 mL of 0.1 M sodium sulfite to terminate the reaction and were cooled down for 15 min. The absorbance was measured at 420 nm and the DH was calculated from following equation:

$$\%\mathrm{DH} = \frac{(L_t - L_0)}{(L_{max} - L_0)} \times 100$$

where L_t represented the released α-amino acid content of sample at time t. L_0 was the initial amount of α-amino acid in gelatin hydrolysate. L_{max} was the maximum amount of α-amino acid in gelatin hydrolysate after acid hydrolysis. The acid hydrolysis was performed from that described by Benjakul and Morrissey (1997). A 4.5 mL of 6 N HCl was added to 500 µL of gelatin suspension and sealed tightly. The hydrolysis was run at 100 °C for 24 hr and, then, the unhydrolyzed sample was remove by filtered through Whatman paper no. 1. A 6 N NaOH was used to neutralize the supernatant before determination of α-amino acid.

2.5 Fractionation of Nile Tilapia Gelatin Hydrolysate by Ultrafiltration

Amicon® Ultra-15 centrifugal filter devices with MWCO 10,000 Da (Figure 2.3) were applied to fractionate the gelatin hydrolysate. Aliquot of hydrolysate sample (15 mL) was filled into these devices, followed by centrifugation at 3,000×g for 30 min to separate peptides with MW lower than 10 kDA from the higher MW peptide (more than 10 kDa). The filtrates were collected and determined for their bioactivities.

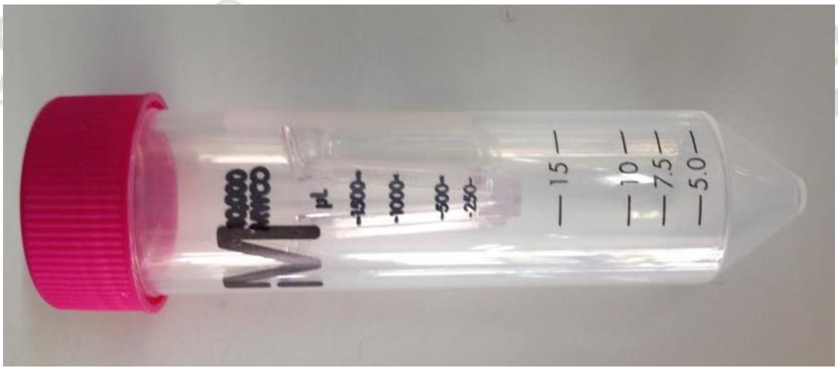

Figure 2.3 Amicon® Ultra-15 centrifugal filter device with MWCO 10,000 Da

2.6 Determination of Bioactivities of Gelatin Hydrolysate

2.6.1 Antioxidant Activity

1) ABTS Radical Scavenging Assay

This assay is based on the reduction of the ABTS radical cation by antioxidant agents (Arts et al. 2004). Briefly, 2.45 mM potassium persulfate was mixed with 7 mM ABTS solution in a ratio of 1:1 (v/v) and kept in the dark for 16-18 hr to generate the radical for $ABTS^{\bullet}$ solution. The dilution of $ABTS^{\bullet}$ solution with Milli-Q water to give an A_{734} around 0.700±0.200 was required before further use. After this, $ABTS^{\bullet}$ solution (980 µL) was added into 20 µL of sample and left at room temperature for 10 min in the dark. The absorbance was measured at 734 nm. Trolox was used as standard antioxidant and the results were calculated as trolox equivalent antioxidant capacity (TEAC) value (µg trolox/mg peptide).

2) Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP method was slightly modified from that described by Griffin and Bhagooli (2004). The working FRAP reagent, consisting of 10 mM TPTZ solution, 20mM $FeCl_2$ and 300 mM acetate buffer pH 3.6 in the ratio of 1:1:10, was freshly prepared and incubated at 37 °C for 30 min. The FRAP reagent (900 µL) was added into sample (100 µL) and left in the dark for 10 min. The absorbance was measured at 593 nm. Trolox was also used as standard antioxidant and the results were reported in the form of FRAP value (mM trolox/mg peptide).

3) Ferrous Ion Chelating Activity Assay

The principle of this method is based on the ability of test samples to trap the Fe^{2+} ion. This assay was slightly modified from the method described by Benjakul et al. (2005). A sample (200 µL) was mixed with 800 µL of distilled water, followed by adding 20 mM $FeSO_4$ (100 µL) and 5 mM ferrozine (200 µL) to form the ferrous ion-ferrozine complex. After leaving at room temperature for 20 min, the mixture was measured the absorbance at 562 nm. The chelating activity percentage was calculated from this formula:

$$\%\text{chelating activity} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where A_{sample} corresponded to the absorbance of ferrous ion-ferrozine complex with the sample presence. $A_{control}$ was the absorbance of ferrous ion-ferrozine complex without sample.

4) Inhibition of Lipid Peroxidation in Linoleic Acid System

This method was slightly modified from that of Yang et al. (2009). The system mixture was composed of sample (0.5 mL), 2 mg/mL linoleic acid in absolute ethanol (2.5 mL), and 0.2 M potassium phosphate buffer pH 7.0 (2.0 mL). The incubation was taken place in the dark at 37 °C for 5 days. After 5 days of incubation, 0.1 mL of aliquot mixture was transferred to new test tube that consisted of 4.7 mL of 75% ethanol and followed by adding 30% ammonium thiocyanate and 20 mM $FeCl_2$ (0.1 mL each). The peroxide value was measured by their absorbance at 500 nm after 3 min, and the inhibition percentage was calculated using the following formula:

$$\%\text{inhibition} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where A_{sample} was the absorbance of mixture with the presence of the hydrolysate sample and $A_{control}$ was the absorbance of mixture without sample.

2.6.2 Antihypertensive Activity

The ACE inhibitory activity was evaluated using a modified method of Park et al. (2003). The hydrolysate sample in 0.1 M phosphate buffer (50 µL) was mixed with 25 mU/mL ACE in 0.01 M phosphate buffer pH 7.0 with 0.5 M NaCl (50 µL) and pre-incubated at 37 °C for 10 min. Then, 100 µL of 6 mM HHL in 50 mM Tris with 300 mM NaCl, the substrate for ACE, was added and incubated for 30 min at the same temperature. The reaction was terminated by adding 200 µL of 1.0 M HCl. Hippuric acid, the product from the ACE activity, was extracted from the solution by ethyl acetate (600 µL), and followed by centrifugation at 4,000 × *g* for 15 min. The upper layer supernatant (200 µL) was transferred to a test tube and evaporated at 95 °C to remove ethyl acetate. One mL of hot distilled water was added to dissolve the hippuric acid and the absorbance was determined at 228 nm. The ACE inhibition was calculated from this equation:

$$\%\text{inhibition} = \left(1 - \frac{A_s}{A_0}\right) \times 100$$

where A_0 was the absorbance of blank mixture (phosphate buffer instead of sample) and A_s was the absorbance of mixture with hydrolysate sample.

2.7 Purification of Bioactive Peptide from Nile Tilapia Skin Gelatin Hydrolysate

Gel filtration chromatography and ion exchange chromatography were applied to purify the hydrolysate with slight modification of Zhang et al. (2012). Sephadex® G-50 was soaked in DI water overnight for maximum swelling before packed into the glass column (2.0 cm × 60 cm). Lyophilized gelatin hydrolysate (1 g) was dissolved in DI water and loaded onto the column. DI water was used as an eluent with a flow rate of 1 mL/min. Eluate was collected 2 mL per tube and monitered at 280 nm using a UV/VIS spectrophotometer. The collecting tubes that had absorbance higher than 0.500 units were pooled and lyophilized. The antioxidant and antihypertensive activities were tested on collected fraction. The fractions with highest antioxidant and antihypertensive activity were further purified by ion exchange chromatography. Before packing the column, SP sephadex® C-25 was equilibrated in 20 mM sodium acetate buffer pH 4.0 overnight. Each hydrolysate fraction (1 g) was dissolved in 20 mM sodium acetate buffer (pH 4.0) and applied to SP sephadex® C-25 column (2.0 cm × 30 cm). The 20 mM sodium acetate buffer with a linear gradient of NaCl of 0-1.0 M at 0.6 mL/min flow rate was used to elute the peptide from the column. The fractions were collected at 2 mL per tube, measured at 280 nm and tested for their bioactivities. The best bioactivities fractions were chosen and lyophilized again for further sequence analysis.

2.8 Identification of Peptide Sequence Using MALDI-TOF/TOF Mass Spectrometer

The sequence of bioactive peptide was analyzed by Proteomics International Pty Ltd (Nedlands, Western Australia). Briefly, the most active purified fraction was subjected to MALDI-TOF/TOF mass spectrometer using a 5800 Proteomics Analyzer (AB Sciex, Framingham, Massachusetts, United States). MS spectra were collected in the molecular mass range 800-8,000 Da. For MS spectra, peak detection signal to noise (S/N) ratio was set to 15. MS/MS spectra were analyzed to identify protein of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR Database.

2.9 Molecular Docking of Identified Peptide on ACE Active Site

The molecular structures of peptides that were identified from MALDI-TOF/TOF were built and energy minimized using SYBYL X Molecular Modeling Software version 1.2 (Tripos Inc., St. Louis, Missouri, United States). The three-dimensional crystal structure of ACE (PDB ID: 4BZR) (Kramer et al. 2014) was retrieved from the Protein Data Bank (PDB) and used as the target receptor for the docking (Figure 2.4). AutoDock Vina program (Trott and Olson 2010) was selected for the docking experiment because of its versatility and flexibility, which easily allow modification and optimization of the docking parameters. Structure of peptide was docked onto the rigid active site of ACE, where a grid box location was centered at the location of the removed original ligand (K-26) binding site. Subsequently, 40 conformations of the docked complexes were automatically ranked based on their estimated binding affinities. Each ligand pose was closely inspected and the molecular interactions between peptide and the ACE were analyzed to confer the most plausible binding mode of the ligands.

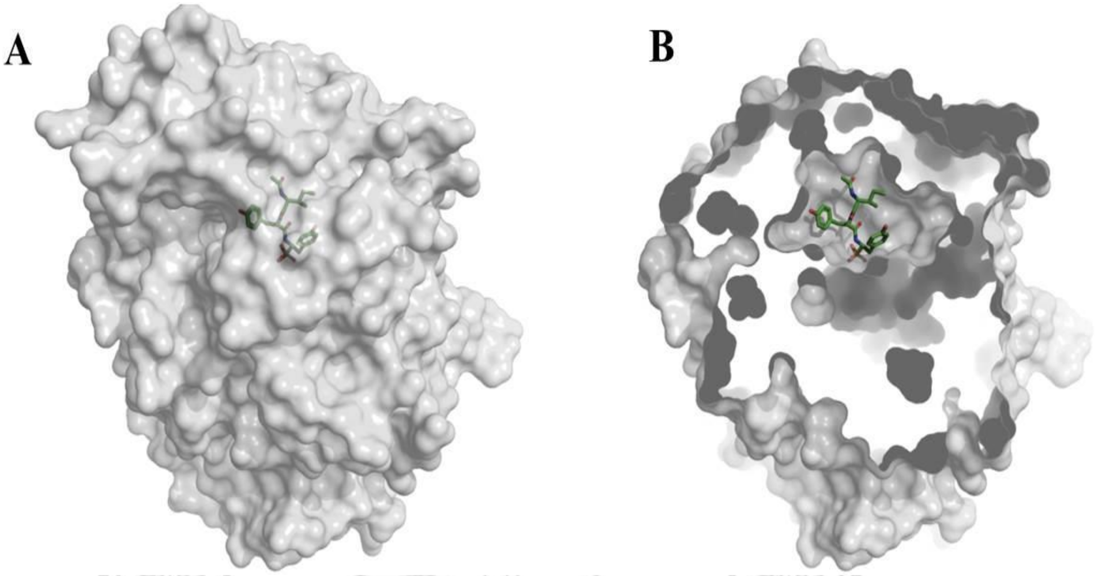

Figure 2.4 Overall 3D structure of the ACE co-crystalized with K-26 inhibitor (PDB ID: 4BZR). (A) Surface model of the crystal structure. (B) The binding site of the embedded K-26 inhibitor which indicated the location of the active site of the enzyme.

2.10 Statistical Analysis

All experiments were performed in triplicates and the result data were given as mean ± S.D. All data were subjected to statistical comparison analysis using the SPSS statistic program (Version 17.0) to assess the differences between samples. The significance level ($p<0.05$) was determined by one-way ANOVA with Duncan's multiple comparison test.