CHAPTER III

EXPERIMENTAL

3.1 Material

3.1.1 Plant material

The young fresh flowers of *Sesbania grandiflora* (Dok khae baan) were purchased from local market in Bangkok, Thailand.

3.1.2 Erythrocytes

Rabbit blood cells were obtained from the faculty of Veterinary medicine of Chulalongkorn University of Thailand. While goat blood cells were obtained from Department of Medical Science of Thailand.

3.1.3 Chemicals

Sodium chloride (NaCl), Potassium hydrogen phosphate (KH_2PO_4), Disodium hydrogen phosphate ($Na_2H_2PO_4$) and Ammonium sulphate ($(NH_4)_2SO_4$) used in extraction and precipitation step were obtaind from Merck (Germany). EDTA was purchased from Fluka (Germany).The most reagents for electrophoresis and the low molecular weight calibration kit, standard molecular weight marker proteins were the product of Amersham Pharmacia biotech (UK). Coomasie brilliant blue and Trichloroacetic acid (TCA), for gel staining was obtained from Sigma (St. Louis, MO, USA). Methanol (CH_3OH), Acetic acid (CH_3COOH) used in destaining reagent, Acetone and Acetonitrile, the solvent for 2-D Electrophoresis and Reverse phase high-performance liquid chromatography (RP-HPLC) , were obtain from Merck (Germany). Media for gel filtration chromatography, Superdex 200 and Superdex 75, and Ion exchange resin, DEAE-cellulose, were the product of Amersham Pharmacia Biotech. For positive test control of Hemagglutinating activity, Concanavalin A (Con A) lectin, substrate and enzyme used in α-glucosidase inhibitory activity,

p-nitrophenyl -α-D-glucopyranoside (PNPG) and α-Glucosidase, respectively, were purchased from Sigma (St. Louis, MO, USA). Solvent use for in-gel digestion and preparation for mass spectrometric techniques were analytical grades obtained from Labscan (Bangkok, Thailand) and Amersham Pharmacia Biotech (Sweden).

3.1.4 Apparatus and Instruments

Immobilized pH gradient strips (IPG strips) pH 3-10 (Amersham pharmacia biotech) was run in Multiphor^{TM} II Electrophoresis Unit (Amersham pharmacia biotech Uppsala Sweden). SDS-PAGE was run in Hoefer TM miniVE (minivertical), 8×9 cm gels (Amersham pharmacia biotech Uppsala Sweden). Desalting Cartridge (Protein macrotrap,Michrom BioResource Inc.) and Dialysis bag (SnakeSkin Dialysis Tubing, Pierce, USA.) were used for protein desalting. Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometer (BIFLEX Bruker, Germany) was used to analyze protein mass spectra. For Column Chromatography, the protein samples were run on AKTA prime (Amersham pharmacia biotech wikstroms, Sweden) with COLUMN XK1.6×15 cm and COLUMN XK1.6×56 cm Amersham pharmacia biotech wikstroms, Sweden) which the buffer system in this technique was adjust pH with pH meter (Denver Instrument USA. system).And Reverse phase high-performance liquid chromatography (RP-HPLC) run on Finnigan Spectra system (Thermo electron Corporation, Massachusetts, USA.) with Aquasil C_{18} column (Thermo electron, USA.).To concentrate protein, Speed vacuum (MAXI dry plus, Heto vacuum centrifuge, Denmark) and Freeze-dryer have been used. Microtiter plate reader was used in measurement absorbance of protein quantities, Bradford test, and α-glucosidase inhibitory activity. The other apparatus and instruments used in this research were Siliconized eppendorf (Axygen sciencetific Inc.), Pipette tips (Bioline USA.), Micropipette (Pipetteman, Gilson, France.), Vortex mixer (Vortex-genie2, Sciencetific Industries.), Sonicate (DHA-100; Branson, U.S.A.), Water Bath Shaking (Memmert, Germany), and Orbital Shaker (Kika-Werke GMBH&Co., Germany).

3.2 Methods of protein purification

3.2.1 Protein extraction and precipitation

All extraction and precipitation steps were carried at 4°C, *Sesbania grandiflora* flowers (2 kg) were homogenized in 50mM phosphate buffer, pH 7.2, containing 0.1 M NaCl and 1mM using a blender. The homogenate was extracted with isolation buffer 8 L by stirring overnight at 4 °C. The light green homogenate was filtered through cheesecloth and then centrifuged for 15 min at 5000 rpm at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant up to 20% saturation (114 g/liter). After 1 h the precipitate was removed by centrifugation (5000 rpm, 4°C, 15 min). $(NH_4)_2SO_4$ was then added to the supernatant to 90% saturation. The suspension was stirred for 1 h and the precipitate, collected after 1 h of centrifugation at 5000 rpm 4°C 15 min, and was dissolved in distilled water. Then the solution was desalted by dialyzed against deionized water and was poured into freeze-drying glass bottle and frozen overnight in preparation for freeze-drying. The bottle was then placed in the freeze-dryer for over 24-36 h. The dried crude protein (a brown fluffy powder) was then collected into plastic bottle, and placed in a refrigerator until used [61].

3.2.2 Determination of protein concentration

Bradford assay was used for determine protein concentration using bovine serum albumin (BSA) as a protein standard to generate a standard curve. For the quantitative of the protein in this research, micro assay method using a microplate reader has been used. The total volume of modified assay is 210 µl by pipetting 10 µl of protein sample into 96 well plates before adding 200 µl of the Bradford working solution (Table 3A, Appendices A). After addition the protein reagent, the absorbance of each sample measure between 2-60 min at wavelength 595 nm [62,63].

3.2.3 SDS-PAGE

SDS-PAGE (15%T, 3%C) was performed according to the Laemmli method. It was carried out in a 1 mm vertical slab gel (8 × 9 cm) consisting of stacking gel mix, (5%T,3%C) and main separating gel mix, 15% acrylamide, prepared in 2 M Tris–HCl, pH 8.8 and allowed gel to polymerize by adding 10% ammonium

persulfate (APS) and tetramethylene diamine (TEMED). Four parts of samples were mixed with one part of reducing buffer, namely 60 mM Tris–HCl, pH 6.8, containing 25% glycerol, 0.1% bromophenol blue, 2% SDS and 14.4 mM 2-mercaptoethanol and incubated at 100 °C for 3 min. Electrophoresis was carried out with electrophoresis buffer containing 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 at 280 V, 20 mA constant current for 2 h. Protein bands were stained with coomassie brilliant blue G-250 (see Table 2A in Appendices A) for 30 min. After that pour out the staining solution and add destaining which is a mixture of methanol and acetic acid in water. The apparent molecular masses of protein were estimated using the low molecular weight marker kit which contains known protein standard, phosphorylase b, bovine serum albumin, egg albumin, carbonic anhydrase, soybean trypsin inhibitor, and lactalbumin, with apparent molecular masses of 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 kDa, respectively [62].

3.2.4 TWO-Dimensional Electrophoresis

3.2.4.1 Protein extraction for 2D Electrophoresis

The proteins were extracted by TCA /acetone precipitation based on the method developed by Granier. Frozen flowers cooled with liquid nitrogen were ground in a mortar with a pestle. The cold (20°C) 10% TCA in acetone containing 0.07% mercaptoethanol was added to the powdered sample. The sample was kept at 20°C to allow complete precipitation. The sample was centrifuged (15 min at 4000 rpm) in a centrifuge, then washed three times with acetone containing 0.07% mercaptoethanol .The precipitate was concentrated by speedvacuum for 1 h and suspended with lysis buffer (9.5 M urea, 2% CHAPS (3[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid), 1% dithiothreitol, and 0.8% carrier ampholytes [64].

3.2.4.2 Isoelectrofocusing and SDS-PAGE

All stock solution in this step present in Appendix A. The protein sample from 3.2.4.1 section was brought to a total volume of 130 µl loaded on 7-cm IPG strips pH 3-10 (Amersham Biosciences) consecutively. Isoelectric focusing was performed at

20°C for 80 kVh on an IPGphor (Amersham Biosciences). For SDS-PAGE, first IPG strips were equilibrated for 15 min by 80 mg DTT in 8 ml SDS Equilibration buffer and a second equilibration performed with an iodoacetamide solution (200 mg IAA, 8 ml SDS Equilibration buffer) for 15 min. SDS-PAGE (15% T) using single percentage gel follow in table 3.1 was performed at constant current in two steps, first electrophoresis condition is 10 mA current at 280 V15 min and second apply 20 mA current at 280 V for 90 min or the dye front is approximately 1 mm from the bottom of the gel. Low molecular weight marker proteins were applied on the gel via a small piece of filter paper. The gels were coomassie -stained according section 3.2.3 [49].

Table 3.1 Preparation of single-percentage gel (15%) for SDS-PAGE in second dimension separation of 2-D electrophoresis (preparation of stock solutions is described in Appendix A.

Final Gel Concentration	**Volume**
Monomer solution	5 ml
4× resolving gel buffer	2.5 ml
10% SDS	100 µl
Double distilled water	2.35 ml
10% ammonium persulfate	50 µl
TEMED	3.3 µl
Total volume	10 ml

3.2.5 Column Chromatography

3.2.5.1 Gel Filtration Chromatography

Proteins were dissolved with 20 mM Tris-HCl buffer (pH 8.0) and then fractionated on a column of Superdex 200 (1.6×60 cm) size-exclusion resin

(Amersham Pharmacia Biotech, Piscataway, NJ) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. The flow rate was 0.5 ml/min and maintained at 4 °C. Absorbance of the protein measurements at 280 nm [51,62].

3.2.5.2 Anion - Exchange Chromatography

Fraction containing interesting activity were pooled and dialyzed with buffer A (50 mM Tris-HCl buffer ,pH 8.0).The dialyzate was fractioned on DEAE-cellulose (1.6×15 cm) column. The column was washed with buffer A and eluted with linear gradient or step gradient buffer B (50 mM Tris-HCl buffer ,pH 8.0,0.5 M NaCl).The flow rate was 1ml/min.All procedures were carried out at 4 °C. The protein absorption was monitored at 280 nm [50, 62]

3.2.5.3 Cation-Exchange Chromatography

Protein sample was adjusted to pH 4.0 with acetate buffer (0.02 M sodium acetate, pH 4.0) and applied on a 1.6 ×15 cm CM-Sepharose column, equilibrated with 0.02 M sodium acetate buffer pH 4.0. The column was eluted with a linear gradient of NaCl (0.3-1 M NaCl in 0.02 M sodium acetate buffer pH 4.0) at a flow rate of 1 ml/min [50, 62]

3.2.5.4 Affinity Chromatography

The sample was applied on a column of Affi-gel blue gel (Bio-Rad) which had previously been equilibrated and was eluted with 10 mM Tris-HCl buffer (pH 7.2). After elution of unadsorbed proteins, the adsorbed proteins were desorbed by using a linear concentration gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 1.5 ml/min. After that regenerated the column with binding buffer, 1.5 M Guanidine-HCl [52].

3.3 Biological activity test

3.3.1 Hemagglutination activity

The assay for hemagglutinating activity was performed as follows. A serial twofold dilution of the protein solution in microtiter U-plates (50 ml) was mixed with 50 ml of a 2% suspension of rabbit erythrocytes in phosphate-buffered saline (pH 7.2) at room temperature. The results were recorded after about 1 h when the blank had fully sediment. Concanavalin A (1mg/ml), lectin isolated from the jack bean, was used as positive control. The hemagglutinating titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was taken as one hemagglutination unit. Specific activity is the number of hemagglutinating units per milligram of protein [9].

3.3.2 α-glucosidase inhibitory activity

α-Glucosidase and *p*-nitrophenyl -α-D-glucopyranoside (PNPG) were assayed using 50 mM phosphate buffer at pH 6.7. 10 μl of protein sample at the designated concentration was premixed with 40 μl of enzyme solution (1U/ml) and incubated at 37°C for 10 min. 950 μl of 1mM PNPG as a substrate was then added to the mixture to initiate the enzyme reaction. The reaction was incubated at 37°C for 20 min and stopped by adding 1 ml of 1 M Na_2CO_3. α-glucosidase inhibitory activity was determined by measuring release of the yellow *p*-nitrophenol at 400 nm [65].

3.3.3 Antifungal activity

In the assay for antifungal activity, sterile petri plates (90×15 mm) containing 10 ml of potato dextrose agar were used. After the fungal colony had developed, paper disks (0.625 cm in diameter) were placed 0.5 cm from the rim of the colony. A 20 μl of the test sample in sterile water was applied to each disk. Incubation of the petri plate was carried out at room temperature for 72 h until mycelial growth had enveloped peripheral disks containing the control and had generated crescents of inhibition around disks with antifungal samples. Three fungal species, *Corynespora cassiicola, Fusarium oxysporum,* and *Stemphylium vesicarium,* were examined in the assay .Whereas sterile distilled water (20 μl) was pipetted onto other filter papers as

negative controls and Commercially Captan, Iprodine and Ketoclonazone (1mg/ml, 20 µl) were used as positive controls [9].

3.3.4 Antimicrobial activity

Protein sample was examined for antimicrobial activity on gram-negative bacterial, *Escherichia coli* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, gram-positive bacterial, *Bacillus subtilis* ATCC 6633, *Staphylococus aureus* ATCC 25923, and fungi, *Candida albican* ATCC 10231. The test was done using disc diffusion test. The accuracy of the density of a prepare standard should be verified by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the OD_{625} should be 0.08 to 0.10. A 0.1 ml suspension bactrria in sterile nutrient broth (Table 5A, Appendices A) with an optical density at 625 nm was evenly spread over nutrient agar in a plate. A filter-paper disc carrying protein sample was then placed in the center and incubated at 37 °C. Microbial growth was assessed by measuring inhibition zones between the filter paper disc and the visible microbial growth after 48 h of incubation for fungi and 24 h for bacteria [31].

3.3.5 Antioxidant activity

The Antioxidant activity scavenging activity of protein sample against DPPH radical was measured according to the method of Hou *et al.* or Yamaguchi *et al.* with some modifications. The 1.2 ml sample solution was added to 0.1 ml of 1 M Tris-HCl buffer (pH 7.9) and then mixed with 1.2 ml of 80 μM DPPH in methanol for 20 min under light protection at room temperature. The absorbance at 517 nm was determined. Deionized water was used instead of sample solution for control experiments. The decrease of absorbance at 517 nm was calculated and expressed as ΔA_{517nm} for scavenging activity [66].

3.4 Method of protein identification

3.4.1 Trypsin In-Gel Digestion

Excise protein bands or spots of interest from a coomassie stained polyacrylamide gel and place into a 1.5 ml microcentrifuge tubes. Wash the gel with

100µl of water (5min, 2 times). Remove the solution and destain with 200 µl of 0.1 M NH_4HCO_3 / 50%CH_3CN (or enough to immerse the gel particles). Vortex and incubate at 30 °C in water bath for 20 min. Remove liquid, and repeat this step until color is gone. Dehydrate gel with 100 µl of acetonitrile, 15 min or until gel shrink (white color and smaller in size) at room temperature. Remove acetonitrile and dry the gel pieces at ambient temperature for 2-3 min. Rehydrate gel with 100µl of 0.1 M NH_4HCO_3, 20 min at room temperature. Remove solution and cover gel pieces with 100 µl of 10 mM DTT /0.1 M NH_4HCO_3 vortex spin briefly, reduce proteins for 1 h at 56 °C. Cool to room temperature, remove DTT solution and add 100 µl of 55 mM iodoacetamide (IAA) in 0.1 M NH_4HCO_3, incubate for 45 minutes in dark place at room temperature. Remove idodoacetamide, twice wash gel pieces with 100 µl of 0.1 M NH_4HCO_3, for 5 min with vortexing, and then dehydrate the gel with acetonitrile 15 min, remove acetonitrile and rehydrate gel pieces by adding 100µl of 0.1 M NH_4HCO_3, 20 min repeat this step 2 times. After that dried gels by using speedvac. Rehydrate gel particles in 20 µl of trypsin solution (10 ng/µl trypsin in 50 mM NH_4HCO_3) to cover the gel pieces and place on ice for 5 min. Remove excess trypsin solution and overlay the rehydrated gel particles with 60 µl of 50 mM NH_4HCO_3, digest overnight at 37 °C. Transfer the digest solution supernatant into a clean 1.5 ml tube. Extract the peptide produced by the digestionin three steps, first step add 30 µl of 50 mM NH_4HCO_3 and sonicate for 10 min. Remove supernatant and combine with initial digest solution supernatant. Second, add 30 µl of the extraction buffer (5% formic acid in 50% CH_3CN) and incubate the sample for 10 min with sonicate, and combine the extract in 1.5 ml microcentifuge tube, repeat this step one time. Third, reduce the volume of the extract to < 20 µl by evaporation in a vacuum centrifuge at ambient temperature. Adjust the volume of the digest to ~ 20 µl with 0.1% formic acid. Storage this sample at 4 °C in refrigerator until analysis [67].

3.4.2 Sample preparation for MALDI-TOF

2 µl of sample was mixed with 2 µl of CCA solution (10mg α-cyano-4-hydroxy-cinnamic acid, 50%ACN/0.1%TFA) in eppendorf 0.5 ml and spotted on the target plate. Myoglobin (1mg/ml), Angiotensin II (1mg/ml), and Bovine serum

albumin (1mg/ml) were used as external calibration for peptide and protein molecular mass, respectively.

3.4.3 Protein Identification by Database Searching

Peptide mass spectra and amino acid sequence were acquired using MALDI-TOF MS. Peptide mass fingerprint (PMF) data, obtained from protein digestion, were used to search in databases NCBInr.20030905 for viridiplantae using Mascot program (http://www.matrixscience.com/cgi/index.pl?page=../home.html). The peptide mass fingerprinting of the proteins were scored with the Mowse score.

De novo peptide sequencing was acquired using ESI-Q-TOF MS. Data of peak lists (PKL file) were used to search in NCBInr.20030905 for viridiplantae using Mascot program whereas data of peptide sequences were used to search in MS BLAST (http://dove.embl-heidelberg.de/Blast2/msblast.html).