CHAPTER 2

Materials and Methods

2.1 Chemical and instruments

Chemicals and instruments used in this study were shown in Appendix A and reagent preparation was shown in Appendix B.

2.2 Alzheimer's patients and control subjects

The study population consisted of a total of 157 subjects diagnosed with mild AD (n = 30), moderate AD (n = 30), severe AD (n = 32), MCI (n = 33) and non-demented elderly controls (n = 32). Clinically diagnosed patients with AD and MCI were recruited from Suanprung Psychiatric Hospital. All patients underwent a standard clinical assessment, including neurological, physical and neuropsychiatric examinations. Criteria for controls were absence of memory complaints or other cognitive symptoms, preservation of general cognitive functioning and no active neurological or psychiatric disease. Participants were matched on factors such as age, sex and no evidence of other dementia. The Mini-Mental State Examination-Thai Version 2002 score (MMSE-Thai score) (Appendix C) was added for investigation and classification of cognitive function in AD, MCI and normal subjects (NS). Informed consent was obtained from all participants. The study was approved by the Ethics Committees at Suanprung Psychiatric Hospital and by the Faculty of Associated Medical Sciences, Chiang Mai University.

2.3 Sample collection

Five milliliters of venous blood samples were collected from all participants. After clotting, blood was centrifuged at 1,800 g for 10 minutes. After centrifugation, serum samples were divided into 1 ml aliquots and stored immediately at -70°C for subsequent serum protein analysis, $A\beta_{40}$, $A\beta_{42}$, clusterin and p97.

2.4 Measurement of serum protein levels by ELISA technique

An ELISA technique was used to determine serum protein biomarkers, including $A\beta_{40}$, $A\beta_{42}$, clusterin and p97 in serum samples from NS, MCI and AD patients according to the manufacturer's instructions. Samples were measured in duplicate. All samples had undergone only one freeze-thaw cycle and were analyzed within 16 months of collection.

A. Amyloid-β peptide ($A\beta_{40}$ & $A\beta_{42}$)

Serum $A\beta_{40}$ and $A\beta_{42}$ concentrations were assayed by a commercially available sandwich ELISA kit (Invitrogen, Camarillo, USA). The kit was supplied with a 96 well microplate, pre-coated with monoclonal antibody specific for the NH_2-terminus of human Aβ. Serum samples were diluted 2-fold with Standard Diluent Buffer to reduce interference from certain component in sample.

Briefly, 50 µl of Aβ peptide standards, controls, and diluted serum samples were added into the microplate wells. Fifty microliters of human $A\beta_{40}$ or $A\beta_{42}$ detection antibody were then pipetted into the wells and incubated for 3 hours at room temperature (RT) with agitation. After 3 hours incubation, a solution of 100 µl of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (dilution 1:100) was added and incubated for 30 minutes at RT with shaking. Finally, 100 µl of TMB Stabilized Chromogen was added and the absorbance was read at 450 nm on a microplate reader (Metertech, Taipei, Taiwan). A standard curve was plotted with optical density (OD) of each standard on Y axis against concentration (pg/ml) on X axis. The concentration of $A\beta_{40}$ and $A\beta_{42}$ in serum was calculated.

B. Clusterin

Human clusterin competitive ELISA Kit (Adipogen, Incheon, Korea) was used to assess the levels of clusterin in serum samples. The wells of a 96 well ELISA plate were coated with human clusterin recombinant protein. The 1:500 dilution of test serum was prepared by adding 10 µl of serum in 990 µl of clusterin diluent to final volume of 1:100 dilution, and then adding 100 µl of dilute serum in 400 µl of clusterin diluent to a final

volume of 1:500 dilution. Fifty microliters of the final diluted sample were used for ELISA.

Briefly, 50 µl of diluted serum sample, human clusterin standard and QC sample were added to microplate wells followed by addition of 50 µl of clusterin detection antibody. After 1 hour incubation at 37 °C, the wells were washed three times with wash buffer to remove any unbound detection antibody. After washing, 100 µl of HRP-conjugated anti-rabbit IgG antibody (dilution 1:100) were added to the wells and incubated for 1 hour at 37 °C. Finally, 100 µl of TMB substrate were added and the absorbance was read at 450 nm on a microplate reader (Metertech, Taipei, Taiwan). Generation of the standard curve by plotting the average absorbance was obtained for each standard concentration on the vertical (Y) axis versus the corresponding concentration (µg/ml) on the horizontal (X) axis. Clusterin concentration of samples was calculated by interpolation of the regression curve formula.

C. p97

Serum p97 levels were quantified using a sandwich ELISA kit (USCN Life Science Inc, China). The 96-well ELISA plates were pre-coated with anti-melanotransferrin antibodies (p97). The samples were diluted to 50-fold dilution by adding 10 µl of serum to 490 µl of the 0.02 mol/L PBS (pH 7.0-7.2).

Briefly, 100 µl of dilutions of standard blanks and diluted samples were added to the wells. After 2 hours incubation at 37 °C, 100 µl of p97 biotin-conjugated detection antibody were pipetted into the wells and then incubated for 30 minutes at 37 °C. Next, the plate was washed three times with buffer for removing any unbound detection antibody. After washing, a solution of 100 µl of avidin conjugated to HRP was added and incubated for 30 minutes at 37 °C. Finally, 90 µl of TMB substrate were added and the absorbance was read at 450 nm on a microplate reader (Metertech, Taipei, Taiwan). The concentration of p97 in the samples was determined by comparing the OD of the samples to a standard curve.

2.5 Analysis of serum proteomics by 2-DE

Individual fresh serum samples for proteomics investigation consisted of 15 subjects diagnosed with MCI (n = 5), AD (n = 5) and cognitive normal subjects (n = 5) were selected randomly as a representative from each group of patients.

2.5.1 Study design

Abundant proteins, such as albumin and immunoglobulin G (IgG), were removed from serum samples. The concentration of protein in each sample was then determined based on Folin-Lowry methods. Serum albumin and IgG depletion proteins were separated with 2-DE. After electrophoresis, gels were stained with silver stain and then were scanned with an image scanner. Spot proteins of interest were detected and matched by image analysis software (**Figure 2.1**).

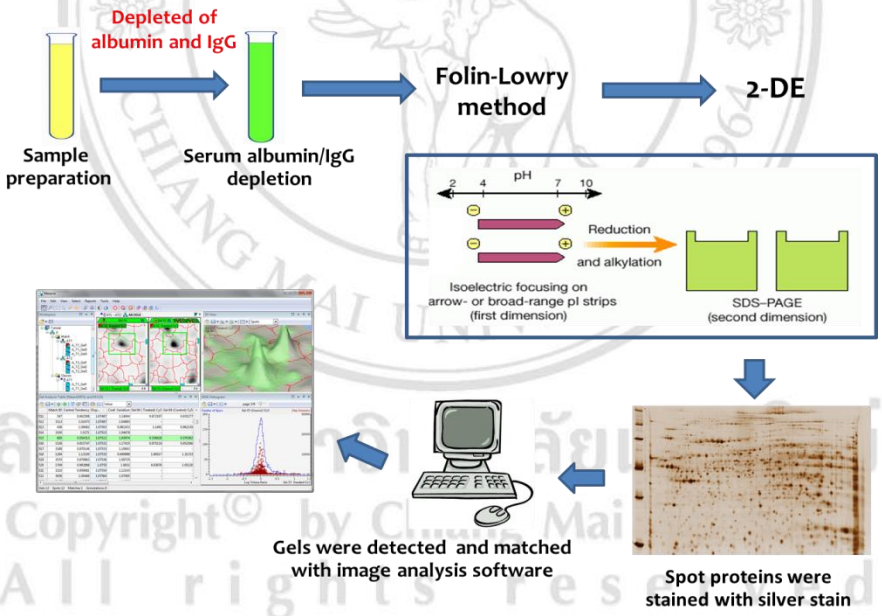

Figure 2.1 Schematic diagram of the study design

2.5.2 Sample preparation

Before each 2-DE run, the serum samples were depleted of albumin and IgG using the ProteoPrep Blue Albumin &IgG Depletion kit (Sigma, St. Lewis, MO) according to the manufacturer's instructions. The ProteoPrep Blue Albumin &IgG Depletion Medium is a mixture of two media: 1) a proprietary blue matrix and 2) Protein G agarose. The protocol used protein G agarose beads to remove IgG, followed by ethanol precipitation of non-albumin proteins; more than 85% of albumin and 70% of IgG were removed from serum samples.

Briefly, the depletion packed media were equilibrated by adding 400 µl of the suspended medium in a spin column and then centrifuged in a collection tube at 10,000 rpm for 10 seconds. Next, 400 µl of Equilibration Buffer were added to the column, and centrifuged at 10,000 rpm for 10 seconds. The buffer was removed and the spin column was placed in a new collection tube. This step was repeated once. In the serum depletion of albumin and IgG step, 100 µl of serum were applied on top of the packed medium bed and incubated at room temperature for 5-10 minutes. The sample was immediately adsorbed into the medium. After incubation, it was centrifuged at 10,000 rpm for 60 seconds. The flow through were reapplied to the column again and incubated for 5-10 minutes, and then centrifuged at 10,000 rpm for 60 seconds. After centrifugation, the twice eluted (unbound proteins) in the collection tube were again collected. In addition, the remaining unbound proteins from the spin column were washed by adding 100 µl of Equilibration Buffer to the on top of the packed medium bed and centrifuged 60 seconds. This flow through was collected and pooled with the twice eluted proteins from the previous step. The albumin/IgG depleted sera were stored at -20 °C and were analyzed within 3 months of collection.

Next, a volume of sample containing 100 µg of total protein determined by the Folin-Lowry assay method were suspended with rehydration buffer to a final volume of 125 µl. The rehydration buffer composition is shown in **Table 2.1**.

Table 2.1 Rehydration buffer composition used for 2-DE experiment

Reagents	Concentration
Urea	8M
Thiourea	2M
CHAPS	4% (w/v)
Bromophenol blue	0.002% (w/v)
IPG buffer pH 3-10 (linear)	0.5 % (w/v)

2.5.3 Protein determination: Folin-Lowry assay

For 2-DE, the Folin-Lowry assay was used for determination of protein concentration in this study. The principle is based on the interaction of Cu^{2+} and protein in alkaline solution, resulting in reduction of Cu^{2+} to Cu^{1+}. The Cu^{1+} can be detected with Folin-Ciocalteu phenol reagent (phosphomolybdic-phosphotungstic acid). Cu^{1+} reduction of Folin-Ciocalteu phenol reagent produces a blue color that can be read at 750 nm. The amount of color produced is proportional to the protein concentration. In this study, Bovin Serum Albumin (BSA) was used as a standard protein, because of its low cost, high purity and ready availability.

The protein concentration in samples was compared to the BSA protein standard curve. The BSA protein standard curve could be constructed by preparation of various concentrations of BSA from stock solution of 1 mg/ml as shown in **Table 2.2**. Twenty microliters of each protein sample were added into 480 µl PBS. Then 2.5 ml of alkaline copper solution were added and mixed. After 10 minutes incubation at room temperature, 250 µl of Folin phenol reagent were added and further incubated at room temperature for 30 minutes. Finally, the concentrations of BSA standard and sample protein were determined at 750 nm using a spectrophotometer.

Table 2.2 Preparation of Bovine serum albumin (BSA) standard solution

BSA concentration (μg/ml)	Stock BSA 1 mg/ml (μl)	Deionized distilled water (μl)
0	0	500
25	25	475
50	50	450
75	75	425
100	100	400
125	125	375
150	150	350
175	175	325
200	200	300

2.5.4 Two dimensional gel electrophoresis

A. First dimension: IEF

A total of 125 μl of sample with rehydration buffer were loaded carefully into a 7 cm strip holder. The gel side of the IPG strip (pH 3-10, linear, GE Healthcare) was placed over the sample. Then 1 ml of immobiline Dry Strip Cover Fluid was added over the IPG strip to prevent evaporation of sample and also to stop precipitation of urea. Isoelectric focusing (IEF) was performed using a Multiphore II apparatus (GE Healthcare) at 20 °C. Appropriate parameters for 7 cm strips were selected according to the manufacturer's instructions (**Table 2.3**).

Table 2.3 Running conditions for 7 cm, pH 3-10 linear of IPG strips

Voltage mode	**Voltage (V)**	**Time (h:min)**	**kVh**
1. Step and Hold	300	4:00	0.3
2. Gradient	1000	1:00	0.3
3. Gradient	5000	1:20	4.5
4. Step and Hold	5000	0:25	3.0

After IEF process, IPG strips were used immediately for the second dimensional analysis or stored at -70°C. Equilibration was performed prior to the second dimension.

B. IPG strip equilibration

The IPG strips must be equilibrated before SDS-PAGE. The strips were placed in individual tubes and then incubated in two changes of SDS equilibration buffer. Firstly, 5 ml of first equilibration buffer containing DTT (100 mg per 10 ml) were added to the IPG strips and incubated in a shaker for 15 minutes at RT. Then the strips were re-equilibrated in the same buffer for 15 minutes and DTT was replaced by IAA (250 mg per 10 ml). After equilibration, the strips were applied to the second dimension to separate proteins in a polyacrylamide gel (142, 143).

C. Second dimension: SDS-PAGE

Proteins were separated based on their mass using SDS-PAGE in the second dimension. Briefly, equilibrated strips were transferred to 12.5% polyacrylamide gels in the well. Strips were overlaid with 0.5% (w/v) low melting point agarose sealing solution containing 0.002% (w/v) bromophenol blue and 3µl of protein marker were loaded in the marker lane. The SDS-PAGE was performed at 100 V for 1-2 hours until bromophenol blue reached the end of the gel (142, 143).

2.5.5 Staining: Silver staining method

The gel bands were visualized by silver staining. Initially, the gel was fixed with 50% methanol, 12% acetic acid and 0.05% formalin for 1.5 hours or overnight. After washing twice with 35% ethanol for 5 minutes, the gel was sensitized by 2 minutes incubation in 0.02% sodium thiosulphate ($Na_2S_2O_3$). After sensitizing, the gel was washed twice with deionized water for 5 minutes then incubated in 0.2% silver nitrate for 20 minutes. After staining, the gel was washed with deionized water for 60 seconds. Then the gel was developed in 6% sodium carbonate (Na_2CO_3), 0.02%$Na_2S_2O_3$ along with 0.0005% formalin solution. Finally the reaction was terminated by adding stop solution (1.46% Na-EDTA) and incubated for 20 minutes. Gels were stored in 0.1% acetic acid at 4 °C or RT (142, 143).

2.5.6 Image analysis

After staining, the gels were scanned using Image scanner software (Amersham Biosciences) in transparency mode with resolution 150-300 dpi (dot per inch). The image of 2-DE gels was analyzed using Imaging Master 2D-platinum software, version 6.0 (GE Healthcare). This software matches and analyzes visualized protein spots among different gels and compares peaks of protein between control and experimental gel images from both quantitative (amount of protein expressed) and qualitative (presence/absence of a protein) points of view.

Briefly, the gel images were added to an import folder in the program. Each gel can be adjusted before analysis of spot detection. Next, the protein spots in selected gels were detected according to the setting parameters. The detected spot shape could be changed in this step. Then protein spots were matched and compared to reference gel controls at the same location. There was background subtraction after manual matching. This software can calculate approximate p*I* and MW values. Reports of match count and spot detection were created and exported to Microsoft Excel®. Pick lists for spots of interest were searched for protein identification by p*I* and MW from SWISS-SDPAGE database, available at http://world-2dpage.expasy.org/swiss-2dpage. A diagram for comparisons of protein spot detection in this study is shown in **Figure 2.2**

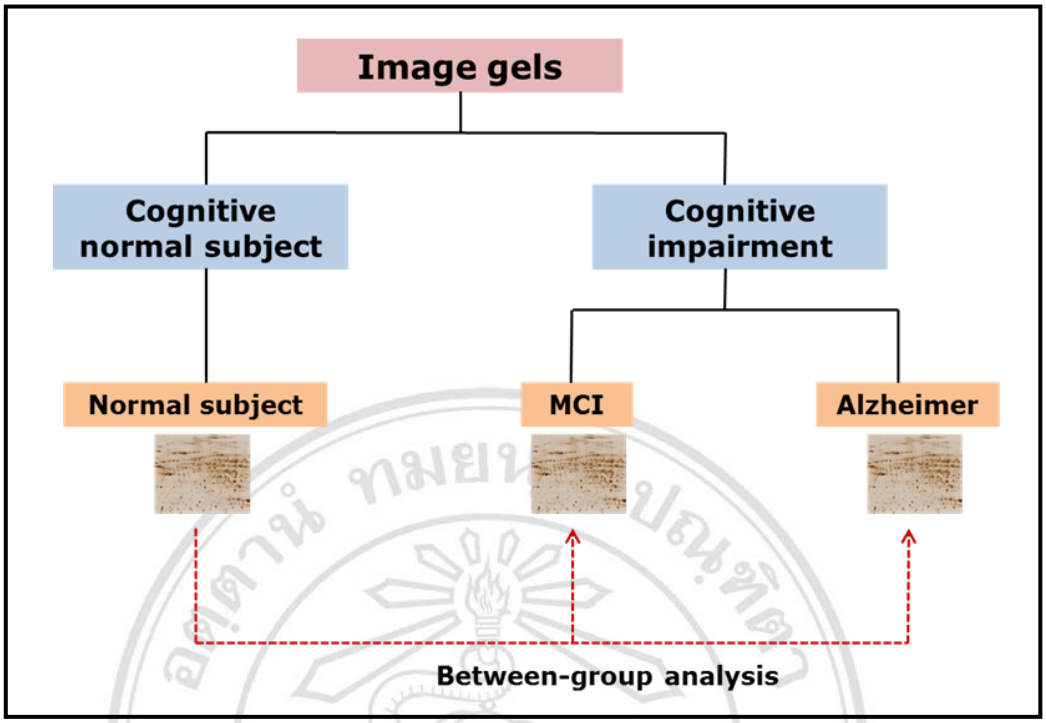

Figure 2.2 Diagram for comparisons of protein spot detection between cognitive impairment (MCI and AD) and cognitive normal subjects in this study.

2.6 Statistical analysis

The data were analyzed using SPSS for Windows version 17 software. Statistical analysis used to compare differences in means between factor levels of age, sex, and MMSE-Thai were one-way analysis of variance (One-Way ANOVA). The significant differences of $A\beta_{40}$, $A\beta_{42}$, clusterin and p97concentrations between AD groups, MCI group and cognitive normal subject were evaluated using non-parametric Kruskal-Wallis and Mann-Whitney U tests. Results of the analyses were considered significant at *p*-values less than 0.05. The relationship between sensitivity and specificity for the different groups of cognitively impaired patients (AD and MCI) versus NS or AD versus MCI and NS was described using receiver operating characteristic (ROC) curve analysis (147). The areas under the ROC curves (AUC) as measures of diagnostic accuracy were calculated using the ROC Curve. The ROC curve was calculated with 95% confidence intervals (CI). In proteomics, measures of central tendency and dispersion from each detected spots in 2-DE gel images were used for descriptive statistics of spot analysis. The mean 100% and the mean squared deviation (M.D.S) 100% are the most commonly used statistics.