CHAPTER III

EXPERIMENTAL

3.1 Materials

3.1.1 Plant Materials

P. dulce pods were bought from Khlong Toei market (Bangkok, Thailand). A voucher specimen (BK48622) is deposited at the Bangkok Herbarium (BK) of the Plant Variety Protection Division, Department of Agriculture (Bangkok, Thailand). Ripe Pods, which the color becoming spiral and reddish-brown, were peeled and seed pulps were removed. The black seeds were manually separated and stored at -80°C until use.

3.1.2 Fungi

Macrophomina phaseolina (Charcoal rot), *Phymtotrichopsis omnivora* (Cotton rot), and *Fusarium avicenariam* were obtained from Plant Biology division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, U.S.A.

3.1.3 Chemicals

All reagents for 2-DE were obtained from Bio-Rad (Hercules, CA, USA). All chemicals used were analytical grade. HPLC grade acetonitrile and water were purchased from Burdick & Jackson (Muskegon, MI, USA). Methanol (CH_3OH), Acetic acid (CH_3COOH) used in destaining reagent and Acetone, the solvent for 2-D Electrophoresis were obtained from Merck (Germany). Tris base, Trichloroacetic acid (TCA), Polyvinylpolypyrrolidone, formic acid, iodoacetamide, endonuclease and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) was purchased from United States Biochemical Corp (Cleveland, OH, USA). Ammonium sulphate ($(NH_4)_2SO_4$) used in extraction and precipitation step was obtained from Merck

(Germany). The Mark[12] protein standards molecular weight marker for electrophoresis was the product of invitrogen (USA). Coomassie brilliant blue R-250 for gel staining was obtained from Sigma (St. Louis, MO, USA). A sequencing grade modified bovine trypsin use for in-gel digestion was purchased from Roche Diagnostics (Germany). Potato Dextrose, Bacto peptone, Yeast extract and Malt extract for Potato dextrose agar media used in antifungal activity testing were obtained from Fluka (Germany).

3.1.4 Apparatus and Instruments

Immobilized pH gradient strips (IPG strips) pH 3-10, 18 cm (Amersham pharmacia biotech) was run in Ettan IPG II Electrophoresis Unit (Amersham pharmacia biotech Uppsala Sweden). SDS-PAGE were run in Ettan Daltsix (for large vertical system), 26×20 cm gels and Hoefer™ miniVE (for mini-vertical), 8×9 cm gels (Amersham pharmacia biotech Uppsala Sweden). Desalting Cartridge (Protein macrotrap, Michrom BioResource Inc.) and Dialysis bag with molecular weight cut off 3,500 Da (SnakeSkin Dialysis Tubing, Pierce, USA.) were used for protein desalting. For mass spectrometers, ABI QSTAR Pulsar *i* hybrid Q-TOF mass spectrometer (Applied Biosystems, USA) equipped with a nano-electrospray ionization source (Protana, USA), a nanoscale HPLC system (LC packing, San Francisco, CA) consisting of an autosampler (Famos, USA), a precolumn switching device (Switchos, USA), and an HPLC pump system (Ultimate, USA) was used to analyze protein mass spectra. Gel images were acquired on a UMax Astra 2400S scanner (Umaz Technologies, TX, USA). Protein spots were detected, numbered and analyzed with Genomic Solutions HT Analyzer software (Genomic Solutions, AnnArbor, MI, USA). For Column Chromatography, the protein samples were run on AKTA prime (Amersham pharmacia biotech wikstroms, Sweden). An anion exchange chromatography column of Resource Q (6 ml) and gel filtration chromatography of Superdex 200 (16×60cm) were purchased from Amersham Biosciences (Piscataway, NJ, USA). For necessary apparatus, there are laboratory centrifuge (Biofuge pico Heraeus; Kendro, Germany), micropipettes adjustable from 2 to 1,000 μl (Gilson, France), pipette tips (Bioline, USA), eppendroff (Axygen Scientific, USA), vortex

mixer (Vortex-Genie2, Scientific Industries, USA), orbital shaker (Kika-Werke GMBH&Co., Germany), sonicator (BHA-1000, Branson, USA), freeze dryer (Labconco, USA), speed vacuum centrifuge (Heto-Holten, Denmark).

3.2 Methods

3.2.1 Protein Profiling of *Pithecellobium dulce* Seeds Using 2-DE and MS/MS

3.2.1.1 Extraction and Precipitation[108]

Seeds (3g) of *P. dulce* were ground in liquid nitrogen and extracted with 10 ml of buffer containing 40 mM Tris-HCl (pH 9.5), 50mM $MgCl_2$, 2%(w/v) polyvinylpolypyrrolidone, 1mM phenylmethylsulfonyl fluoride, and 125 units/ml endonuclease followed by sonication. The extracts were centrifuged at 5000 × *g* for 10 min at 4 °C, and the supernatant was recovered. The supernatant was brought to a final concentration of 12.5% (w/v) trichloroacetic acid (TCA) plus 0.1% (v/v) β-mercaptoethanol and incubated at -20 °C for 1 hr. Precipitated protein was recovered by centrifugation at 15,000 × *g* for 15 min. The protein pellets were washed with 200 μl of a cold solution of 80% acetone and 20% water containing 0.05% (v/v) β-mercaptoethanol three times to remove residual TCA, air-dried, and resuspended in 250 μl of 2-DE solubilization buffer consisting of 5 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 2% (v/v) Triton X-100, 20 mM DTT, and 0.5% ampholytes.

3.2.1.2 Protein Quantification[109, 110]

The protein concentration was determined by the Bradford assay 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 a commercial dye reagent from Bio-Rad. After addition the protein reagent, the absorbance of each sample measure between 2-60 min at wavelength 595 nm.

3.2.1.3 Two-Dimensional Gel Electrophoresis[111]

For isoelectric focusing method, Immobilized pH gradient (IPG) strips (Immobiline™ Dry Strips, 24 cm, pH 3-10 non-linear) were passively rehydrated overnight with *P. dulce* seed protein solution mixed with rehydration solution (8 M urea, 2% CHAPS and 2% IPG buffer pH 4-7, 0.2% DTT and bromophenol blue (3 mg of protein in total volume 450 μL). Isoelectric focusing (IEF) of proteins was performed using the following step gradient: 500 volt for 1 h, 1,000 volt for 1 h, and 8000 volt until a total of 76,000 V-h had been achieved. After IEF, strips were equilibrated in buffer containing 7 M urea, 2% SDS, 375 mM Tris (pH 8.8), and 10% glycerol plus either 50mM DTT for reduction or 100 mM iodocetamide for alkylation. For SDS-PAGE, SDS-PAGE was run in Ettan Daltsix (for 26×20 cm gel) and carried out with electrophoresis buffer (see Table 1A in Appendix A). Equilibrated IPG strips were loaded onto a 10 % acrylamide gel (Table 3.1), sealed with 1 % agarose, and electrophoresed at 2 watts/gel until the dye front is approximately 1 mm from the bottom of the gel. The Mark[12] protein standards molecular weight markers were applied on the gel via a small piece of filter paper.

Table 3.1 Preparation of single-percentage gel (10%) for SDS-PAGE of 2-DE (preparation of stock solutions is described in Appendix A)

Final Gel Concentration	**Volume**
Monomer solution	40 ml
4× resolving gel buffer	25 ml
10% SDS	1 ml
Double distilled water	33.55 ml
10% ammonium persulfate	500 μl
TEMED	50 μl
Total volume	100 ml

3.2.1.4 Gel Image Analysis

2-DE gels were stained overnight with Coomassie Briliant Blue R-250 (1g/L) in 55% water, 40% MeOH, and 5% acetic acid. The gels were destained three times with 55% water, 40% MeOH, and 5% acetic acid. The destained gels were preserved in 5% acetic acid. Gel images were acquired on a UMax Astra 2400S scanner at 300 dpi and saved as a gray scale TIFF file. Protein spots were detected, numbered and analyzed with Genomic Solutions HT Analyzer software.

3.2.1.5 In-Gel Trypsin Digestion

Stained protein spots were manually excised from the gels as 1.5 mm diameter plugs. Gel plugs were transferred to polypropylene 96-well plates, sealed, and stored at -80 °c until further processing. To each well, 25 µl of a 1:1 (v/v) solution of 50 mM ammonium bicarbonate and acetonitrile was added, and the mixtures were incubated at room temperature for 15 min. This process was repeated until all gel spots were completely destained. The spots were then dehydrated with 25 µl of acetonitrile for 15 min at room temperature. After acetonitrile removal, the gel spots were dried under vacuum and rehydrated in 20 µl of sequencing grade modified bovine trypsin (10 ng/µl in 25 mM ammonium bicarbonate). After rehydration for 20 min, excess trypsin solution was removed, and 15 µl of 25 mM ammonium bicarbonate was added to each well to prevent dehydration during incubation. Proteolysis was allowed to continue overnight at 37 °C and stopped by adding 15 µl of 10% formic acid. The supernatant was recovered, and the spots were extracted twice more with 25 µl of a 1:1 (v/v) solution of acetonitrile and 25 mM ammonium bicarbonate and once more with 25 µl acetonitrile. The extracts were then combined and concentrated under vacuum to a final volume of 25 µl.

3.2.1.6 Protein Identification Using Tandem Mass spectrometry

3.2.1.6.1 LC-ESI-MS/MS

Separations of the protein digests were achieved using a nanoscale HPLC system (LC packing, San Francisco, CA) consisting of an autosampler (Famos), a precolumn switching device (Switchos), and an HPLC pump system

(Ultimate). Samples (5 μl) were loaded onto a C_{18} precolumn (0.3-mm inner diameter × 1.0 mm, 100 Å, PepMap C_{18}, LC Packings) for desalting and concentrating at a flow rate of 50 μl/min using mobile A (5% acetonitrile and 95% water containing 0.1% formic acid). Peptides were then eluted from the precolumn and separated on a nanoanalytical C_{18} column (75-μm diameter × 15 cm, 100 °A, PepMap C_{18}, LC Packings) at a flow rate of 200 nl/min. Peptides were eluted with a linear gradient of 5-40% mobile phase B (95% acetonitrile and 5% water containing 0.08% formic acid) over 40 min. The separated peptides were directly analyzed with an ABI QSTAR Pulsar *i* hybrid Q-TOF mass spectrometer equipped with a nano-electrospray ionization source (Protana). The nano-electrospray was generated using a PicoTip needle (10-μm inner diameter, New Objectives, Woburn, MA) maintained at a voltage of 2400 V. TOF-MS and tandem mass spectral data were acquired using information-dependent acquisition (IDA) with the following settings: charge state selection from 2 to 5, an intensity threshold of 10 counts/s for tandem experiment and a collision energy setting automatically determined by the IDA based on the *m/z* values of each precursor ion. Following IDA data acquisition, precursor ions were excluded for 90 s using a window of 6 amu to minimize the redundancy in tandem mass spectra.

3.2.1.6.2 Data Analysis and Mascot Searching

The acquired mass spectra data were queried against the NCBInr protein database using MASCOT[112] (version 2.2, Matrix Science Ltd., London, UK) search engine with a mass tolerance of 150 ppm, one trypsin miscleavage allowance and two variable amino acid modifications, *i.e.*, methionine oxidation and cysteine carbamidomethylation. Taxonomy was limited to green plants. To qualify the mass spectra data as a positive identification, the molecular weight search (MOWSE) score was equal to or exceeded the minimum significant score of 64. Only statistically significant protein identifications with at least two peptides matched are reported in this study.

3.2.1.6.3 *De novo* Sequencing and Sequence-Similarity Searches

The *De novo* sequencing was performed manually using the peptide-sequencing software BioAnalyst (MDS Sciex). The resulting peptide sequences were

merged into a single query string and submitted to an MS-BLAST (MS driven BLAST)[113] search against nr database (nrdb95) at the web-accessible server with default settings (http://genetics.bwh.harvard.edu/msblast /).

3.2.2 Purification of Antifungal Protein from *Pithecellobium dulce* Seeds

3.2.2.1 Extraction and Precipitation

Seeds (100g) of *Pithecellobium dulce* were soaked in deionized water and homogenized in 100 mM Tris-HCl buffer pH 8.0 using blender, then stirring overnight at 4 °C. The extracts were filtered through cheesecloth and centrifuged at 5000 × *g* for 30 min at 4 °C and the supernatant was recovered. The supernatant was brought to 80% saturation with ammonium sulfate and centrifuged as above. The precipitate was dissolved and dialyzed with SnakeSkin dialysis tubing, molecular weight cutoff of 3.5 kDa (Pierce Biotechnology, Rockford, IL, USA) in deionized water at 4 °C overnight. The crude proteins were freeze-dried and keep at -80 °C for further purification. The protein concentration was determined by the Bradfored method using a commercial dye reagent (Bio-Rad) as mentioned in section 3.2.1.2.

3.2.2.2 Biological Activity Test

3.2.2.2.1 Hemagglutination Activity[114]

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. 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

3.2.2.2.2 α-Glucosidase Inhibitory Activity [115]

α-Glucosidase and *p*-nitrophenyl -α-D-glucopyranoside (PNPG) were assayed using 50 mM phosphate buffer at pH 6.7.The protein sample at the designated

concentration was premixed with 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. The IC_{50} value was defined as the concentration of α-glucosidase inhibitor to inhibit 50% of its activity under the assay conditions.

3.2.2.2.3 Antioxidant Activity [116]

The antioxidant activity scavenging activity of protein sample against DPPH radical was measured according to the method of Hou *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.

3.2.2.2.4 Antifungal Activity[117]

Antifungal activity was performed using sterile Petri plates containing potato dextrose agar (Table 3.3). After the mycelial colony developed, sterile paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm from the rim of the mycelial colony. An aliquot (40 μl) of the fractions and 20 mM Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl (the negative control) were added to a disk. The plates were incubated at 28 °C for 72 h until mycelial growth from the central disk had enveloped peripheral disks containing the control and had formed crescents of inhibition around disks with antifungal samples. Three fungi *Macrophomina phaseolina* (Charcoal rot), *Phymtotrichopsis omnivora* (Cotton rot), and *Fusarium avicenariam* were examined in this assay. For thermal stability testing, the thermal stability of the purified antifungal protein was conducted by using the same sterile Petri plates containing potato dextrose agar method as mentioned above. The purified

antifungal protein after incubation for 15 min at various temperatures from 20 to 100 °C were investigated at pH 8.0 using 20 mM Tris-HCl buffer containing 0.5 M NaCl.

Table 3.2 Composition of potato dextrose agar
(25ml/ Petri plate- 15 mm in deep × 90 mm in diameter)

Chemical	**Amount**
Potato Dextrose	18 g
Yeast extract	1 g
Bacto peptone	1g
Malt extract	1g
Double distilled water	to 1000 ml

3.2.2.3 Protein Purification by Column Chromatography[47,48]

The crude protein of *Pithecellobium dulce* seeds was dissolved in 20 mM Tris-HCl buffer pH 8.0 and then was loaded on an anion column of Resource Q (6 ml; Amersham Biosciences; Piscataway, NJ, USA) previously equilibrated with the same buffer. Following removal of unadsorbed proteins, the column was eluted with linear gradient of 0–0.5 M NaCl in the same buffer (120 ml) at 4 °C. The flow rate was 6 ml/min. The absorbance of the eluate was monitored at 280 nm. The active proteins in unbound fraction containing antifungal activity (Q1) was pooled and lyophilized. The lyophilized active fraction obtained from Resource Q chromatography was separated on a Superdex 200 gel filtration chromatography column (16×60cm; Amersham Biosciences), equilibrated with 20 mM Tris-HCl pH 8.0 at 4 °C with a flow rate of 1 ml/min. The absorbances of all fractions were monitored at 280 nm. Antifungal activity was determined for all of the collected fractions. The active fractions were collected and lyophilized. The fourth eluted peak (G4) represents a purified antifungal protein from *Pithecellobium dulce* seeds.

3.2.2.4 SDS–PAGE

SDS-PAGE (12%T, 4%C) was performed in Hoefer™ miniVE (for mini-vertical), 8×9 cm gels, according to the method of Laemmli and Favre.[118] SDS gel was 1 mm thick and consisted of a 6 cm separation gel of 15% w/v acrylamide overlaid with a 0.5 cm stacking gel of 4% w/v acrylamide. The resolving and stacking gel consisted of solutions in the Table 3.2. Samples were resuspended in sample buffer (62.5 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS), were heated at 100°C for 3 min and then 30 μl of sample solution was loaded into each sample well. Electrophoresis was carried out with electrophoresis buffer containing 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3. The electrophoresis condition is 10 mA current at 280 V for 15 min and then 20 mA current at 280 V for 90 min or the dye front is approximately 1 mm from the bottom of the gel. Gel image analysis and in-gel trypsin digestion were performed as described in section 3.2.1.4 and section 3.2.1.5.

Table 3.3 Composition of the gel solutions for one 8×9 cm gel (preparation of stock solutions is described in Appendix A)

Stock solution	**12% *T* Resolving gel**	**4% *T* Stacking gel**
30% T, 2.6 % C monomer (ml)	3.2	0.130
Resolving buffer (ml)	2	-
Stacking gel buffer (ml)	-	0.250
Double distilled water (ml)	1.98	0.610
10% Ammonium persulfate (μl)	40	5
10% SDS (μl)	80	10
TEMED (μl)	4	1
Total volume (ml)	8	1

3.2.2.5 Protein Identification

The purified antifungal protein was identified using tandem mass spectrometry. The LC-ESI-MS/MS, data analysis and Mascot searching method were described in section 3.2.1.6.1 and section 3.2.1.6.2.