CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Equipments

Instrument	Model	Company
Autoclave	ES-315	Tomy
Balance (2 digits)	PG5002-S	Mettler
Balance (4 digits)	AB204-S	Mettler
Centrifuge	JA-30.50 Ti	BECKMAN
Ettan IPGphor 3 (with ImageMaster™ 2D Platinum)	Ettan IPGphor 3(11003364)	GE Healthcare
Microcentrifruge	CF-10	WiseSpin
Microcentrifruge	micro 4214	ALC
Microcentrifruge	Mikro 200R	Hettich
Orbital shaker	MS-OR	Major Science
Orbital shaker	SH 30	FINEPCR
Oven	240 litre	Binder
pH meter	731	Metrohm
Power supply	MP-300 N	Major science
Power supply	MP-300 V	Major science
Power supply	MP-500 V	Major science
Scanner	Powerlook 1120	UMAX
Vertical gel electrophoresis	Mini Protean tetra cell	Bio-Rad
Spectrophotometer	8453	Hewlett-Packard
Vivaspin 500 (5kDa MWCO)		GE Healthcare
Vortex-mixer		WIGGEN
Vortex-mixer	VX 100	Labnet

1.2 Chemicals & Reagents

1.2.1 Analytical grade

Chemical	Company
Absolute ethanol	J.T.Baker
Acetic acid	Lab Scan
Acrylamide	AMRESCO
Acrylamide	Fluka
Agarose	GenePure
Ammonium persulfate	Bio-Rad
Ammonium sulfate	Lab Scan
Bis-acrylamide	Fluka
Bovine serum albumin	Sigma
Bromophenol blue	Fisher
Citric acid	AR
Coomassie brilliant blue G-250	USB
Cupric sulfate	J.T.Baker
di-Sodium hydrogen phosphate	J.T.Baker
Dithiothreitol	Bio-Rad
Glucose (Dextrose)	RANKEM
Glycerol	Normapur
Glycine	Fisher
Hydrochloric acid	J.T.Baker
Immobiline^{TM} DryStrip pH 3-10	GE Healthcare
Immobiline^{TM} DryStrip pH 4-7	GE Healthcare
Iodoacetamide	GE Healthcare
IPG buffer pH 3-10	GE Healthcare
IPG buffer pH 4-7	GE Healthcare
Methanol	Lab Scan
Mineral oil (DryStrip Cover Fluid)	GE Healthcare
Phosphoric acid	J.T.Baker
Sodium chloride	Lab Scan

Sodium citrate	UNILAB
Sodium dihydrogen orthophosphate	BDH
Sodium dodecyl sulfate (SDS)	Finechem
Sodium hydroxide	Lab Scan
Tetramethylethylenediamine (TEMED)	Sigma Aldrich
Tris (Hydroxymethyl)- methylamine	USB
Tris	Vivantis
Urea	UNILAB

1.2.2 Reagent kits

Reagent	Company
Albumin & IgG Depletion SpinTrap	GE Healthcare
2-D Protein Extraction Buffer (component V)	GE Healthcare

2. Methods

2.1 Blood collection and preparation of human plasma

Plasma samples of patients with depressive disorder before the treatment with fluoxetine were kindly provided by the Rajanukul institute, Bangkok, Thailand. Whole blood of healthy volunteer was collected following the procedure for routine plasma blood draw with ACD as an anticoagulant. It was centrifuged at 2,000 xg for 10 min at 4 °C, and the plasma was collected, aliquot, and kept at -20^{o}C until use. The concentration of total protein in the plasma was determined by spectrophotometric biuret method (Itzhaki and Gill, 1964). The patients were classified into fast-response (FR), slow-response (SR) and non-response (NR) based on their psychopathologic status according to the Hamilton Depression Rating Scale (HAM-D) (Hamilton, 1960).

2.2 Depletion of major protein(s) in plasma

Since major or high abundance proteins in particular albumin and IgG can mask signals of low concentration proteins in plasma during the analysis by 2-DE. The depletion of albumin and IgG from plasma samples was carried out with the depletion SpinTrap column (GE Healthcare), pre-packed with anti-HSA Sepharose,

and followed the manufacturer's instruction. In brief, 25 to 50 µl of plasma was diluted with a binding buffer containing 20 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4, to a final volume of 100 µl prior application onto the column. The separation was accomplished by centrifugation at 800xg for 30 s. The flow through was collected, and the step was repeated 3 times. All of the flow through was concentrated to 50 µl in Vivaspin 500 (GE Healthcare) column (5kDa molecular weight cutoff), and it was immediately used or kept at -20^{o}C until use.

2.3 Determination of protein concentration

Total protein concentration of plasma was determined by biuret assay (Itzhaki and Gill, 1964) the reaction mixture comprised of 0.001 ml of protein sample and 1 ml of reagent (0.0131 mg/ml Cupric sulfate, 0.30 mg/ml Sodium Hydroxide). The mixture was well mixed using a vortex mixer, and the dye-protein complex formation was allowed at room temperature for 10 min prior the measurement for an absorbance at 310 nm. Bovine serum albumin (BSA) was used in generating a standard curve.

2.4 Two dimensional gel electrophoresis (2DE)

2.4.1 First dimensional electrophoresis

The separation in first dimension of proteins in plasma was performed with Immobiline^{TM} Drystrips (7 cm, pH3-10, pH 4-7; GE Healthcare). Aliquot (1-3 µl; 180 µg) of was diluted to a final volume of 130 µl with rehydration buffer containing 5 M urea, 2 M thiourea, 2% Chaps, 2%, 0.3% DTT, 1% of IPG buffers (GE Healthcare Bio-Science) and 1.2% of DeStreak ^{TM} reagent (GE Healthcare Bio-Science). Then, an IPG strip was passively rehydrated in the mixture overnight (or at least 12 h) at room temperature prior isoelectric focusing was performed in Ettan IPGphor 3 (GE Healthcare). The focusing was carried out at 20°C in five steps: 300 V for 0.3 h, 1000 V for 0.3 h, 5000 V for 1.20 h, 5000 V for 0.25 h and 100 V for at least 0.3 h. Thereafter, proteins in the strip were equilibrated at room temperature in a buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol and 2% SDS) containing 1% DTT for 15 min. Then, they were alkylated in the buffer containing 2.5% iodoacetamide and bromophenol blue for 15 min.

2.4.2 Second dimensional electrophoresis

Separation of the proteins in the second dimension was performed by SDS-PAGE on 12.5% polyacrylamide gel. The electrophoretic separation steps were at 25 mA/gel for 15 min, and then at 60 mA/gel until the dye front reached to the gel bottom edge (usually took 1.30 h in total). The 2DE was performed at least in triplicate for each plasma sample.

2.5 Staining of proteins in 2DE gel

2.5.1 by Coomassie Brilliant Blue (CBB) G-250

The polyacrylamide gel was stained with some modified from Neuhoff et al., 1988. Protein fixation was performed by incubating gel in a 40% v/v ethanol and 10% v/v acetic acid solution for at least 30 minutes. Decant the fixer and place the gel in colloidal stain (100-300 ml per gel depending on size). According to Neuhoff et al., gel was immersed in a staining solution consisting of 8% ammonium sulfate, 20% methanol, 0.08% of CBB G-250, and 0.8% phosphoric acid for 14 to 18 hr or overnight. To remove excessive residual stain, the gel was repeatedly rinsed and incubated with gently shaking in distilled water at room temperature for 6-8 h. Gel was sealed in a plastic bag and stored at 4°C.

2.6 Image analysis

Digitized image of stained gel was scanned using UMAX Powerlook 1120 at a resolution of 300 dpi and 16 bit grayscale pixel depth, and spots of protein were analyzed using ImageMaster 2D Platinum version 5.0 (GE Healthcare). Spot detection and matching of the gels were performed using the gel with highest number of spots as reference. The matched and unmatched protein spots were manually rechecked. The intensity volumes (V) of the individual spots were quantified and normalized with the total intensity volume of all the spots present in each gel (%V). Differences of >1.5 in expression (ratio of %V) between matched spots were considered significant in the comparison.

2.3.5 In-gel digestion and protein identification by Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The protein spots of interest were manually excised from the colloidal Coomassie blue G-250 stained 2-DE gels with pipette tip, transferred into a 1.5 ml tube containing MilliQ water and subjected to in-gel digestion and MALDI-TOF MS at Biotec, Thailand. In brief, after protein bands were excised, the gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, Then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and gels incubated at room temperature for 10 min in a shaker. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

Spectra was internally calibrated, and protein(s) were identified by peptide mass fingerprinting (PMF) with Mascot MS/MS Ions searches of the National Center for Biotechnology Information nonredundant (NCBInr) database (www.matrixscience.com). The important parameters those selected for proteins identification including Database(s) = NCBInr, Enzyme = Trypsin, Taxonomy = Homo sapiens (human), Fixed modifications = Carbamidomethyl C, Variable modifications = Oxidation M, Peptide tolerance ± 1.2 Da, MS/MS tolerance ± 0.6 Da Peptide charge = 1+, 2+ and 3+, Data format = Micromass (.PKL) and Instrument = ESI-QUAD-TOF. All mass searches were performed using a mass window between 0 and 100 kDa.