CHAPTER 3

MATERIALS & METHODS

3.1 Maintenance and cultivation of *G. lucidum* mycelia

Ganoderma lucidum culture (KUM50079) was kindly provided by Mushroom Research Centre, University of Malaya, Kuala Lumpur. It was maintained on malt extract agar (Oxoid Ltd., United Kingdom) slants at 25 °C for 7 days. Afterward, the slants were transferred to 4 °C refrigerator for storage. Mycelial culture for inoculum was prepared by centrally inoculating on media containing 2 % (w/v) brown sugar, 1 % (w/v) spent brewer's yeast (Carlsberg Brewery Bhd., Malaysia) and 2 % (w/v) agar (Oxoid Ltd., United Kingdom) at pH 5 in Petri plates and incubated at 25 °C for 12 days.

Mycelia plugs (10 mm diameter) were cut at the periphery of the colony and 10 plugs were transferred into sterile liquid medium (100 mL) consisting of 2 % (w/v) brown sugar and 1 % (w/v) spent brewer's yeast at pH 5. The cultivation was performed in 500 mL Erlenmeyer flasks incubated at room temperature and agitated at 140 rpm on a rotary shaker (Jeio Tech-Lab Companion, Korea).

Following 7 days of cultivation, the mycelia were harvested. Mycelia biomass was separated from broth culture by employing vacuum filtration. On the part of removing broth culture's remnants, the mycelia biomass was washed with large amount of distilled water. Later on, the mycelia biomass and the broth culture were sent for freeze-drying separately.

3.2 Preparation of mycelia and broth crude water extracts

10 g of freeze-dried mycelia were crushed using mortar and pestle to break the cell wall. Subsequently, the mycelia were added with 200 mL of distilled water (1: 20 ratio) and the mixture was stirred for 4 hours before being subjected to centrifugation (Hermle Laboratechnik GmbH, Germany) at 4 °C and 5000 rpm for 20 minutes. The

pellet formed was discarded while the supernatant was sent for freeze-drying and later used as mycelia crude water extract. Stock crude water extract for the mycelia was prepared by dissolving 10 mg of crude water extract in 1 mL of Milli-Q water. A serial dilution was done to prepare test concentrations of 0, 0.625, 1.25, 2.5, 5.0 and 10.0 mg/mL for IC_{50} determination.

On the other hand, 20 g of freeze-dried broth was dissolved in 200 mL of distilled water (1: 10 ratio). The solution was subjected to centrifugation at 5000 rpm and 4 ºC for 20 minutes. The pellet which formed at the bottom of the centrifuge tube was discarded while the remaining supernatant was sent for freeze-drying and labelled as broth crude water extract. Stock crude water extract for the broth was prepared by dissolving 10 mg of crude water extract in 1 mL of Milli-Q water. Preliminary screening of ACE inhibitory activity was carried out for stock crude water extracts of the mycelia and broth (10 mg/mL).

3.3 Angiotensin I-Converting Enzyme (ACE) inhibitory activity

3.3.1 ACE inhibitory assay principle

In vitro study to evaluate ACE inhibitory activity of the mycelia and broth crude water extracts was done using ACE Kit-WST (Dojindo, Japan) where the inhibition activity is measured by the detection of 3-hydroxybutyric acid and involves series of enzyme reaction (Figure 3.1). 3-hydroxybutyric acid is detected using a water-soluble tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-1). In the presence of 3-Hydroxybutyric acid, WST-1 is reduced to WST-1 formazan which is yellow in colour and can be measured at 450 nm.

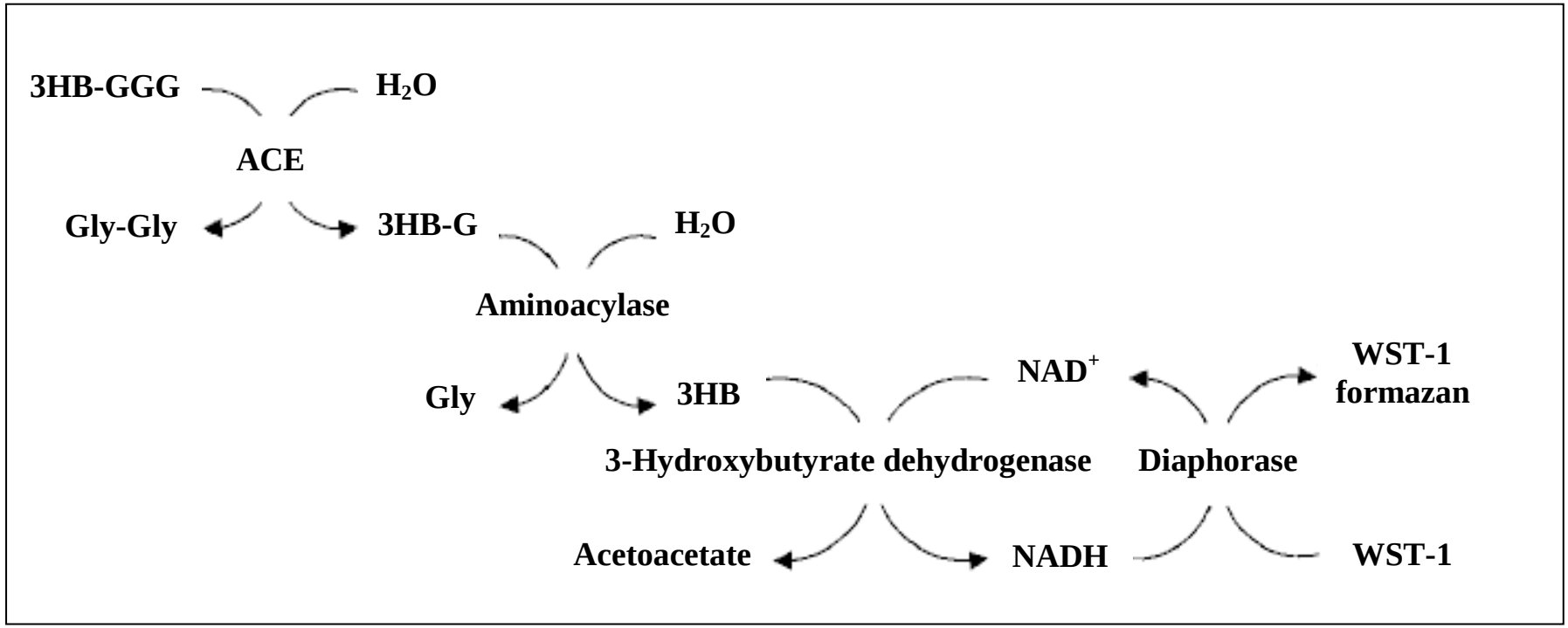

Figure 3.1 : ACE inhibitory assay principle applied for ACE-Kit WST

Adapted from Lam *et al.* (2008).

3.3.2 Preparation of positive control solution

Captopril was selected as the positive control for this assay. Captopril's stock solution of 10 mg/mL was prepared by dissolving 10 mg of the ACE inhibitor powder in 1 mL of Milli-Q water. For IC_{50} determination, the solution was subjected to 10 times dilution sequentially until the lowest concentration of 1×10^{-11} mg/mL.

3.3.3 Preparation of enzyme working solution

Enzyme working solution was prepared by following the manufacturer's instruction (Appendix 4). 2 mL of Milli-Q water was added to enzyme B vial using a syringe in order to prevent dispersal of enzyme powder. The vial was shaken slowly to dissolve the powder. After it has fully dissolved, 1.5 mL of enzyme B solution was added to enzyme A vial using a syringe. To ensure enzyme B and enzyme A were entirely mixed, the vial was shaken slowly for a few minutes. Enzyme working solution was prepared on ice bath to avoid enzyme denaturation.

3.3.4 Preparation of indicator working solution

Indicator working solution was prepared as referred to the manufacturer's instruction (Appendix 4). By using a syringe, 3 mL of Milli-Q water was added to enzyme C and coenzyme vial, respectively. 2.8 mL of enzyme C solution and coenzyme solution were added to indicator solution. The preparation of indicator working solution was done on ice bath.

3.3.5 ACE inhibitory assay protocol

The ACE inhibitory assay was conducted using microplate procedure that offered several advantages with due to lesser consumption of reagents and rapid

evaluation process. The inhibitory assay was started by loading 20 µL of Captopril, mycelia (10 mg/mL) and broth (10 mg/mL) crude water extracts into sample wells respectively. On the other hand, 'blank 1' and 'blank 2' wells were loaded with 20 µL of Milli-Q water. This was followed by the addition of 20 µL of substrate buffer to each well. To initiate the enzymatic reaction, 20 µL of enzyme working solution was added meticulously to each sample well and 'blank 1' well. Since 'blank 2' well represented reagent blank, it was added with 20 µL of Milli-Q water instead of enzyme working solution. 'Blank 1' was a positive blank in which 100 % of enzymatic reaction occurred without any inhibition.

The microplate was shaken carefully to mix the solution before being incubated at 37 ºC for 1 hour to allow optimum enzymatic reaction. Detection of 3-hydroxybutyric acid was performed by adding 200 µL of indicator working solution into each well. The microplate was left incubated at room temperature to allow thorough colour development. After 10 minutes, absorbance reading at 450 nm was measured using a microplate reader (Tecan, Austria). ACE inhibition rate was calculated by applying the following equation:

$$\text{ACE inhibition \%} = [(\text{abs. blank 1} - \text{abs. sample}) / (\text{abs. blank 1} - \text{abs. blank 2})] \times 100$$

3.4 Fractionation of proteins from the mycelia of *G. lucidum*

Following ACE inhibitory assay, mycelia crude water extract was found to exhibit stronger inhibition on ACE activity compared to broth crude water extract. Hence, mycelia crude water extract was chosen for further study. Proteins were fractionated from mycelia crude water extract by employing ammonium sulphate precipitation method or also known as salting out. Ammonium sulphate has been used

widely to fractionate proteins from solutions based on the proteins solubility. An increase of the salt concentration will lower the solubility of proteins especially those which are hydrophobic.

Mycelia crude water extract was prepared as described in section 3.2. The supernatant obtained was kept for salting out process. Appropriate amount of crystallized ammonium sulphate was weighed to obtain final salt saturation of 10 %. The amount of salt required was determined using a nomogram in Table 3.1. Ammonium sulphate was then added imperceptibly to the supernatant with gentle stirring. It is important to note that sudden addition of salt could cause protein degradation.

Once all the salt has been added, gentle stirring of the solution continued for another 45 minutes to bring the salt saturation into equilibrium. This was then followed by centrifugation at 4 °C and 10,000 rpm for 15 minutes. Pellet was collected and dissolved in 4 mL of distilled water. Salting out was continued for 20 % salt saturation using the supernatant and this process was repeated until 100 % salt saturation accomplished.

Semi-purified proteins which have been fractionated through the salting out process might contain residual ammonium sulphate bound to those proteins. Removal of salt was done by employing dialysis procedure using SnakeSkin pleated dialysis tubing (Thermo Fisher Scientific, USA) with 3,500 Dalton (Da) molecular weight cut-off. An appropriate length of dialysis tubing membrane was measured and cut, according to the volume of solution needed to be dialysed. The dialysis tubing was pre-soaked in a beaker of distilled water for 30 minutes. After it has been removed from the beaker, a tight knot was tied at one end of the tube.

Table 3.1 : Nomogram for determining ammonium sulphate saturation

		Final saturation of ammonium sulphate (% saturation)									
		10	20	30	40	50	60	70	80	90	100
		Amount of ammonium sulphate (g) need to be added to 1 L of solution									
Initial saturation of ammonium sulphate (% saturation)	0	56	114	176	243	313	390	472	561	662	767
	10		57	118	183	251	326	406	494	592	694
	20			59	123	189	262	340	424	520	619
	30				62	127	198	273	356	449	546
	40					63	132	205	285	375	469
	50						66	137	214	302	392
	60							69	143	227	314
	70								72	153	237
	80									77	157
	90										79

Protein solutions obtained from salting out process were poured into individual dialysis tubing and another tight knot was tied at the other end. The outer surface of the dialysis tubing was rinsed with distilled water to remove any fluids. The dialysis tubing filled with protein sample was placed in a beaker containing 500 mL of distilled water. The tubing was left stirring at 4 °C for 48 hours with 4 times buffer changes. Afterwards, all dialysed protein samples were removed out from individual tubing and freeze-dried. All freeze-dried samples were labelled as 10 % to 100 % semi-purified protein fractions. The concentration of the proteins was determined and the proteins' profiling was performed by SDS-PAGE.

Note: 3.5 kDa proteins were fully retained in the sample solution while smaller molecules, such as salt and small peptides, were dialysed out into the buffer solution exhibiting low concentration of salt.

3.5 Estimation of mycelial proteins concentration

In order to determine the concentration of proteins obtained from the mycelia crude water extract, an assay was performed using Pierce BCA Protein Assay Kit. The method of the assay is based on the combination principle of the reduction of Cu^{+2} to Cu^{+1} by protein occurs in an alkaline medium, which is known as the biuret reaction, with the highly sensitive colorimetric detection and quantitation of cuprous cation, Cu^{+1}, by bicinchoninic acid (BCA) (Smith *et al.*, 1985).

3.5.1 Preparation of standard protein

The protein concentrations were determined with reference to a standard protein, bovine serum albumin (BSA). In purpose to produce a standard protein curve, BSA was

prepared in a series of dilutions range from 2000 µg/mL to 0 µg/mL and assayed together with the mycelial proteins. The concentrations of the mycelial proteins were then determined based on the standard curve. Preparation of the BSA dilutions is shown in Table 3.2.

3.5.2 Preparation of BCA working reagent

BCA working reagent was prepared from combination of reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and reagent B (containing 4 % cupric sulphate), as referred to manufacturer's instruction (Appendix 4). 50 parts of reagent A was mixed together with 1 part of reagent B. The volume of working reagent was calculated and prepared sufficiently based on the number of samples to be assayed. The preparation was done at room temperature and the working reagent was stored in a closed container.

3.5.3 Protein estimation protocol

25 µL of each standard protein and mycelial proteins was pipetted into a microplate well. Each of the well was added with 200 µL of working reagent which has been prepared earlier. The plate was covered and agitated for about 30 seconds to mix the content. Then, it was incubated at 37 ºC using water bath for even heat transfer. After 30 minutes, the plate was cooled at room temperature before its absorbance at 562 nm could be measured using a microplate reader (Tecan, Austria).

Table 3.2 : Preparation of BSA serial dilutions

Vial	Volume of distilled water (µL)	Volume of BSA (µL)	Final BSA concentration (µg/mL)
A	0	300	2000
B	125	375	1500
C	325	325	1000
D	325	325 (of vial C dilution)	500
E	325	325 (of vial D dilution)	250
F	325	325 (of vial E dilution)	125
G	400	100 (of vial F dilution)	25
H	400	0	0

3.6 Analysis of mycelial proteins by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The mycelia's crude water extract as well as semi-purified protein fractions were subjected to SDS-PAGE analysis to obtain a protein band profile. SDS-PAGE is a one dimensional gel electrophoresis, whereby it is widely used in separating proteins on the basis of their molecular weight. All solutions for SDS-PAGE were prepared based on the discontinuous buffering system by Laemmli (1970). The buffer system engages different buffers for the tank and gel, which contrasts to continuous buffering system. Variation of the buffers contributes to major advantage as it improves resolution of the protein bands compared to continuous system.

3.6.1 Preparation of SDS-PAGE solutions

(a) Solution A- Monomer acrylamide and bisacrylamide

30 g of acrylamide and 0.8 g of N, N'-methylenebisacrylamide were dissolved in 100 mL of distilled water. A small quantity of Amberlite IRN-150L was added to remove ionic impurities from the mixture. After 1 hour of stirring, the solution was filtered to discard the Amberlite. The solution was stored in a dark bottle at 4 ºC.

(b) Solution B- 4x running gel buffer (1.5 M Tris-HCl, pH 8.8)

18.17 g of Tris base was dissolved with 100 mL of distilled water. The pH of the solution was adjusted to pH 8.8 using concentrated HCl and stored at 4 ºC.

(c) Solution C- 10 % (w/v) Sodium Dodecyl Sulphate (SDS)

10 g of SDS was weighed and added with 100 mL of distilled water. The solution was stored in a Schott bottle at room temperature.

(d) Solution D- Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

6.06 g of Tris base was mixed with 100 mL of distilled water and stirred until it dissolved. By adding concentrated HCl, the pH of the solution was adjusted to pH 6.8. Solution D was kept at 4 ºC.

(e) 10 % (w/v) Ammonium persulphate (APS)

0.1 g of ammonium persulphate was dissolved in 1 mL of distilled water. The solution was prepared freshly prior to use.

(f) N, N, N', N'-tetramethylenediamine (TEMED)

TEMED was directly used from the purchased bottle and kept at room temperature.

(g) 4x SDS-PAGE sample buffer

10 mg of bromophenol blue, 200 mg of SDS, 100 mg of dithiotreitol, 1 mL of glycerol and 1.25 mL of Solution D were mixed and made up to 10 mL with distilled water. The prepared sample buffer was stored at -20 ºC in aliquots.

(h) Running buffer (25 mM Tris, 198 mM Glycine, 0.1 % (w/v) SDS, pH 8.3)

3.03 g of Tris base, 14.4 g of Glycine and 1 g of SDS were weighed and dissolved in distilled water until the final volume reached 1 L. The buffer was stored at room temperature and ready to be used.

3.6.2 Preparation of separating gel

The 18 % separating gel was prepared based on the recipe shown in Table 3.3. Final volume of 10 mL was enough to prepare 1 gel with 0.75 mm thickness. 10 % APS and TEMED were added prior to gel pouring.

Table 3.3 : Preparation of 18 % separating gel

Solution	**Volume**
Solution A (mL)	6.00
Solution B (mL)	2.50
Solution C (µL)	100.00
Distilled water (mL)	1.345
10 % APS (µL)	50.00
TEMED (µL)	3.30
Final volume (mL)	**10.00**

The gel solution was mixed well and poured slowly into the gel casting apparatus by using a pipette. To ensure formation of even gel surface, distilled water was overlaid gently on the surface of the gel. Later, the gel was left overnight to allow thorough polymerization.

3.6.3 Preparation of stacking gel

The 4 % stacking gel was prepared based on the recipe in Table 3.4. To prepare 1 gel with 0.75 mm thickness, total volume of approximately 5 mL was required. 10 % APS and TEMED were added prior to gel pouring. The solutions were mixed well by gentle stirring.

Distilled water which was overlaid on the separating gel was poured off and the gel surface was rinsed with 2 mL of stacking gel solution. By using a pipette, the stacking gel solution was poured slowly to overlay the polymerized separating gel. A comb consisting of 14 wells was inserted carefully to prevent bubble formation and the gel was left to polymerize for 1 hour. Soon after the gel had completely polymerized, the wells were rinsed with electrophoresis running buffer to remove remnant of stacking gel solution.

3.6.4 Running the SDS-PAGE

Polymerized gel was clamped to the electrophoresis chamber and running buffer of pH 8.3 was poured to fill up the chambers. Appropriate concentration of mycelia crude water extract and ten semi-purified mycelial protein fractions were mixed with sample buffer in the ratio of 3:1. Then, protein marker and the mixtures were heated at 90 ºC for 5 minutes prior to electrophoresis run to allow complete protein denaturation in the sample.

Table 3.4 : Preparation of 4% stacking gel

Solution	**Volume**
Solution A (mL)	0.325
Solution B (mL)	0.625
Solution C (µL)	25.0
Distilled water (mL)	1.525
10 % APS (µL)	12.5
TEMED (µL)	2.5
Final volume (mL)	**2.50**

3 µL of protein marker together with 12 µL of crude water extract and semi-purified protein fractions were loaded meticulously into respective wells. It was noteworthy to avoid samples from diffused out and entered adjacent wells. Tank cover and power leads were appropriately attached to the electrophoresis chamber (C.B.S Scientific, USA). Electrophoresis was started at a constant voltage of 60 V for the first 10 minutes followed by increasing the voltage up to 100 V. Once the blue dye reached approximately 1 cm from the gel bottom, electrophoresis will end. The gel was removed from the casting apparatus and was immediately subjected to Coomassie Brilliant Blue staining.

3.6.5 Preparation of Coomassie Brilliant Blue staining solutions

(a) Staining solution (0.1 % Coomassie Briliant Blue R-250 in 10 % acetic acid and 40 % methanol)

1.0 g of Coomassie Brilliant Blue R-250 was dissolved in 400 mL of methanol and 100 mL of acetic acid. The mixture was stirred well and filled up with distilled water until final volume reached 1 L. The staining solution was kept in dark storage at room temperature.

(b) Destaining solution (10 % acetic acid)

100 mL of acetic acid was added with 900 mL of distilled water. The destaining solution was kept at room temperature.

3.6.6 Coomassie Brilliant Blue staining protocol

Through a delicate procedure, stacking gel was discarded and separating gel was placed in Coomassie Brilliant Blue staining solution. The staining step required

gentle shaking on a rotary shaker in order to develop a satisfactory staining visualization. After 1 hour, the staining solution was discarded and replaced with destaining solution. The gel was incubated with several changes of destaining solution until the protein bands were nicely visualized and the gel's background turned transparent. Later on, the destaining solution was substituted with distilled water.

3.6.7 Preparation of modified silver staining solutions

(a) Fixing solution

40 mL of ethanol was added with 10 mL of acetic acid glacial. Double distilled water was added to the solution until the final volume reached 100 mL.

(b) Sensitizing/incubating solution

6.8 g of sodium acetate and 0.2 g of sodium thiosulfate were dissolved in 30 mL of ethanol. The solution was added with double distilled water until the final volume reached 100 mL.

(c) Staining solution

0.25 g silver nitrate was measured and dissolved in 100 mL of double distilled water.

(d) Developing solution

2.5 g of sodium carbonate was weighed and dissolved in 50 mL of double distilled water. 0.04 mL of 37 % formaldehyde and 2.8 µL of 5 % sodium thiosulfate were mixed into the solution. A final volume of 100 mL was obtained by adding double distilled water.

(e) Stopping solution

1.46 g of EDTA-Na_2H_2O was dissolved in 100 mL of double distilled water.

Note: The modified silver staining solutions were prepared prior to use.

3.6.8 Modified silver staining protocol

Stacking gel was detached carefully and separating gel was incubated in 100 mL of fixing solution for 30 minutes with gentle shaking on a rotary shaker. The fixing solution was replaced with 100 mL of sensitizing solution and left to be agitated. After 30 minutes, the sensitizing solution was substituted with distilled water. The gel was washed for 5 minutes and the washing step was repeated 3 times.

Subsequently, the gel was assigned to incubation in 100 mL of silver staining solution. During staining step, gentle shaking was imperative in order to permit uniform binding of silver ions to the proteins. The staining solution was removed after 20 minutes of incubation and once again the gel was subjected to washing step for 30 seconds with 2 times repetition.

For the development phase, the gel was placed in 100 mL of developing solution until the protein bands started to visualize. This stage was critical as the previous silver ions that attached to proteins were reduced and resulted in the development of gel image. Once the interested protein bands profile appeared, the reaction was terminated by replacing the developing solution with 100 mL of stopping solution. 10 minutes incubation in stopping solution was later trailed with 3 times of 10 minutes washing using distilled water.

3.6.9 Grouping of semi-purified mycelial proteins

Upon visualization of Coomassie Brilliant Blue stained gel, the 10 % to 100 % semi-purified mycelial proteins were grouped together based on their protein bands profile. These protein groups were labelled as fractions A, B, C, D and E. Each fraction

was evaluated for their ACE inhibitory potential according to the procedure described in section 3.3.

3.7 Purification of selected ACE inhibitory protein fractions by reversed phase High Performance Liquid Chromatography (RP-HPLC)

3.7.1 Preparation of stock solution for selected protein fractions

Protein fractions with good anti-ACE potential (A and C) were prepared at a concentration of 1 mg/mL by dissolving them in distilled water. Protein solutions were subjected to centrifugation (Eppendorf, Germany) at 1000 rpm for 2 minutes as to remove un-dissolved proteins. Afterwards, the supernatant formed was filtered through a 0.45 µm membrane filter (Lubitech Technologies, Shanghai, China) and ready to be used for HPLC analysis.

3.7.2 Preparation of mobile phase system

(a) 0.1 % Trifluoroacetic acid (TFA) in water

1 mL of TFA was added to 1 L of distilled water. The solution was filtered using 0.45 µm cellulose membrane filter (Whatman, United Kingdom) and kept in a Schott bottle at room temperature.

(b) 100% Acetonitrile (ACN)

1 L of ACN was taken directly from purchased bottle. The solvent was filtered using 0.45 µm nylon membrane filter (Whatman, United Kingdom) and kept in a Schott bottle at room temperature.

3.7.3 HPLC analysis

The chromatography was performed using Shimadzu SCL-10A VP system controller, equipped with a vacuum degasser (DGU-12A), a binary pump (LC-10AT VP), a sample inlet, a diode array detector (SPD-M10A VP) and connection to a computer system. The separation was carried out using Atlantis® T3 C_{18} column (5 µm particle size, 250 mm × 4.6 mm) from Waters Corporation, Ireland.

Optimization of the solvent system was accomplished using 0.1 % (v/v) TFA in water as mobile phase A and 100 % ACN which represented mobile phase B. Elution for fraction A was performed using 5 % - 90 % B within 0 to 45 minutes while protein from fraction C was eluted using 5 % - 90 % B within 0 to 40 minutes with a flow rate of 1 $mLmin^{-1}$. 200 µL of 1 mg/mL protein solution that had been prepared earlier was injected into the sample inlet. The elution was monitored at 220 nm and 254 nm.

Six and seven HPLC peaks were collected from fractions A and C respectively. The HPLC peaks were left under a liquid nitrogen flow in order to remove ACN. Afterwards, the HPLC peaks were introduced to freeze-drying.

3.8 Assessment of ACE inhibitory activity of HPLC peaks post HPLC purification

Freeze-dried HPLC peaks of both protein fractions were dissolved with appropriate amount of Milli-Q water in order to prepare sample concentration of 25 µg/mL. Assay for determining ACE inhibitory activity was performed according to protocol explained in section 3.3.

3.9 SDS-PAGE analysis of active anti-ACE HPLC peaks

Protein profile for HPLC peaks labelled 3, 4 and 5 from fraction C were developed by SDS-PAGE. The electrophoretic study was carried out by following the

procedure elucidated in section 3.6 and the SDS-PAGE gel obtained was stained using modified silver staining method.

3.9.1 Determination of molecular weight for visualized protein bands

Molecular weight for the protein bands were determined by comparing the migration of the unknown proteins to the migration of known protein markers. A standard curve of log molecular weight against R_f value of known proteins was plotted properly. Molecular weight for the unknown proteins was determined by interpolating the R_f value of the unknown proteins into the standard curve. The R_f value was defined as following equation:

R_f value = Distance of protein migration / Distance of dye front migration

3.10 Characterization and identification of potential anti-ACE proteins

Matrix Assisted Laser Desorption/Ionization-Time of Flight/Time of Flight Mass Spectrometry **(**MALDI-TOF/TOF MS) was implemented in this study for characterizing and identifying the anti-hypertensive proteins. The identification was performed by peptide mass fingerprint (PMF) and tandem MS analysis. The essential components of MALDI TOF/TOF MS include sample inlet, ion sources, mass analysers and ion detector.

3.10.1 Preparation of solutions for sample digestion

(a) 100 mM ammonium bicarbonate solution

0.791 g of ammonium bicarbonate was dissolved in 100 mL of Milli-Q water and kept in a Schott bottle at room temperature.

(b) 100 % ACN

100 mL of ACN was taken directly from purchased bottle and kept in a Schott bottle at room temperature.

(c) Destaining solution (15 mM potassioum ferricyanide in 50 mM sodium thiosulphate)

Stock solution of 30 mM potassium ferricyanide was prepared by mixing 98.78 mg of potassium ferricyanide with 10 mL of Milli-Q water. On the other hand, 248.21 mg of sodium thiosulphate was dissolved in 10 mL of Milli-Q water to make a 100 mM sodium thiosulphate stock solution. 500 µL from both stock solution was mixed together to formulate the working solution. Since the working solution was unstable, its preparation was done prior to digestion.

(d) Reducing solution (10 mM dithiothreitol in 100 mM ammonium bicarbonate)

15.42 mg of dithiothreitol (DTT) was weighed and dissolved in 10 mL of Milli-Q water. The solution was prepared prior to digestion.

(e) Alkylating solution (55 mM iodoacetamide in 100 mM ammonium bicarbonate)

50.86 mg of iodoacetamide (IAA) was weighed and dissolved in 5 mL of 100 mM ammonium bicarbonate. The solution was prepared in advance of digestion.

(f) Washing solution (50 mM ammonium bicarbonate in 50 % ACN)

20 mL of 100 mM ammonium bicarbonate was mixed thoroughly with 20 mL of 100 % ACN.

(g) Trypsin working solution (6 ng/µL of trypsin in 40 mM ammonium bicarbonate)

0.1 µg/µL trypsin stock solution was prepared by adding 200 µL of trypsin suspension buffer to 20 µg of trypsin. The solution could be stored at -20 °C for up to two months. A serial dilution was done in order to prepare trypsin solution of 6 ng/µL.

3.10.2 Sample digestion protocol

Protein bands of HPLC peaks C3, C4 and C5 from SDS-PAGE gel were excised. The gel plugs were placed in micro centrifuge tubes and soaked in 50 µL of destaining solution until became crystal clear. The destaining solution was discarded carefully and replaced with 150 µL of reducing solution. The gel plugs were incubated in a water bath with temperature of 60 °C for 30 minutes and later were left to cool at room temperature before the reducing solution was removed.

150 µL of alkylating solution was added and the gel plugs were incubated in dark environment for 20 minutes. The addition of alkylating solution purposely to prevent the formation of disulphide bonds by binding to the thiol group of cysteine. Subsequently, the alkylating solution was discarded and replaced with 500 µL of washing solution in order to remove remnants of DTT as well as IAA. After 20 minutes, the solution was discarded and this washing step was repeated three times.

Upon dehydration step, the gel plugs were soaked in 50 µL of 100 % ACN and subjected to drying process performed using vacuum concentrator (Thermo Fisher Scientific, USA). Proteins in the dried gel plugs were digested by adding 25 µL of trypsin working solution. It was crucial to ensure that the gel was completely covered with the enzyme solution. The digestion activity was carried out overnight under optimal temperature at 37 °C to obtain maximal peptide recovery.

Supernatant for each gel was withdrawn and placed in a new sterile micro centrifuge tube labelled tube A. Extraction for digested peptide was initiated by adding 50 µL of 50 % ACN to the gel plugs. After 15 minutes of soaking, the supernatant was transferred into tube A. Once again, the gel plugs were allowed to incubate in 100 µL ACN. Following 15 minutes of incubation, the supernatant was removed and pooled with supernatant in tube A. The combined supernatant was dried down in a vacuum concentrator and the process took approximately 2 to 2.5 hours. The dried extracts could be stored in -20 °C for several months.

3.10.3 Preparation of solutions for sample desalting

(a) Wetting solution (50 % ACN)

2 mL of 100 % ACN was mixed with 2 mL of Milli-Q water.

(b) Equilibrating and washing solutions (0.1 % TFA)

10 µL of TFA was pipetted into 9.99 mL of Milli-Q water in order to prepare a final concentration of 0.1 % TFA.

(c) Eluting solution (0.1 % TFA in 50 % ACN)

0.2 % of TFA was prepared by mixing 10 µL of 100 % TFA with 4.99 mL of Milli-Q water. Afterwards, 2 mL of the solution was dissolved in 2 mL of 100 % ACN.

3.10.4 Sample desalting protocol

The dried extracts were dissolved in 10 µL of 0.1 % TFA prior to desalting on a Millipore Zip Tip (C18). 10 µL of wetting solution was aspirated and dispensed several times through the C18 resin followed with equilibrating solution, sample solution and washing solution. Later, 1.5 µL of eluting solution was aspirated and dispensed

repeatedly to elute the cleaned up samples which bind to the immobilized resin at the end of Zip Tip.

3.10.5 MALDI-TOF/TOF MS analysis and database search

Following desalting, the peptide mixtures were dissolved uniformly in 1.5 µL of matrix consisting of saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma Chemical Co., St. Louis, USA). The matrix was prepared in acetonitrile/water (1:1) containing 0.25 % (v/v) of TFA. Afterwards, 0.7 µL of the mixture were spotted appropriately on a stainless-steel sample target plate and left to dry at room temperature before being loaded into 4800 Plus MALDI-TOF/TOF MS analyser (Applied Biosystem/MDS Sciex, Toronto, Canada). Mascot (http://www.matrixscience.com) and ProFound (http://prowl.rockefeller.edu) programmes were adopted for protein identification.

Mascot database search parameters used were: trypsin as the enzyme; one missed cleavage allowed; fixed modification/carbamidomethyl (cysteine); variable modification/oxidation (methionine); mass tolerance for precursor ion/peptide tolerance: 50 ppm and mass tolerance for fragment ion/MS/MS tolerance: 0.1 Da. The cut-off score for accepting individual MS/MS spectra was set at .29 for homology and .38 for matched identity. Search parameters for ProFound were as follows: Fungi as taxonomic category; protein mass range of 0-100 kDa; protein pI range of 3-10; one missed cleavage site; charge state of MH+; mass tolerance of 0.5-1.15 Da.

3.11 Statistical analysis

Data analysis was performed using Minitab statistical software (Minitab Incorporation, USA). The effect of ACE inhibitor on the activity of ACE was tested by one-

way ANOVA. Means were accepted as significantly different at 95 % confidence level ($p < 0.05$).