CHAPTER 3

EXPERIMENTAL

3.1 Plant material

The seed of *Parkia apeciosa* Hassk from the family Leguminosae, which grow wild in temperate region, were collected from the south of Thailand. Fresh seed of of *Parkia apeciosa* was purchased from local markets in Bangkok, Thailand (Pak Khlong Talat).

3.2 Chemicals and reagents

Concanavalin A (ConA) and Dextrose agar were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Methyl-α-D-manopyranoside, EDTA, Guanidine HCl were purchased from Fluka (Germany). Affi gel blue gel was obtained by Amersham Bioscience. The most reagent and staining kit used in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Plusone Pharmacia Biotech (Uppsala, Sweden). The low molecular weight calibration kit was used as standard molecule weight marker proteins and purchased from Amersham Pharmacia biotech (UK). Brilliant Blue G and Trichloro acetic acid was the product of Sigma Chemicals Co. (St. Louis, MO, USA) and Merck (Germany). Potassium hy drogen phosphate (KH_2PO_4), Disodium hydrogen phosphate ($Na_2H_2PO_4$), Ammonium sulphate $(NH_4)_2SO_4$ and Sodium chloride (NaCl) were obtained from Merck (Germany).

3.3 Solvents

Methanol (MeOH), Acetone, Acetonitrile, Acetic acid was purchased from Merck (Germany). Solvents used for in-gel digestion and preparation for mass spectrometric and sequencing techniques were analytical grades and were obtained from Labscan (Bangkok, Thailand) and Amersham Pharmacia Biotech (Upppsala, Sweden). Double distilled water was used in this research was prepared with Glass water Sills (GFL Gesellschaft für labortecilk mbH, Germany).

3.4 Apparatus and Instrument

SDS-polyacrylamide gel electrophoresis gels were run in Hoefer^{Tm} miniVE (minivertical), 8x9 cm gels (Amersham pharmacia biotech, Uppsala, Sweden).

Mass spectra of sequencing protein were acquired from Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometric (MALDI-TOF MS) (BIFLEX Bruker, Germany) and Electrospray Quandrupole-Time of Flight (Model Q-TOF II, Micromass, UK).

1.5 and 0.5 ml. siliconized eppendorf and pipette tips were purchased from Axygen sciencetific Inc. and Bioline U.S.A.

Desalting Cartridge (Protein macrotrap, Michrom Bioresource Inc.) and Dialysis bag were used for desalt *Parkia apeciosa* fraction.

Liquid chromatography run on AKTA prime (Amersham pharmacia biotech, wikstroms, Sweden)

Micropipette (pipetteman, Gilson, France)

Vortex mixer (Vortex-genie 2, Sciencetific Industries)

pH meter (Denver Instrument U.S.A. system)

Speed vacuum (MAXI dry plus, Heto vacuum centrifuge, Denmark)

Sonicate (DHA-1000, Branson, U.S.A.)

Orbital Shaker (Kika-Werke GMBH&Co., Germany)

Refrigarated centrifuge (Himac CR20B2, HITACHI, Japan)

Water Bath Shaking (Memmert, Germany)

Power Supply (EPS 3500 XL, Pharmacia, England)

Aquasil C_{18} Thermo hypersil-keystone (Thermo electron, USA.)

Reverse phase column run on Finnigan Spectra System (Thermo electron Corporation,Massachusetts, USA.)

Dialysis bag (Rockford, USA)

Syringe 500µl (Hamilton, Reno, Nevada, USA.)

3.5 Procedures

3.5.1 Extraction and precipitation [15, 17]

One kilogram of the *Parkia speciosa* seeds was defatted with acetone at -20 °C and extracted overnight at 4° C with 50 mM phosphate buffer, pH 7.5 containing 0.1 M NaCl and chelating agent such as EDTA (0.1 mM). After centrifugation (8,500 x g, 15 min, 4°C), protein in the supernatant were precipitated with 25%, 40%, 60 %, 90% amonium sulphate, respectively. The proteins were dissolved and dialyzed in distilled water then were freezed dry.

3.5.2 Determination of protein concentration [3]

Protein concentration was determined by the method of Bradford using Bovine serum albumin as a standard and color reagent from Amersham bioscience.

3.5.3 Purification

3.5.3.1 Affinity column

Concanavalin A (Con A) [5, 14]

Dried crude extract was dissolved in binding buffer (20 mM Tris-HCl pH 7.4 containing with 0.5 M NaCl, 1mM $CaCl_2 \cdot 2H_2O$, 1mM $MnCl_2$). Insoluble proteins were removed by centrifugation and the solution applied to affinity column (1.6 x 5cm) of concavalin A equilibrates in the same buffer. The lectin which bound to the matrix was eluted with 20 mM Tris-HCl pH 7.4 containing with 0.1-0.5 M Methyl-α-D-manopyranoside and regenated with 0.5 M NaCl in 20 mM Tris-HCl buffer pH 8.5 and 20mM acetate buffer pH 4.5.The column was eluted at flow rate 1.5 ml/min with the same buffer and maintained at 4°C. The protein absorption was monitored at 280 nm. The fractions were collected and concentrated using a freeze dryer, and then kept at -20°C.

Affi-gel blue-gel [1, 30]

Dry crude extract was dissolved in binding buffer (1mM Tris-HCl pH 7.2). Insoluble proteins were removed by centrifugation and the solutions applied to affinity column (1.6 x 10 cm) of affi-gel blue gel equilibrates in the same buffer. The protein bound to the matrix was eluted with 10 mM Tris-HCl pH 7.2 containing with 0.5 mM NaCl and regenerated with 1.5 M Guanidine-HCl in binding buffer. The column was eluted at flow rate 1.5 ml/min with the same buffer and maintained at 4°C. The protein absorption was monitored at 280 nm. The fractions were collected and concentrated using a freeze dryer, and then kept at -20°C.

3.5.3.2 Gel filtration [2]

Protein was dissolved in Double distilled water and the solution applied to column (1.6 x 60 cm) of Superdex – 200 equilibrates in the water. The column was eluted at flow rate 0.5 ml/min and maintained at 4°C. The protein absorption was monitored at 280 nm. The fractions were collected and concentrated using a freeze dryer, and then kept at -20°C.

3.5.3.3 High Performance Liquid Chromatography

Protein were separated by using mobile phase are acetonitrile : water (20 :80) and the column was eluted at flow rate 0.5 ml/min. The protein absorption was monitored at 280 and 230 nm. The fractions were collected and concentrated using a freeze dryer, and then kept at -20°C.

3.5.3.4 SDS-PAGE [24]

SDS-polyacrylamide gels were cased and run in the compact Hoefer miniVE Vertical Electrophoresis system (Amersham Pharmacia Biotech). The SDS-PAGE procedure was performed exactly as described in Chapter 5 of Protein method book. The stock solutions are 30% acrylamide, 0.8% bis-acrylamide, stacking gel buffer(0.5 M Tris-HCl, pH 6.8, 0.4% SDS), separating gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4% SDS), 10% ammonium persulfate solution (APS), electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS). The SDS-polyacrylamide gels (8 x 9 cm gels; 1mm thick; 10 wells) were prepared as discontinuous gel.

1-D gel electrophoresis [16]

For 12.5 %SDS-PAGE, the composition of 4.17ml acrylamide, bis stock solutions, 2.5 ml of separating gel buffer, 3.33 ml of water, 50 µl of 10% APS, and 5 µl of TEMED. The 5% stacking gel solutions are 0.67 ml of acrylamide, bis stock solution, 1.0 ml of stacking gel buffer, 2.3 ml water, 30 µl of 10% APS, and 5 µl of TEMED. First, 3.4 ml of separating gel solution was pipetted into the gel cassette. Then, 1.2 ml of stacking gel solution was carefully applied like an overlay. The comb was inserted without trapping air bubbles for making the sample wells. These two gel solutions were polymerized together for one day before use. For sample preparation, the 0.3 mg of *Parkia apeciosa* was dissolved in 300 µl of Tris buffer (10mM Tris-HCl, pH 7.3, 50 mM NaCl). The resulting solution was centrifuged at 5000 rpm for 15 min; the supernatant was collected and used as the *Parkia apeciosa* stock solution. The 26 M reducing sample buffer contains 50 µl of 2.6 M dithiothreitol (DTT; 30 mg of DTT in 60 µl water) and 5 ml of non-reducing sample buffer (1.0 g of SDS, 3 mg of EDTA, 10 mg of bromophenol blue, 2.5 ml of resolving gel buffer, 25 ml glycerol, make up to 100 ml with water). The diluting samples were prepared from the mixture of 5 µl of the *Parkia apeciosa* stock solution and 10 µl of the 26 M reducing sample buffer for loading gel samples and the contents of a vial of Low Molecular Weight (LMW; molecular mass range 14.4 to 97.0 kDa) SDS Calibration kit (Amersham Pharmacia Biotech) was dissolved in 415 µl of the 26 M reducing sample buffer for LMW marker. Then, the diluting samples were boiled for 3 min. After cooling, 1 µl of 2.6 M DTT was added to each sample. Before electrophoresis starting, the comb was removed from gel, the cathode buffer was poured in to buffer chamber, 10 µl of diluting samples were loaded into the wells using the gel loading tip (Bio-Rad, USA), the safety lid was placed, and the system was connected to power supply. The electrophoresis was carried out at 280 V, 20 mA, and 6 W for one gel and then was terminated when the dye front reached close to the bottom of the gel.

Staining a Gel with Coomassie Blue [19]

After electrophoresis, proteins in the gel were stained by coomassie blue. Remove the polyacrylamide gel from between the glass plates and immersed in 50 mL of coomassie blue staining solution (1 liter: 1.0 g Coomassie blie R-250, 450 ml methanol, 450 ml water, 100 ml glacial acetic acid) Placed on a rotary shaker and gently shake the gel 20 minutes. Removed the staining solution and rinse the gel with coomassie blue destain solution (1 liter: 100 ml methanol, 100 ml glacial acetic acid, 800 ml water) 30 minutes and change coomassie blue destain solution and agitate overnight.

In-Gel Digestion Protocal [13]

The protein spots were manually excised from the SDS-polyacrylamide gels and transferred to 0.5 ml microcentrifuge tube. Add 500 µl of the water (5 min, ca. 2-3 times). Removed the liquid and add acetronitile/ 0.1 M NH_4HCO_3 (1:1, ca 3-4 times) wait for 10- 15 min until gel become white. Spin gel down and remove liquid. Dry down gel particle in a vacuum centrifuge. Swell the gel in 10 mM dithiotreiol/0.1M NH_4HCO_3/1mM EDTA until cover gel and incubate 45 min at 60°C to reduce the protein. In-gel reduction was recommended even if proteins were reduced prior to an electrophoresis. Removed excess dithiotreitol solution and add 100 mM iodoacetamide/0.1 M NH_4HCO_3. Incubate for 30 min at room temperature in the dark. Remove iodoacetamide solution. Wash the gel particles with 150-200 µl of 0.05 M Tris-HCl pH 8.5/50% acetonitrile (*ca.* 3-4 times). Rehydrate gel particles in the 180 µl digestion buffer (containing 100 µl of 0.1 M Tris-HCl, pH 8.5, 2 µl of 100 mM $CaCl_2$, 20 µl of ACN and 78µl of distilled water) and 20 µl of trypsin solution. Incubate for overnight at 37 °C. After overnight incubate keep 100 µl supernatant in a microcentrifuge tube (0.5 ml or 1.5 ml Eppendorf) and add 100 µl of 2% TFA incubate for 30 min at 60 °C. After incubate, combine supernatant in before microcentrifuge tube and dry in a vacuum centrifuge. Add 30 µl of digestion buffer to the tube containing the gel pieces incubate for 10 min at 30 °C and sonicate 5 min. After then add ACN 30 µl in extraction buffer, incubate for 10 min at 30 °C and sonicate 5 min. Remove and combine supernatant in before tube containing supernatant. Add 5%formic acid/ACN in gel, incubate for 10 min at 30 °C and sonicate for 5 min. Combine all supernatant in tube and dry in a vacuum centrifuge.

3.5.4 Assay for Hemagglutinating activity [28, 29]

A solution of diluted 2-fold lectin (50µl) were mixed with 2% of rabbit erythrocytes in 10 mM phosphate buffer pH7.2 at romm temperature.The result were recorded approximately after 1 hour when the blank had fully sedimented. The hemagglutination titer is defined as reciprocal of the highest dilution exhibiting hemagglutination and is equivalent to one hemagglutinatiion unit. Specific hemagglutination activity is expressed as the number of hemagglutinating units per mg protein.

3.5.5 Assay for alpha glucosidase inhibitor [25, 12]

∝-glucosidase (0.0075) was mixed with protein. 3 mM p- nitrophenyl glucopyranoside (pNPG) as a substrate in phosphate buffer was added to mixture to start the reaction. The reaction was incubated at 37°C for 30 min and stopped by adding 2 ml of 0.1 M Na2CO3. The ∝-glucosidase activity was determined by measuring the p-nitrophenol release from pNPG at 400 nm.

3.5.6 Sample preparation for MALDI-TOF [22]

One microlittre of sample was mixed with 10 µl of ∝-cyano-4-hydroxy-cinnamic acid (CCA) in 50% ACN/ 0.1% TFA in eppendorf 0.5 ml and spotted on the target. Myoglobin 1µM was used for external standard to calibration.

3.5.7 Protein Sequencing [22]

Peptide mass spectra and amino acid sequencing were acquired using MALDI-TOF mass spectrometer, respectively. Peptide mass spectra were searched database via MASCOT (http://www.matrixscience.com).