Antidepressant resistant protein markers in Thai

Surasak Supuntee

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Thesis Title	Antidepressant resistant protein markers in Thai
Author	Mr. Surasak Supuntee
Major Program	Biochemistry

Major advisor

…….

(Dr. Ladda Leelawatwattana)

Examining Committee:

……..……Chairperson

(Verayuth Prapunpoj, M.D.)

……

(Assoc. Prof. Dr. Nunta Churngchow)

……

(Dr. Ladda Leelawatwattana)

Co-advisor

…….

(Assoc. Prof. Dr. Porntip Prapunpoj)

……

(Assoc. Prof. Dr. Porntip Prapunpoj)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Biochemistry

……...

(Assoc. Prof. Dr. Teerapol Srichana)

Dean of graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……....Signature

(Dr. Ladda Leelawatwattana)

Major advisor

……....Signature

(Mr. Surasak Supuntee)

Candidate

I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

……....Signature

(Mr. Surasak Supuntee)

Candidate

ชื่อวิทยานิพนธ์	โปรตีนบ่งชี้การต้านยาโรคซึมเศร้าในคนไทย
ผู้เขียน	นายสุรศักดิ์ สุปันตี
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บทคัดย่อ

โรคซึมเศร้า (depressive disorder) เป็นโรคที่ถูกคาดการณ์ว่าจะเป็นสาเหตุของปัญหาสุขภาพในศตวรรษที่ 21 ถึงแม้จะมีหลายวิธีในการรักษาโรคนี้ แต่การใช้ยาต้านโรคซึมเศร้า (antidepressant) มักถูกเลือกเป็นขั้นตอนแรกในการรักษา อย่างไรก็ตามปัญหาที่พบคือการที่ผู้ป่วยตอบสนองต่อยาค่อนข้างน้อย ทำให้อัตราการหายจากโรคอยู่ในระดับต่ำ ดังนั้นเพื่อเป็นการลดระยะเวลา รวมถึงเพิ่มโอกาสประสบความสำเร็จในการรักษาโรค การค้นหาตัวบ่งชี้ทางชีวภาพที่สามารถใช้ในการวินิจฉัยรวมถึงบ่งบอกประสิทธิภาพในการรักษาโรค โดยเฉพาะในช่วงต้นของการรักษา นับเป็นงานที่ท้าทายต่อนักวิจัยเป็นอย่างมาก วิทยานิพนธ์นี้ได้ทำการศึกษาเพื่อหาโปรตีนบ่งชี้การต้านยาโรคซึมเศร้า Fluoxetine ซึ่งเป็นยาในกลุ่ม serotonin reuptake inhibitor (SSRI) โดยใช้เทคนิคการแยกโปรตีนแบบสองมิติ (2D gel electrophoresis, 2DE) และ เทคนิค Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) โดยการทดลองจะเริ่มจากการเก็บตัวอย่างพลาสมาจากคนปกติจำนวน 20 คน และจากผู้ป่วยโรคซึมเศร้ารุนแรง (Major depressive disorder, MDD) จำนวน 48 คน ซึ่งถูกแบ่งตาม HAM-D score ออกเป็น 3 กลุ่ม คือกลุ่มผู้ป่วยที่มีการตอบสนองต่อยาอย่างเร็ว (fast-response; FR) จำนวน 34 คน กลุ่มผู้ป่วยที่ตอบสนองต่อยาช้า (slow- response; SR) จำนวน 9 คน และกลุ่มผู้ป่วยที่ไม่ตอบสนองต่อยาดังกล่าว (non-response; NR) จำนวน 5 คน หลังจากนั้นแบบแผนของโปรตีนทั้งใน whole plasma รวมทั้งในพลาสมาที่มีการแยก albumin และ IgG ออกแล้ว จะถูกนำมาวิเคราะห์ในมิติที่หนึ่งด้วย Isoelectric focusing (IEF) โดยใช้ IPG strip ขนาด 7 ซม. และทำการแยกโปรตีนในมิติที่สองด้วย SDS-PAGE ผลการเปรียบเทียบร้อยละโดยปริมาตร (%V) ของ spot โปรตีน จากกลุ่มผู้ป่วย MDD กับคนปกติ พบการแสดงออกของโปรตีน α1-antitrypsin, fibrinogen, haptoglobin, transcritption factor, apoliporpotein AI (apo AI), apolipoprotein AII (apo AII), apolipoprotein E (apo E), HDL associated protein และ IgG light chain ที่สูงในกลุ่มผู้ป่วย MDD มากกว่าในคนปกติ นอกจากนี้พบโปรตีน complement C3 (C3) มีการแสดงออกสูงในผู้ป่วย MDD แต่แสดงออกต่ำมากในคนปกติในระดับที่ไม่สามารถตรวจสอบได้โดย 2-DE ซึ่งการแสดงออกที่แตกต่างกันของโปรตีนเหล่านี้ ยกเว้น apo AI, apo AII, และ C3 พบว่าสอดคล้องกับงานวิจัยอื่นที่เคยรายงานไว้ ส่วนผลการเพิ่มขึ้นของระดับโปรตีน apo AI, apo AII และ C3 ในผู้ป่วย MDD ที่พบในวิทยานิพนธ์นี้ มีความขัดแย้งกับงานวิจัยอื่นๆ จึงจำเป็นต้องมี

การทดลองเพิ่มเติมเพื่อยืนยันผล เมื่อวิเคราะห์ spot โปรตีนจากวิธี 2-DE ในกลุ่มผู้ป่วย MDD พบโปรตีนที่เกี่ยวข้องกับการอักเสบและระบบภูมิคุ้มกัน เช่น α1-antitrypsin, apo AI, C3, haptoglobin และ IgG light chain มีการแสดงออกสูงในกลุ่ม NR มากกว่ากลุ่ม FR และ SR ในทางตรงกันข้ามการแสดงออกของ transcription factor และ Rap 1A จะต่ำในกลุ่ม NR เมื่อเปรียบเทียบกับกลุ่ม FR และ SR ผลการทดลองที่ได้ชี้ให้เห็นว่าโปรตีนในพลาสมาที่กล่าวมา โดยเฉพาะ Rap 1A จะสามารถใช้เป็นตัวบ่งชี้ในเบื้องต้นถึงการตอบสนองต่อ fluoxetine ซึ่งคาดว่าจะมีประโยชน์ต่อการรักษาโรค MDD ต่อไป

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Author	Mister. Surasak Supuntee
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ABSTRACT

The depressive disorder has been predicted to be the most burdensome disease in the world in the 21st century. Although, several treatments strategies have been developed for the patients, pharmacotherapy using particular antidepressant is oftenly the first line of treatments. However, an inadequate response following the treatment with antidepressant as well as low remission rates have been reported. To reduce time and increase succeed of the treatment, finding out an indicator for diagnosis and treatment efficacy since early time of treatment is a challenge work for many researchers. In this thesis, protein biomarkers in plasma for the prediction of patient responsiveness to fluoxetine, an antidepressant in the class of serotonin reuptake inhibitors (SSRIs), were identified by two-dimensional gel electrophoresis (2DE) and Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). The plasma samples were collected from 20 normal and 48 major depressive disorder (MDD) patients, which their responses to the antidepressant were classified into fast response (FR; n = 34), slow response (SR; n = 9) and non-response (NR; n = 5) according to HAM-D score. The separation of the proteins either from whole plasma or after depletion of albumin and IgG was performed in the first dimension by isoelectric focusing (IEF) in IPG strip (7 cm long), and the second dimension of separation was carried out by SDS-PAGE. By comparing %volume (%V) of the protein spots on the gels between MDD and the normals, it showed that the expression levels of α1-antitrypsin, fibrinogen, haptoglobin, transcritption factor, apoliporpotein AI (apo AI), apolipoprotein AII (apo AII), apolipoprotein E (apo E), HDL associated protein and IgG light chain were higher in MDD than in the normal. In addition, while the expression of complement C3 (C3) in MDD was detected, the expression in normal group was very low and could not be detected by 2DE. The differences in the expressions of these proteins except apo AI, apo AII, and C3 were well agree with the previous reports. Higher

expressions of apo AI, apo AII and C3 in MDD observed in this thesis were controversial to the previous reports and need to be validated. The matching and analysis of 2DE spots from the plasma of NR, FR and SR revealed that the expressions of proteins in the inflammatory and immune systems, i.e. α1-antitrypsin, apo AI, C3, haptoglobin and IgG light chain, in NR were higher than in FR and SR. In contrast, the expressions of transcription factor and Rap 1A were low in NR compared to FR and SR. The results indicated that these proteins in particular Rap 1A would be used as biomarkers in plasma for early prediction of the responsiveness to fluoxetine, which be valuable for the treatment of MDD.

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LIST OF ABBREVIATIONS AND SYMBOLS

BSA	=	bovine serum albumin
CBB	=	Coomassie Brilliant Blue G-250
cm	=	centimeter
DTT	=	dithiothreitol
dpi	=	dot per inch
g	=	gravitational acceleration
h	=	hour
HCl	=	hydrochloric acid
IEF	=	isoelectric focusing
kDa	=	kilodalton
mA	=	milliampere
mg	=	milligram
min	=	minute
ml	=	milliliter
M	=	molar
mM	=	millimolar
MALDI-TOF MS	=	Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry
°C	=	degree Celsius
PAGE	=	polyacrylamide gel electrophoresis
%	=	percentage
2DE	=	Two dimensional gel Electrophoresis
SDS	=	sodium dodecyl sulfate
s	=	second
V	=	volt
µg	=	microgram
µl	=	micromolar

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURES

Introduction

"Mental illness" or "mental disorder" normally refers to conditions such as major unipolar depression (or depression or depressive disorder), schizophrenia, and manic depression (Murphy, 2000; Simon et al., 2005). Worldwide, more than one in four people suffer from mental disorder at some point in their life. Based on disability-adjusted life year (DALY), a standard measurement of overall disease burden which expressed as the number of years lost due to ill-health, disability or early death, depressive disorder is predicted to become the second most important cause of DALY lost by 2020. The depressive disorder is characterized by a profound and persistent feeling of sadness and/or loss of interest in things that once preferable. There are 2 main categories classified basing on during of the symptom, acute (or major depressive disorder, MDD) and chronic (or dysthymic disorder) depressions. The causes of depressive disorder are complex. The molecular mechanisms that cause and maintain the symptom are still unknown. Several factors have been hypothesized to be involved, including the decrease in function of monoamines, particularly serotonin (5-hydroxytryptamine, 5-HT) and noradrenaline (NA) in the brain (for review see Porcelli et al., 2010). Several treatment strategies have been explored, however, the use of antidepressant drugs (ADs) for treatment is well established. Up to date, many antidepressants have been developed and most of them act on 5-HT and NA (Nutt et al., 2006). Several selective 5-HT reuptake inhibitors such as fluoxetine (for reviews see Carrasco and Sandner, 2005; Cipriani et al., 2009) and sertraline (for review see Carrasco and Sandner, 2005; Cipriani et al., 2010) have been introduced to reduce the symptom by enhancing activity and increasing levels of 5-HT. However, patients may still on their depressive symptoms after first medication, since this treatment requires 4-8 weeks to determine whether the patients respond to the drug (Kemp et al., 2008). Moreover, an inadequate response following the treatment with antidepressant has been reported occurring among patients and the treatment outcome remains disappointing with remission rates of maximal 37% (Warden et al., 2007).

These problems, led to attentions on seeking for predictors especially those could be matched patients with right medication at early time point of the treatment since the early detection may reduce suffering, prevent the development of negative attitudes as well as increase the chance for successful treatment. Although several indicators including clinical, psychophysiological and neuropsychological were reported could improve response prediction, they could not be used in practice due to poor prognostic sensitivity and specificity (for review see Kemp et al., 2008; Esteve et. al., 2010).

Molecular biomarkers are measurable indicators of a particular biological state that relevant to the risk, the presence or the stage of disease. They can be in many forms including transcriptional profiling and DNA methylation (Ramaswamy and Perou, 2003), metabolomics (Fernie et al., 2004), and protein domain. Till now, many novel and improved proteomic technologies have been developed which resulted in major efforts towards the discovery of new protein biomarkers those can be used in clinical practice to identify risk or diagnose a disease, stratify patients, assess disease severity or progression, predict prognosis, or guide treatment. The peripheral markers for psychiatry disorders have been explored for many years to predict the treatment efficacy, which generally requires knowing in advance whether or not depressive disorder patients are responding to the drugs. However, the specific biomarker(s) for prediction of an individual patient's responsiveness to a particular antidepressant have not been identified. Among the human body sources, plasma is an ultimate source of biomarker discovery since it is the most comprehensive human proteome which represent to all body tissues and to both physiological and pathological processes (Anderson and Anderson, 2002). In this thesis, protein biomarker(s) for prediction of responsiveness to an antidepressant in particular serotonin reuptake inhibitor was identified from plasma by 2DE and mass spectrophotometry (MS).

Review of literatures

1. Mental disorder: characteristics and types

The terms "mental illness" and "mental disorder" normally refer to conditions such as major unipolar depression, schizophrenia, manic depression, and obsessive compulsive disorder (Murphy, 2000; Simon et al., 2005). Principles for

diagnosis and classification of mental disorders are outlined in terms of unit of classification, threshold for illness, clinical significance, syndrome similarity, underpinning biological mechanisms and need for an ontological structure which allows both categorical and dimensional models and expression of co-morbidity (for review see Austin, 2010)

There are many different conditions that are recognized as mental illnesses. The common types include the disorders of anxiety, mood, psychosis, eating, impulse control and addiction, personality, adjustment, dissociation, factitiousness, sex and gender, somatisation and mortor tics (Stengel, 1959)

Anxiety disorders: This type of disorders involves an apprehensive anticipation of future danger or misfortune accompanied by a feeling of dysphoria or somatic symptoms of tension (American Psychiatric Association, 1994). People with anxiety disorders respond to certain objects or situations with fear and dread, as well as with physical signs of anxiety or nervousness, such as a rapid heartbeat and sweating. An anxiety disorder is diagnosed if the person's response is not appropriate for the situation, if the person cannot control the response, or if the anxiety interferes with normal functioning. There are many types of anxiety disorders including generalized anxiety disorder (GAD), post-traumatic stress disorder (PTSD), obsessive-compulsive disorder (OCD), panic disorder (PD), social anxiety disorder (SAD), and specific phobias (SP).

Mood disorders (affective disorders): These disorders are mental health problems that can be found in children and adolescents, as well as adults. At any age, mood disorders put individuals at risk for other conditions that may persist long after the initial episodes of depression are resolved (http://www. hopkinsmedicine. org/ healthlibrary/conditions/mental_health_disorders/overview_of_mood_disorders_85,P 00759). The most common mood disorders are depression, mania, and bipolar disorder (Austin, 2010). The symptoms are different depend on age and type of mood disorder present. However, the most common symptoms involve persistent feelings of sadness or periods of feeling overly happy, or fluctuations from extreme happiness to extreme sadness. Mood disorders are multifactorial inherited in which genetic and environment are considered to be involved. Causes of the disease are not well understood until now, however, imbalances in certain neurotransmitters including

serotonin, norepinephrine, and dopamine as well as life events may also contribute to depress mood.

Psychotic disorders: Psychotic disorders involve distorted awareness and thinking. Two of the most common symptoms of psychotic disorders are hallucinations which experiences of images or sounds that are not real, such as hearing voices and delusions false beliefs which the ill person accepts as true, despite evidence to the contrary. Schizophrenia is an example of psychotic disorder.

Eating disorders: Eating disorders involve extreme emotions, attitudes, and behaviors involving weight and food. Anorexia nervosa, bulimia nervosa and binge eating disorder are the most common eating disorders.

Impulse control and addiction disorders: People with impulse control disorders are unable to resist urges, or impulses, to perform acts that could be harmful to themselves or others. Pyromania (starting fires), kleptomania (stealing), and compulsive gambling are examples of impulse control disorders. Alcohol and drugs are common objects of addictions. Often, people with these disorders become so involved with the objects of their addiction that they begin to ignore responsibilities and relationships.

Personality disorders: People with personality disorders have extreme and inflexible personality traits that are distressing to the person and/or cause problems in work, school, or social relationships. In addition, the person's patterns of thinking and behavior significantly differ from the expectations of society and are so rigid that they interfere with the person's normal functioning. Examples include antisocial personality disorder, obsessive-compulsive personality disorder, and paranoid personality disorder. Other, less common types of mental illnesses include.

Adjustment disorder: Adjustment disorder occurs when a person develops emotional or behavioral symptoms in response to a stressful event or situation. The stressors may include natural disasters, such as an earthquake or tornado; events or crises, such as a car accident or the diagnosis of a major illness; or interpersonal problems, such as a divorce, death of a loved one, loss of a job, or a problem with substance abuse. Adjustment disorder usually begins within three months of the event or situation and ends within six months after the stressor stops or is eliminated.

Dissociative disorders: People with these disorders suffer severe disturbances or changes in memory, consciousness, identity, and general awareness of themselves and their surroundings. These disorders usually are associated with overwhelming stress, which may be the result of traumatic events, accidents, or disasters that may be experienced or witnessed by the individual. Dissociative identity disorder, formerly called multiple personality disorder, or "split personality", and depersonalization disorder are examples of dissociative disorders.

Factitious disorders: Factitious disorders are conditions in which physical and/or emotional symptoms are created in order to place the individual in the role of a patient or a person in need of help.

Sexual and gender disorders: These include disorders that affect sexual desire, performance, and behavior. Sexual dysfunction, gender identity disorder, and the paraphilias are examples of sexual and gender disorders.

Somatoform disorders: A person with a somatoform disorder, formerly known as psychosomatic disorder, experiences physical symptoms of an illness even though a doctor can find no medical cause for the symptoms.

Tic disorders: People with tic disorders make sounds or display body movements that are repeated, quick, sudden, and/or uncontrollable. (Sounds that are made involuntarily are called vocal tics.) Tourette's syndrome is an example of a tic disorder.

2. Depressive disorders

Depressive disorders are one type of mood disorder that include major depressive disorder (MDD), the depressed phase of bipolar disorder, and dysthymic disorder. MDD and dysthymia differ primarily in chronicity and severity of symptoms. Amongst, MDD is the most prevalent disorder.

2.1 Major depressive disorder (MDD)

Major depressive disorder (MDD) is a common psychiatric syndrome that can interfere an individual’s thoughts, feelings and behaviors. This disease has been predicted to be the most burdensome disease in the world in the 21st century (World Health Organization, 2004). According to Diagnostic and Statistical Manual of

Mental Disorders (DSM), MDD is characterized by one or more major depressive incidents that are persistent and abnormally depressed mood states that last at least 2 weeks (Diagnostic and Statistical Manual, 4th ed. (DSM-IV)). An incident is generally accompanied by a significant persistent and pervasive depression, irritability and loss of interest or pleasure in almost all activities. It is also associated with other symptoms, such as disruption in appetite, sleep, energy, and ability to concentrate; feelings of worthlessness or excessive guilt; or recurrent thoughts of death, all of which have a negative impact on interpersonal and academic functioning. MDD is often chronic and recurrent (Judd et al., 1998). About 40% of youths who have recovered have a recurrence before 2 years, and 70% before 5 years (Kovacs et al., 1984). Several studies have reported that the clinical presentation of symptoms in women markedly differs from those reported by men. The incidence is higher and more severe in female than in male (Thase et al., 1994), and associated with a greater functional impairment (Angst and Dobler-Mikola, 1984). In addition, the symptom first onset peaks during the childbearing years (Weissman and Olfson, 1995; Kessler et al., 2005). However, although women appear to have a higher risk of new-onset incidence, they have similar recurrence and episode length as men (Eaton et al., 1997).

Patients with MDD have a great deal of heterogeneity in the level of several symptoms such as depression severity, number of earlier episodes, anxiety disorders, chronic medical disorders, co morbidity with personality disorders and maladaptive coping styles (such as high levels of neuroticism), and socioeconomic status (Gaynes et al., 2007; Gopinath et al., 2007). These differences have been found to significantly affect patient outcomes (Trivedi et al., 2006; Gaynes et al., 2007).

Because MDD is a relatively common mental disorder, several investigations have been conducted to identify possible underlying causes. There are several factors i.e. genetic, environment and biology that are identified. The genetic is consider to be involved in early onset, severity and recurrent of the disease (Kendler, Gardner and Prescott, 2000). Although, there is no specific reliable molecular risk factor for occurrence of MDD, however, some chromosomal region i.e. on chromosome 15q25-q26 has been reported can link to recurrent and early onset of depression (Holmans et al., 2007). Besides, environmental factors such as personal losses, economic status

and poor interpersonal relationship are also considered to relate to depression of a person. Other causes of MDD are biological factors. Abnormalities in the neuroendocrine systems, neurotransmitters , and neuroanatomy of the brains are found in both children and adult with MDD (http://www.minddisorders.com/Kau-Nu/Major-depressive-disorder.html). Deficiency of norepinephrine and serotonin due to inhibition of tyrosine hydroxylase, depletion of tryptophan, polymorphism of serotonin reuptake transporter as well as improper functioning of norepinephrine receptors, all have been found in patients with MDD (for review see Belmaker and Agam, 2008). Moreover, abnormalities in the frontal lobe as well as lower levels of electrical activity in the left frontal cortex were suggested to be found in depressed subjects (http://www.minddisorders.com/Kau-Nu/Major-depressive-disorder.html).

2.2 Treatment of depressive disorder

There are various types of treatment for mental disorders, however, the two commons are psychiatric medication and psychotherapy. Pharmacotherapy is often used as first line treatment for depression and there are several medications that have been proven to be effective in treating MDD (Stahl, 2002; Rush et al., 2006). Psychological treatment showed to have significant effects on depression in women with postpartum depression (Lumley, Austin, and Mitchell, 2004), and patients with both depression and general medical disorders (Mohr and Goodkin, 1999; Hackett, Anderson and House, 2004). In comparison, the effects of pharmacotherapy are somewhat superior to those of psychotherapy, especially in case of chronic major depression (Cuijpers et al., 2010). However, studies on success rates of psychotherapy versus medication on treating of depressive disorder revealed that combined treatments are more effective than either use alone especially in case of more severe depressed patients (Friedman et al., 2004; Pampanolla et al, 2004; De Maat et al, 2007; Cuijpers et al., 2010).

The treatment of depressive disorder consists of three phases (for review see American Psychiatric Association, 2000, Karasu et al., 2000; Gartlehner et al., 2007).

1. An acute phase, which is the phase of remission induction. This period takes 6-12 weeks in length. The treatment options i.e. pharmacotherapy, psychotherapy or the combination may be chosen depend on several factors i.e.

severity of symptoms and patient preference. If the antidepressants have been used for initial treatment, the careful monitoring for drug response and side effects are necessary. In case that there is no clinical response after 4-8 weeks of first medication, evaluation for factors that may be cause of non-response as well as adjusting the treatment should be considered.

2. A continuation phase, which remission is preserved. This phase usually takes 16-20 months. The patient should be maintained with antidepressants to prevent relapse.

3. A maintenance phase, this phase takes one or more years to prevent patients against the recurrence of major depressive episodes. Continued medication in this phase may or may not include if patients have single episode of MDD, however, in case of the recurrent MDD patients, maintenance on an antidepressant for years are recommended.

2.3 Antidepressants for treatment of depressive disorder

At the first time, antidepressants including tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) have been approved for the treatment of depression. However, they are no longer used because of their multiple side effects that many patients find intolerable (for review see Gartlehner et al., 2007). Due to drawbacks of these drugs, the second generation of antidepressants with better side effect profile (Williams et al., 2000) were introduced. There are several types of drug in this generation which can be classified by mechanism of action or by chemical structure, however, most of them are in class of selective serotonin reuptake inhibitors (SSRIs) or serotonin norepinephrine reuptake inhibitors (SNRIs) (Liang and Richelson, 2008). These antidepressants act by inhibiting reuptake of serotonin and/or norepinephrine at the presynaptic neuron membrane leading to increasing level of this neurotransmitter and consequently enhance the transmission of neuron signals at the synapse. In general, the clinical responses and remission rates of first- and second-generation antidepressant medications is similar (Liang and Richelson, 2008), however, since SSRI and SNRIs act more specifically, these drugs therefore provide fewer side effects when compare to TCAs and MAOIs (Richelson, 2003).

SSRIs are predominantly prescribed in the treatment of depression, due in part to their limited side-effects particular in adults. They include fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram, and escitalopram. Although these drugs are much more similar, each has a distinct side-effect profile including a predilection to cause gastrointestinal side effects and either daytime sedation or stimulation (for review see Olivier et al., 2010). Furthermore, SSRI are also prescribed to children and adolescents in the treatment of depression and other neurodevelopmental disorders (for reviews see Williams et al., 2010). Potential hazards of SSRIs in the pediatric population involve increases in suicidal ideation and behavior. These adverse outcomes are not observed during adult SSRI treatment, suggesting that SSRIs exert age-dependent effects, with negative outcomes during early life. Amongst, fluoxetine (Prozac) is the preferred SSRI because it is the only SSRI that is registered for treatment of the pediatric population (Bhatia and Bhatia, 2007).

Although large number of antidepressant have been used widely to cure patients who suffered from depression. It had been found that response rate of patient with an initial antidepressant medication are approximately 55% and only 30% of those treated for MDD had achieved remission (Trivedi et al., 2006). Moreover, some residual symptoms are found in patients who achieve remission (Nierenberg et al., 1999). There are two treatment strategies which can be used for the patients who showed inadequate response to the first medication. They include (1) optimization of dosage and duration of antidepressant treatment and (2) switching to another antidepressants (for review see Ministry of Health Malaysia, 2007). Moreover, strategies including drug optimization, augmentation, combination as well as switching were used to treat patient who did not respond to several antidepressant trials (also called difficult to treat patients) (for review see Fleck and Horwath, 2005).

3. Biomarker(s) and proteome of human plasma

In medicine, a biomarker is normally referred to a protein measured in blood (serum or plasma) whose concentration reflects the severity or presence of some disease state, or is anything that can be used as an indicator of a particular disease state or some other physiological state of an organism. They can be classified based

on characteristics to imaging biomarkers and non-imaging biomarkers (or molecular biomarkers). Molecular biomarkers are the biomarkers that have biophysical properties, which allow their measurements in biological samples such as plasma, serum, and cerebrospinal fluid. These include nucleic acids-based biomarkers (such as gene mutations or polymorphisms and quantitative gene expression analysis), peptides, proteins, lipids metabolites, and other small molecules. The biomarker(s) associated with mental disorders have been identified so far involve structural and functional neuroimaging, genomics, neurophysiologic studies, and plasma analysis. For examples, apolipoprotein E (ApoE) gene is found to be a major risk factor for dementia. The presence of both depressive symptoms and APOE-4 allele increased several fold the risk for dementia across subtypes (Irie et al., 2008). The association of the APOE-4 allele with specific genetic variants of other genes such as CYP2D6 negatively modulates the therapeutic response to multi-factorial treatments affecting cognition, mood, and behavior. Besides, a polymorphism in the brain-derived neurotrophic factor (BDNF) gene has been reported in some studies to relate with depression (Sklar et al., 2002; Sen et al., 2003). The finding that single bilateral infusion of BDNF into the brains of animals has been shown to produce an anti-depressant effect in two animal models of depression supported those studies (Siuciak et al., 1997; Shirayama et al., 2002). Moreover, genetic variants in the promoter region of the serotonin transporter gene (SLC6A4) have also focus to be involved in the vulnerability to depression (for review see Kemp et al., 2008).

The proteomic approach is a powerful tool that can provide protein expression profiles, which useful to predict clinical events, therapeutic response, or to probe underlying mechanisms of several diseases such as autoimmune disorders, cardiovascular diseases, and cancers. The urgent need for the proteomic based discovery of novel disease markers and/or evaluating factors associated with specific diseases together with the development of high-throughput techniques that provide highly sensitive analyses of the protein content in cells, tissues, and organisms, as well as of different body fluids, such as plasma and urine, has opened a completely new chapter in biomarker discovery. Biomedical proteome research aimed at biomarker discovery is mainly based on expression proteomics, which analyzes the quantity of certain proteins in different conditions, therefore, the proteomic studies

that aid characterization of proteins and selection of optimal proteomics technologies are key factors in propelling the discovery of novel biomarkers (Bahk et al., 2010). Large numbers of candidate biomarkers are typically identified and have been reported (for review see Kingsmore, 2006). These candidates must be further evaluated to identify those markers that have a statistically significant association with disease (for reviews see Rifai et al., 2006). Once it is identified, the biomarker must be undergo verification/validation phase, and concentrations of candidate proteins must be measured across clinical cohorts (Rifai and Gerszten, 2006).

In plasma, the most abundant protein is albumin which presents at about 40 mg/ml. Besides, other 22 highly abundant proteins exist, including immunoglobulins (IgGs), transferring and fibrinogen. These highly abundant proteins comprise about 98% of the protein content of serum (Anderson and Anderson, 2002). Conversely, it is estimated that over 10,000 different proteins are present in plasma and most of them exist in very low quantities (Adkins et al., 2002). In addition, proteins in serum exhibit large dynamic range concentrations, somewhere between 10 and 15 orders of magnitude. Both the great number of proteins and the extended range of their concentrations result in a great challenge for the identification of potential biomarkers. Human plasma is easily collected and it contains large amounts of proteins including many proteins originating from vascularized tissues. Plasma or serum analyses may thus provide relevant information regarding these tissues. For example, some of the proteins released by tissues could be from the cells that undergo cell death, whereas other proteins could be secreted by tumor cells. Notably, these released proteins could be characterized in terms of their presence or absence or by changes in their concentrations between healthy and diseased states. Consequently, a systematic characterization of proteins in human plasma in health and in diseased states has a considerable potential for identifying possible biomarkers for disease diagnosis, for the development of new therapeutic products and for monitoring responses to drug treatments (Richard et al., 2007). Plasma proteins are useful targets for diagnostic, prognostic, and/or therapeutic development. With proteomic tools available recently, profiling of human plasma proteome becomes more feasible in searching for disease-related protein markers (Anderson and Anderson, 2002). The measurement of proteins expression and modification may lead to the detection and

identification of particular proteins in cellular and may correspond to cellular functions often change during different disease states.

For depressive disorder diseases, no biomarkers have sufficient potential utility to be ready for clinical application at present (Leuchter et al., 2010). However, several promising biomarkers have been investigated and reported. An example was BDNF, the presence of low level of this brain-derived neurotrophic factor in MDD patients 's serum combined with the elevation level of BDNF after treating with antidepressants suggested to potential as a clinically use biomarker in future (Duman and Monteggia 2006; Sen, Duman and Sanacoa, 2008; Leuchter et al., 2010). Furthermore, depression has been associated with the activation of the immune system and acute phase response with changed level of acute phase proteins (Maes, 1995; Erdem et al., 2011). In the depressive cases, results for serum concentration of haptoglobin, one acute-phase proteins showed associate to severity of depressive symptoms (Erdem et al., 2011). Although, this finding could not absolutely conclude the use of haptoglobin as biomarker for depression due to small size of samples, however, the idea of using this protein as one of biomarker for MDD treatment in future is interesting.

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Objectives

To explore and identify protein marker(s) in plasma of Thai patients with major depressive disorder for predict the responsiveness to the treatment with antidepressant by 2-DE and mass spectrometry.

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.

CHAPTER 3

RESULTS

1. Blood preparation and concentration of human plasma

The plasma samples of MDD were kindly provided by the Rajanukul institute, Bangkok, Thailand. These include 48 cases of MDD, both before and during the treatment with fluoxetine. The whole blood of 20 normal volunteers was collected following the procedure for routine plasma blood draw with ACD as an anticoagulant. The responsiveness to the antidepressant of MDD was classified according to HAM-D score (as shown in Table 1) into fast response (FR), slow response (SR) and non-response (NR). The concentration of proteins in the plasma was determined by biuret assay using BSA as standard. In average, the concentration of proteins of the plasma used in this study was 109.31 g/L.

2. Two dimensional gel electrophoresis (2DE) and protein staining

2.1 Using IPG strip in the first dimension

One of the most common and effective methods has been used to separate proteins in the first dimension is isoelectric focusing (IEF). The separation of whole plasma by IEF in the first dimension was firstly attempted (n=8) using an immobilized pH gradient (IPG) strip pH 3-10, 7 cm long. After separation in the second dimension on SDS-PAGE, gel was stained with Coomassie blue G-250. The separation pattern of the protein spots was shown in Figure 1A. Only few spots were clearly detected on the gel. Most of them did not distribute evenly on the gel but located within the neutral pH area, slightly to the left of the gel. Upon using the pH 4-7 IPG strip, more protein spots were clearly detected (Figure 1B) and their distribution was nearly covered all the gel area. Since the separation in the first dimension by IEF using 4-7 pH IPG strip provided more information (spots) of protein in the plasma, it was selected for further analyzing all of the plasma samples targeted in this study.

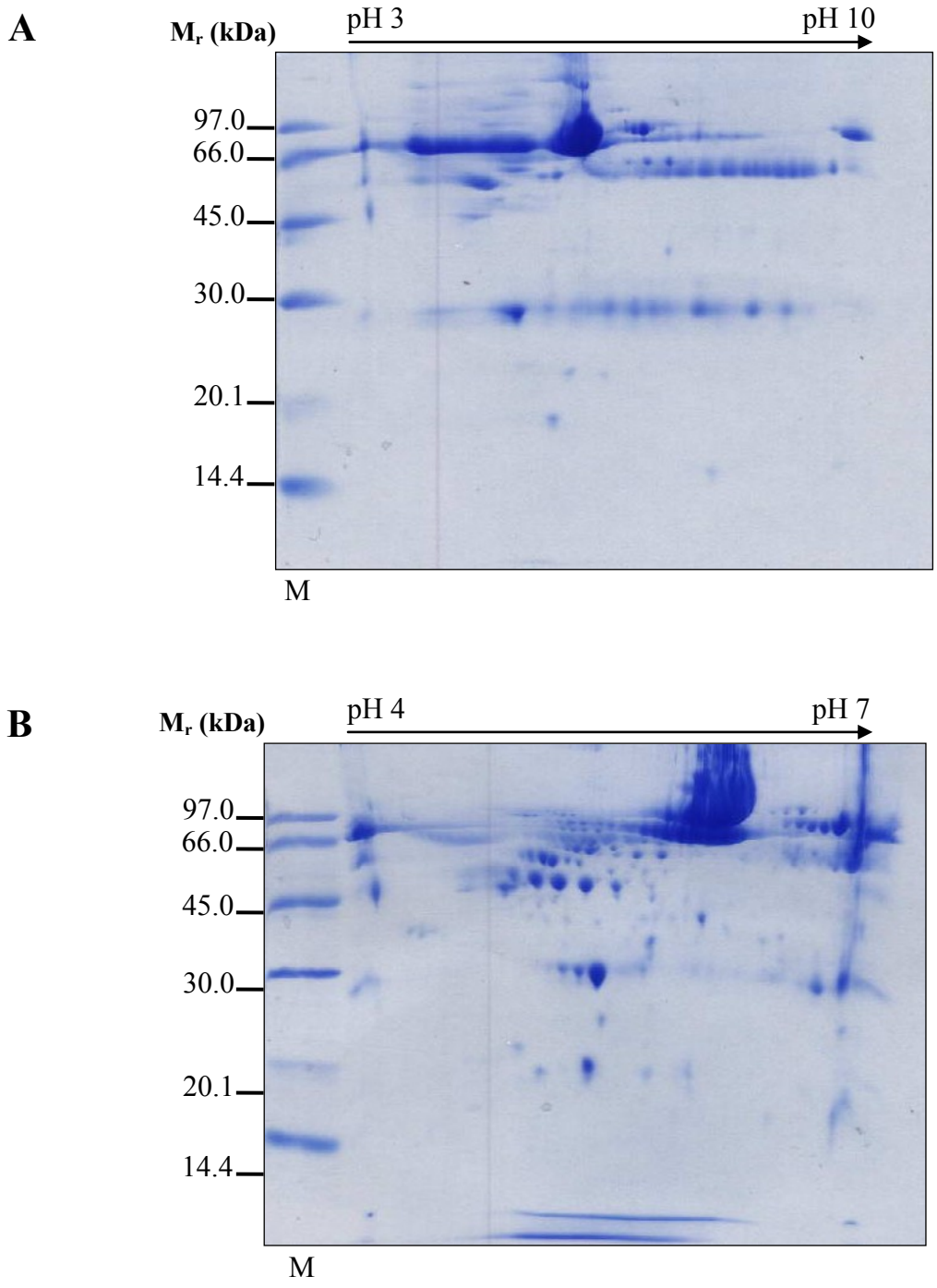


Figure 1 The plasma protein spot patterns separated in the first dimension by using immobilized pH gradient (IPG) strip pH 3-10 (A) and 4-7 (B)**.** 180 μg of plasma proteins were subjected to the analysis. The protein spots were visualized by staining with Coomassie blue G-250. M, low molecular weight protein markers.

2.2 Depletion of high abundant protein(s) in plasma

Human plasma is an important biological material for diagnosis of diseases. However, the wide dynamic range in protein concentration and the presence of high abundance proteins obscured the development of diagnostic assays for a very low concentration of biomarker proteins. To increase efficiency of spot detection of low abundant proteins, whole plasma was subjected to remove out abundant proteins in particular albumin and IgG by using the depletion SpinTrap column (GE Healthcare). In comparing, it showed that most albumin in the plasma was removed (Figure 2). In average, more than 90% of the albumin could be removed by this spin column. The 2DE gel of plasma after albumin and IgG depletion showed more and distribution of protein spots over the gel compared to the whole plasma (Figure 3). Not only had the protein spots masked by albumin shown up after the depletion but also the other low abundant proteins. In general, more than 2 folds of amount was increased for each spot of the proteins.

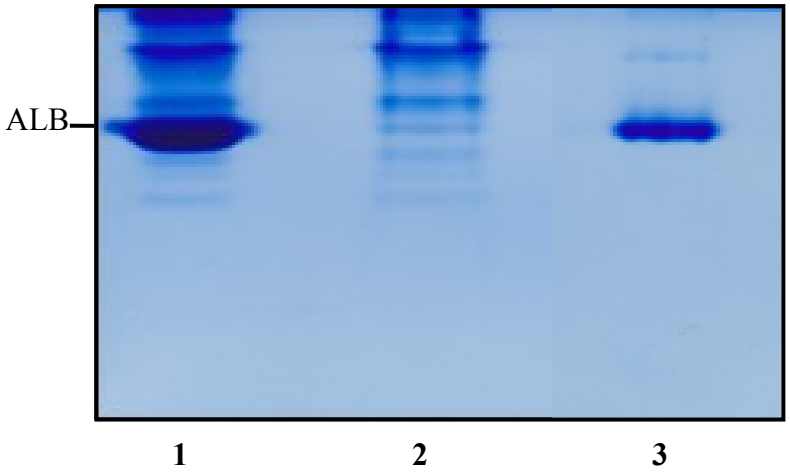


Figure 2 The native PAGE (10 % resolving gel) analysis of proteins from individual plasma samples before and after removal of proteins with Albumin and IgG Depletion Spin Trap (GE Healthcare). The proteins were detected by colloidal Coomassie brilliant blue (CBB) G-250 staining. 1, human plasma; 2, unbound fraction; 3, bound fraction which was eluted with 0.1 molar glycine-HCl pH 2.7.

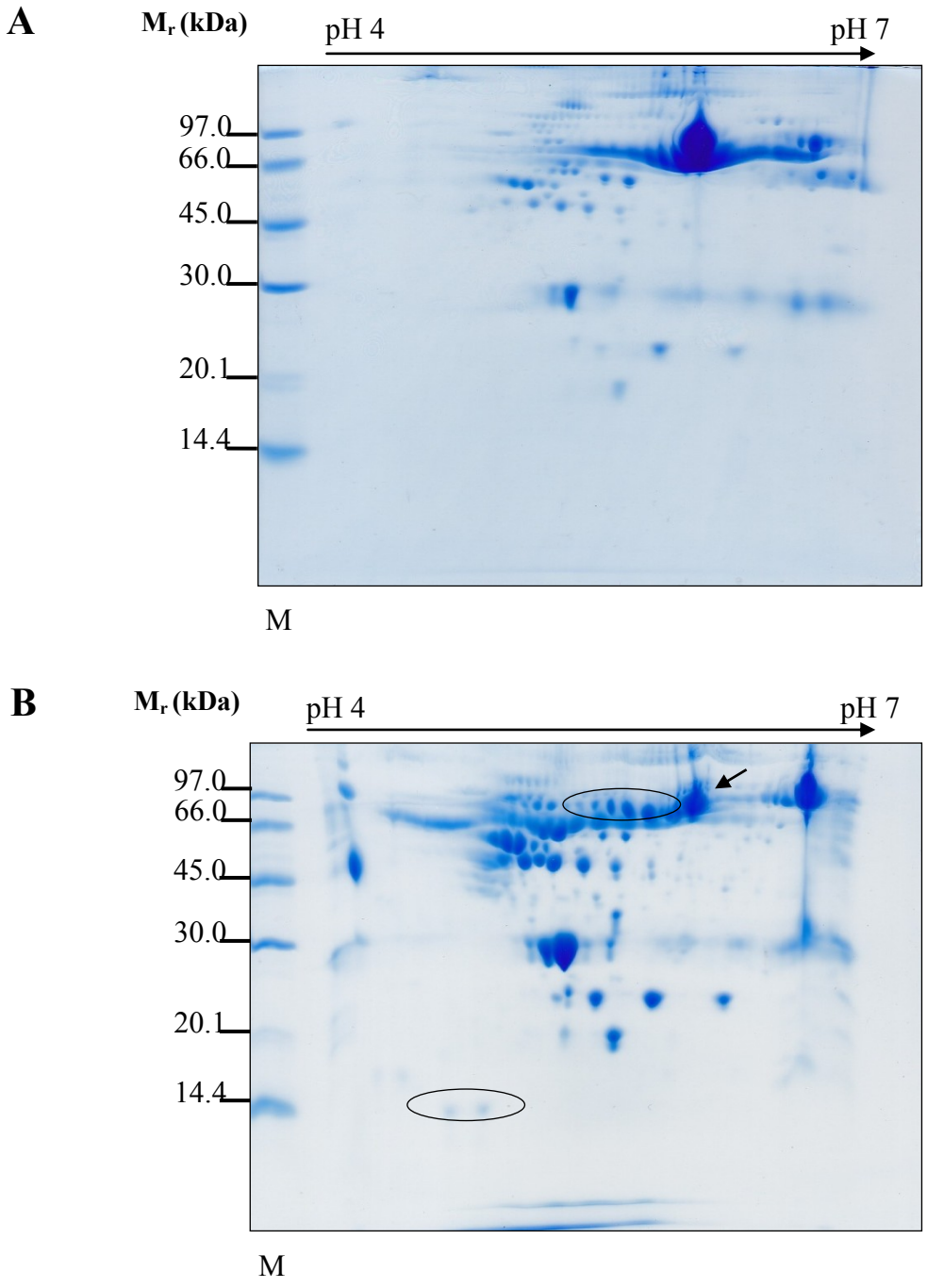
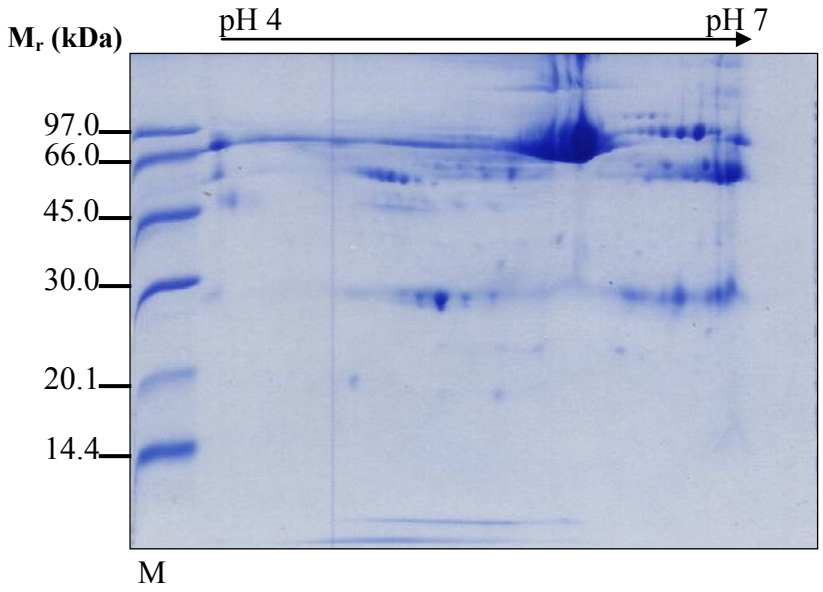


Figure 3 The protein patterns on 2DE gel of plasma before (A) and after (B) removal of major proteins with Albumin & IgG Depletion Spin Trap (GE Healthcare). Aliquots of sample (180 µg and 180 µg of total protein in plasma before and after depletion, respectively). M, low molecular weight protein markers; oval, protein spots that did not detected before the depletion; arrow, the position of albumin in the plasma.

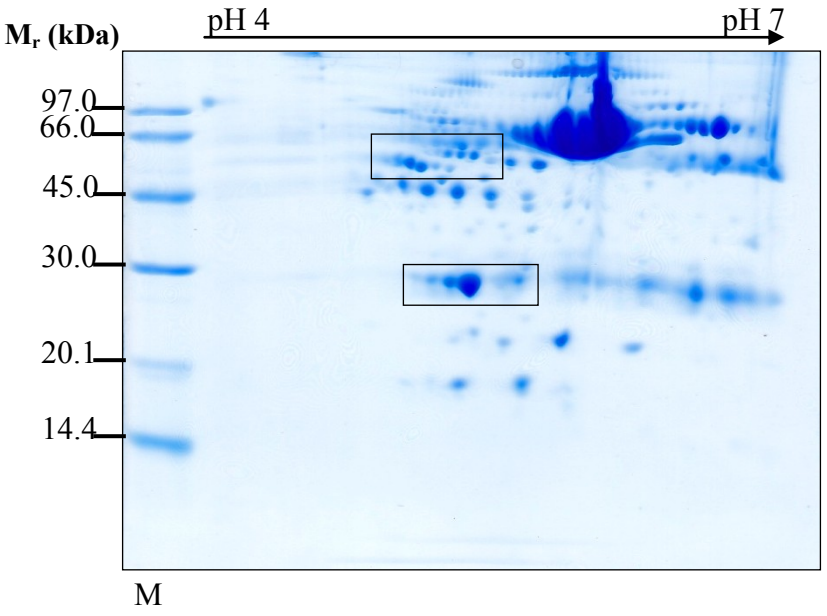
2.3 Gel staining

Obtaining the useful information from the proteomic study of plasma is principally depended on numbers of the protein spots that well separated and clearly detected on a gel. Till now, several detection methods have been developed to trace the spots of even very low amount protein. These include colloidal Coomassie blue G-250 and silver staining. In this study, 3 methods of gel staining were carried out and compared. In comparison, staining with Coomassie blue R-250 showed higher background of the gel but less spots can be observed than that staining with colloidal Coomassie blue G-250 (Figure 4A, B). The staining with silver nitrate, however, provided the highest number of spots. Approximate 20, 45, and 185 spots of the protein in 1-3 μl of plasma (equivalent to ~180 μg of proteins) could be detected after staining gel with Coomassie blue R-250, colloidal Coomassie blue G-250 and silver nitrate, respectively. However, since silver staining has a very high sensitivity, overlapping of the detectable signal occurred among adjacent spots, which resulted to decrease in resolution of an individual spot (Figure 4B, C). In this study, the staining with colloidal Coomassie blue G-250 was selected for all of gels that were further subjected to image analysis and comparison.

A


M_r (kDa)
pH 4
pH 7
97.0
66.0
45.0
30.0
20.1
14.4
M

B


M_r (kDa)
pH 4
pH 7
97.0
66.0
45.0
30.0
20.1
14.4
M

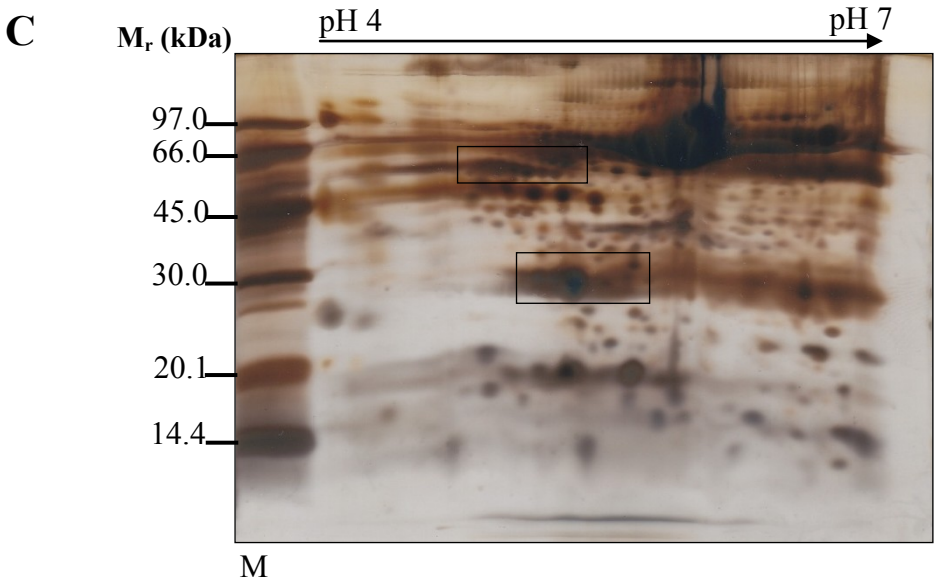


Figure 4 The patterns of protein spots on 2DE gel detected by staining with Coomassie blue R-250 (A), colloidal Coomassie brilliant blue G-250 (B) and silver nitrate (C). M is low molecular weight protein markers; rectangles, examples of spots that resolution of individual spots was decreased when stained with silver nitrate.

2.4 Image analysis: spot detection and gel matching

After staining, an excess dye was removed out by de-staining process until gel background was completely clear. Then, image and intensity of dye attached to each protein spot was recorded by scanning gel at the resolution of 300 dpi pixels. Manipulation of each gel image such as selection of spot area and contrast adjust was carried out to ensure quality and repeatability of each gel. Spot detection was performed on all of the triplicate gels of each sample using three protein spots, which detected in all of the examined gels, as landmark (Figure 5). In overall, approximate 30 to 45 spots could be detected in each gel. Gel matching was later carried out, first on the gels of the samples within the same group, then those between groups. To match gels of the samples within the same group, gel of the sample that displayed the highest spots was selected as a reference. To match gels of the samples in different groups, the reference gel of NR group was selected as a reference. According to spot detection and gel matching, the analysis program reported into four values i.e. intensity, area, volume and %volume. To compare and find out the significantly differences of spots among the sample groups, %volume (%V) was selected in the analysis. The matching of protein spots in the whole plasma of samples among groups showed approximate 20 protein spots in difference (Figure 6). The comparison of %V between the normal and MDD groups revealed the volume of 6 spots were significantly higher in MDD than in the normal, whereas the expression of 2 spots were significantly higher in the normal than in MDD and no expression of 3 spots were detected in the normal samples (Table 1). The comparison among MDD subgroups, i.e. NR, FR and SR, showed the expression of 2 spots were higher in NR than in both FR and SR. In addition, 2 same and 1 different spots were found more expressed in FR and SR than in NR. Moreover, a spot that was presence in both NR and FR but absence from SR (Table 1).

The matching of proteins in the plasma after removing out of albumin and IgG brought up to more than 30 spots that were significantly different among sample groups. Some of them were the same as those spots detected in the whole plasma (Table 1 and 2). Upon the comparison to the whole plasma, the expression of at least additional 8 spots was found higher in the depleted plasma of MDD than in the normal. In addition, the expression of additional 4 spots was higher in the normal than

in MDD, whereas the expression of 2 spots was not detected in samples of the normal (Table 2). Among NR, FR and SR groups, the expression of proteins detected in the depleted plasma of NR revealed additional 3 spots, compared to that detected in the whole plasma, had higher expressed in NR than in FR and SR. In addition, 3 and 4 additional spots were more expressed in FR and SR than in NR, respectively. Moreover, more absence spots (at least 3) were detected in SR (Table 2).

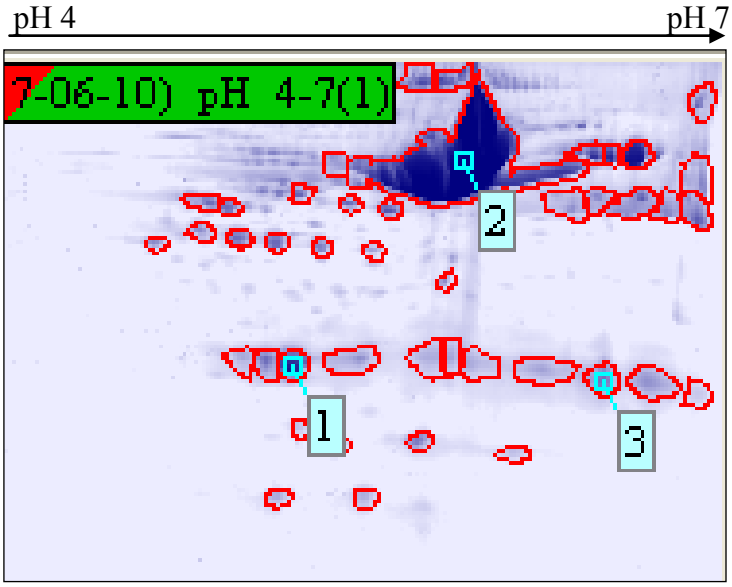
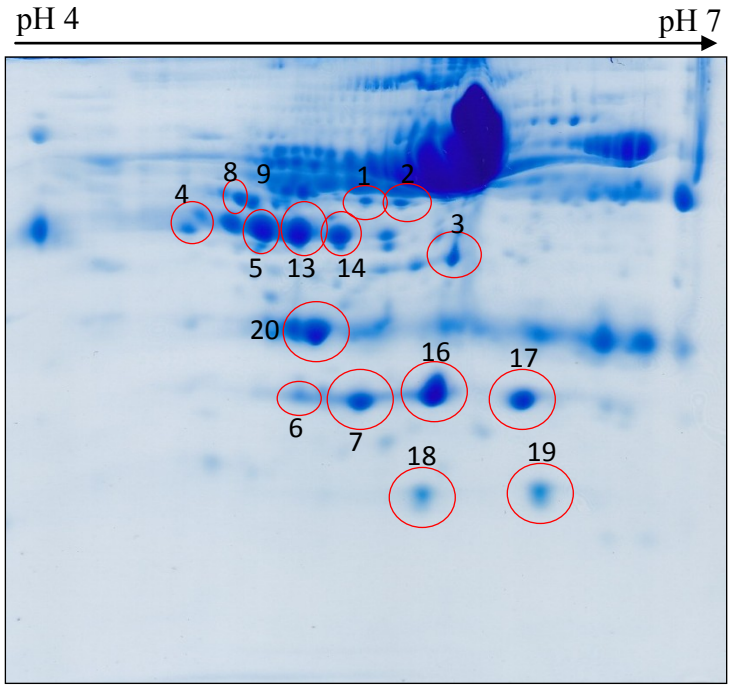
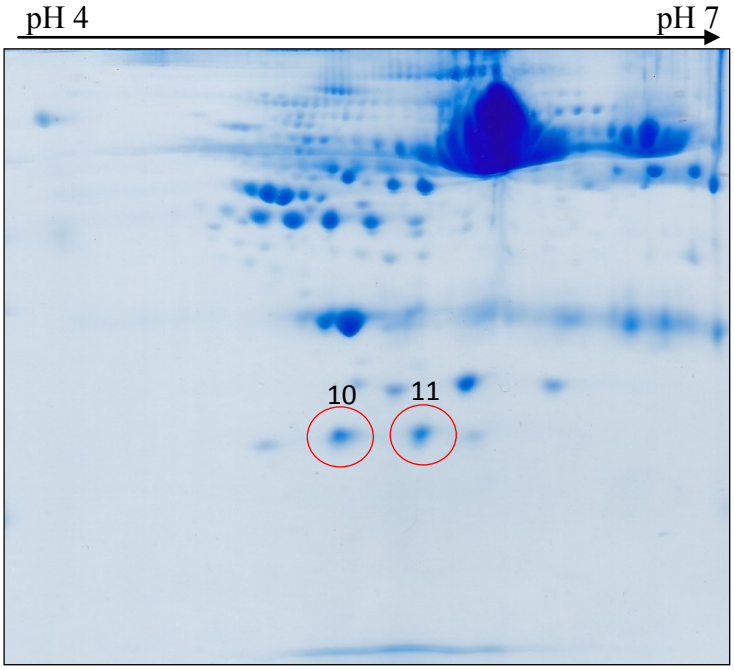


Figure 5 Positions of protein spots that used as landmarks for gel analysis and spot match by 2DE platinum software.

A



B



C

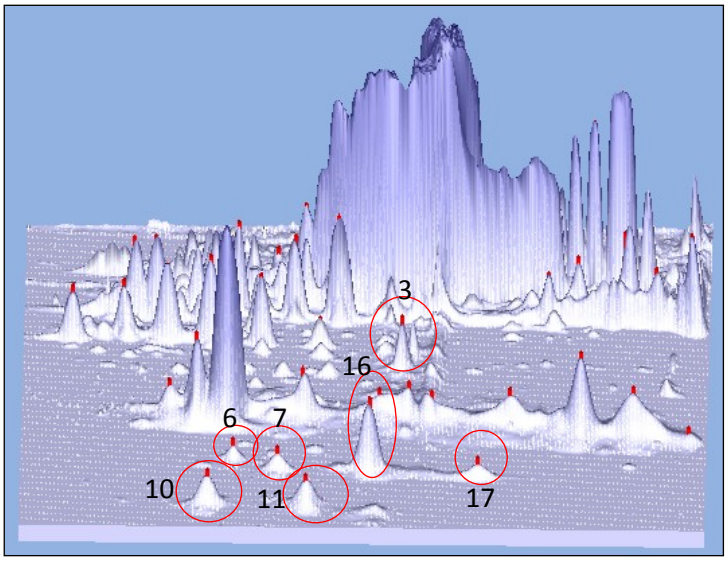


Figure 6 The 2DE protein patterns of whole plasma. Aliquot (180 µg) of plasma was separated in the first dimension on IPG strip pH 4-7, followed by in the second dimension on 12.5% (v/v) SDS-PAGE. The protein detection was performed by colloidal Coomassie blue G-250 staining. The spots that significantly differed among sample groups were circled and numbering (A, B). Three-dimensional (3D) profile which indicates volume of protein spots is shown in C.

Table 1 The comparative ratio of %volume of the protein spots detected in the whole plasma obtained from normal and different groups of MDD before treatment

Protein	Normal / MDD (folds)	MDD / Normal (folds)	NR / FR (folds)	FR / NR (folds)	NR / SR (folds)	SR / NR (folds)
α1-antitrypsin (3, 15)	0.1	**13.0**	1.0	1.0	1.4	0.7
α1-antitrypsin (8)	1.1	0.9	1.3	0.8	1.0	1.0
α1-antitrypsin (9)	1.4	0.7	**1.7**	0.6	**1.7**	0.6
Apolipoprotein AI (20)	1.0	1.0	**1.6**	0.6	**1.6**	0.6
Complement C3 (4)	0.0	∝	1.0	1.0	0.8	1.3
Fibrinogen α-chain (1)	0.6	**1.6**	1.3	0.7	1.1	0.9
Fibrinogen α-chain (2)	1.4	0.7	0.7	1.4	∝	0.0
Haptoglobin precursor (5, 12)	0.7	**1.5**	0.9	1.2	0.7	1.3
Haptoglobin precursor (13)	0.9	1.1	0.7	1.5	0.7	1.4
Haptoglobin precursor (14)	0.8	1.2	0.9	1.1	0.9	1.1
Haptoglobin hp2 (16)	1.2	0.9	0.7	1.5	0.7	1.4
Transcription factor (6)	0.6	**1.8**	0.6	**1.6**	0.6	**1.7**
Transthyretin (11)	**1.9**	0.5	0.9	1.1	1.1	0.9
Rap 1A (17)	1.2	0.8	0.5	**2.2**	0.4	**2.4**
ND	**3.8**	0.3	0.6	**1.7**	0.6	1.5
ND	0.9	1.1	1.2	0.9	1.5	0.7
ND	0.0	∝	0.9	1.2	1.1	0.9
ND	0.0	∝	1.0	1.0	0.6	**1.7**
ND	0.2	**4.3**	0.7	1.4	1.2	0.8
ND	0.2	**5.1**	0.8	1.3	1.1	0.9

The differences of %V ratio which is >1.5 folds between matched spots are in bold and highlighted in green whereas the absence of matched spot is in bold and highlighted in red. The number in blanket which placed after protein is related to the spot number in Figure 6 and Table 3. ND indicates spot that has not been determined yet by MALDI-TOF MS.

Table 2 The ratio of %volume of the protein spots detected in the albumin and IgG depleted plasma obtained from normal and different groups of MDD before treatment

Protein	Normal / MDD (folds)	MDD / Normal (folds)	NR / FR (folds)	FR / NR (folds)	NR / SR (folds)	SR / NR (folds)
Anti-chymotrypsin (31)	**3.2**	0.3	**5.6**	0.2	1.3	0.8
α1-antitrypsin (8)	0.6	**1.7**	0.6	**1.7**	1.5	0.7
α1-antitrypsin (9)	0.6	**1.6**	1.4	0.7	**2.3**	0.4
Apolipoprotein AI (20)	1.0	1.0	1.0	1.0	1.2	0.8
Apolipoprotein A I (61)	0.2	**6.2**	1.2	0.8	1.1	0.9
Apolipoprotein C III (81)	**4.6**	0.2	**7.3**	0.1	∝	0.0
Apolipoprotein A II (83)	0.4	**2.7**	**2.6**	0.4	∝	0.0
Apolipoprotein E (55)	0.1	**14.8**	0.8	1.2	0.2	**4.3**
Complement C3 (4)	0.7	1.4	**1.8**	0.6	**2.5**	0.4
Fibrinogen α-chain (24)	0.1	**7.1**	1.2	0.8	0.4	**2.4**
Haptoglobin α-2 (7)	0.9	1.1	0.9	1.1	0.7	1.5
Haptoglobin 1S (10)	1.0	1.0	0.7	1.4	**7.7**	0.1
Haptoglobin hp2 (16)	0.9	1.1	0.7	1.5	0.5	**1.9**
Haptoglobin precursor (5, 12)	1.1	0.9	1.2	0.9	**2.9**	0.3
Haptoglobin precursor (13)	0.8	1.2	0.6	**1.6**	1.1	0.9
Haptoglobin precursor (14)	0.9	1.1	0.7	1.5	0.7	1.4
HDL associated protein (47)	0.4	**2.6**	**3.0**	0.3	1.4	0.7
HDL associated protein (48)	0.0	∝	1.5	0.7	∝	0.0
IgG light chain (62)	0.7	1.5	**1.6**	0.6	0.8	1.2
IgG light chain (63)	0.9	1.1	**1.7**	0.6	1.3	0.7
IgG light chain (64)	0.4	**2.3**	**1.8**	0.5	1.3	0.8
IgG light chain (67)	0.9	1.2	**2.0**	0.5	**2.0**	0.5
IgG light chain (68)	0.8	1.3	**2.6**	0.4	**2.7**	0.4
IgG light chain (69)	0.0	∝	**3.1**	0.3	1.1	0.9
Pro apolipoprotein AI (66)	**1.6**	0.6	1.1	0.9	**1.8**	0.6
Rap 1A (17)	0.8	1.2	0.6	**1.8**	0.4	**2.4**
Transferrin (12)	0.6	1.5	1.5	0.7	0.5	**2.0**
Transcription factor (6)	0.9	1.1	0.9	1.1	1.1	0.9
Transthyretin (11)	**1.7**	0.6	0.6	**1.6**	1.4	0.7

The %V ratio which is >1.5 folds between matched spots are in bold and highlighted in green whereas the absence of matched spot is in bold and highlighted in red. The number 1-20 in blanket which placed after protein is related to the spot number in Figure 6 and Table 3. The protein which numbered upward from 20 was identified by spot alignment with the available two-dimensional map of human plasma (Natale et al., 2011).

2.5 Protein identification by Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

To identify the proteins which showed significantly differences and had potential to be used as biomarkers, 20 spots of the proteins were cut and subjected to in-gel digestion and analysis by MALDI-TOF MS as described in the section of Materials and Methods. The identification was based on NCBI non-redundant (NCBInr), MSDB and SwissProt database entries with the Matrix Science (Mascot MS/MS Ions Search) search engine. Lists of name of proteins/peptides, mascot score, matching peptide, amino acid sequence coverage (expressed in %), isoelectric point (pI) and molecular weight (MW) were shown in Table 3.

Table 3 List of the protein spots identified in this study by MALDI-TOF MS

Spot No.	Protein name	Mascot score	Matched peptide	Sequence coverage (%)	pI	MW (kDa)
1	Fibrinogens α-chain	191	5	19	5.61	63.09
2	Fibrinogens α-chain	187	3	8	5.61	63.09
3	α 1-antitrypsin	440	17	30	5.51	50.12
4	Complement C3	103	2	1	6.02	54.95
5	Haptoglobin precursor	494	14	25	6.24	54.95
6	Transcription factor	33	2	1	5.07	23.98
7	Haptoglobin α-2	57	3	28	6.46	23.44
8	α 1-antitrypsin	585	18	32	5.43	60.25
9	α 1-antitrypsin	611	19	33	5.43	60.25
10	Haptoglobin 1S	62	1	15	6.08	16.59
11	Transthyretin	210	3	37	5.35	21.87
12	Haptoglobin precursor	518	14	25	6.24	54.95
13	Haptoglobin precursor	437	12	23	6.24	54.95
14	Haptoglobin precursor	558	17	24	6.24	54.95
15	α 1-antitrypsin	152	5	13	5.35	50.12
16	Haptoglobin hp2	306	5	18	6.23	23.98
17	Rap 1A	24	1	9	6..38	24.54
18	Serum amyloid A1	207	4	48	5.27	16.21
19	Serum amyloid A1	118	3	47	5.27	16.21
20	Apolopoprotein A1	691	17	46	5.27	31.62

CHAPTER 4

DISCUSSION

Biomarker(s) and proteome profile of human plasma

2DE has been proved to be an extremely promising method of protein analysis in proteomics (for reviews see Görg et al., 2004; López, 2006). Together with mass spectrophotometry (MS), several proteins/peptides that are related to metabolic systems and diseases including depressive disorder have been identified (for reviews see Steel et al., 2005; Zeugmann et al., 2010; Schiess et al., 2009; Brewis and Brennan, 2010). Many evidences suggested that peripheral fluids such as plasma are rich source of proteins marker (Adkins et al., 2002; for reviews see Anderson and Anderson, 2002) which some of them display complex combinations of post-translational modification, particularly involving glycosylation, that can be discriminated by proteomics techniques in particular 2DE (Gravel et al., 1994, 1996; for review sees Anderson and Anderson, 2002) Some of these proteins reflect a systemic metabolic signature or the changes in plasma secondary indicate to a disease-specific process in the brain, thus the disease stages and disease development may be defined and/or predicted (for review sees Schneider and Prvulovic, 2013). In this study, the proteins in plasma those are high potential to be used as markers for early prediction of the responsiveness to antidepressant (fluoxetine) of MDD were explored by 2DE and these proteins were identified by mass spectrophotometry.

The analysis between the plasma from the normal and MDD groups in this study revealed that the expression of several proteins including α1-antitrypsin, fibrinogen, haptoglobin, transcritption factor, apoliporpotein AI (apo AI), apolipoprotein AII (apo AII), apolipoprotein E (apo E), HDL associated protein and IgG light chain was high in MDD compared with the normal. All of these proteins, except apolipoproteins, function or are related to acute phase and inflammatory responsive systems (for reviews see Janciauskiene et al., 2011; Davalos and Akassoglou, 2012; Quaye, 2008; Salim et al., 2012; Zhu and Parks, 2012), and the increase of these protein levels in MDD well agree with that previous reported (Joyce et al., 1992; Zeugmann et al., 2010; Erdem et al., 2011; Papakostas et al, 2011).

Recently, the examination of the relationship between apo E ε4 (ApoE ε4) and depression in adults and elders showed high association of the symptom with the ApoE ε4 allele (Arnold et al., 2012; Michels et al., 2012). Together with the finding of higher expression of apo E in plasma of MDD than in the normal, it conceived that this apolipoprotein can be a biomarker for MDD, and may be used to classify between MDD and the normal.

Several controversial results have been reported for the relationship between apolipoprotein in particular apo A and depression. Either detection the protein in the serum of depressed patients at lower level of than in control or no significant difference between these two people groups were reported (Severus et al., 2001; Kopf et al., 2004; Sarandol et al., 2006). The severity of depression was also shown correlated with the decrement in serum apo A level (Sadeghi et al., 2011). The result obtained in this study, i.e. the levels of both apo AI and apo AII were higher in MDD than in the normal, therefore, was inversed to the previous reports, and may need to be validated such as by Western blot. However, since the correlation between the high expression of apo A with some diseases such as coronary heart disease has been evidenced (Yang et al., 2012). In addition, the recent searching on markers in plasma for predicting the development of Alzheimer disease (AD) showed that apo AII was one of the significant signatures that can differentiate AD from normal groups (Llano et al., 2012). It suggested that apo A may be a predictor for depression and its increase would be a risk factor for coronary heart disease particular in MDD (Sadeghi et al., 2011).

The absence or very low expression of complement C3 (C3) in the normal groups which led to extremely high expression level of the protein in MDD was observed in this study. The elevation of C3 but not C4 was reported associated with hostility, anger and depression (Boyle et al., 2007). However, it also was reported that no significant differences between the mean levels of C3 could be detected between depressed patients and controls, whereas the levels of C4 and C-reactive protein were significantly raised in the group with a depressive disorder (Berk et al., 1997). The previous controversial findings and the result observed in this study are still needed to be proved. Several observations showed inflammatory mechanisms as one of the causes of MDD (for reviews see Dean, 2011; Muller et al., 2011), and several proteins

in the system have been identified as markers for MDD. In addition, since C3 is one of the mediator in the inflammatory cascade and play important role particular in the alternative pathway of complement activation which provides host defense system and immune regulatory mechanisms (for reviews see Huang et al., 2008; Dinarello, 2010). High elevation of the level of C3 in plasma of MDD, thus, could confirm the association of the inflammatory and immune systems with depression.

To explore for a possible biomarkers in plasma for prediction of the responsiveness to antidepressant, 2DE spots from the plasma of NR, FR and SR were matched and analyzed. It showed the expression of α1-antitrypsin, apo AI, C3, haptoglobin and IgG light chain detected in the plasma of NR was higher than those of FR and SR. This indicated the association of proteins in the inflammatory and immune systems not only with the symptom of depression but also with the response to antidepressant. It seems likely that there was an inverse relationship between these body protective systems and the antidepressant effects. The expressions of transcription factor and Rap 1A, in contrast, were low in NR compared to FR and SR. Serotonin (5-HT) 5-HT1A autoreceptors (5-HT1AautoR) is one of key elements in the regulation of central 5-HT function and the responsiveness to antidepressant drugs in particular SSRIs (for review sees Descarries and Riad, 2012). Negatively regulation of expression of the receptor was shown be a possible clinical approach to improve the treatment of depression with SSRIs (Bortolozzi et al., 2011; for review sees Albert, 2012). It is possible that the transcription factor detected and had high expression in NR observed in this study may play role and associate to the low response to antidepressant of NR. To reveal whether this protein suppressive regulates 5-HT1AautoR or not, further identification and validation of the protein are necessary.

Synaptic plasticity confers adaptability and strength in response to changes of environment through modifications of the connectivity between neuronal cells and circuits. Deficits in synaptic plasticity were shown correlate to stress and depression (for review sees Marsden, 2012). Neurons, glia and many signaling systems including cAMP-protein kinase A complex (cAMP-PKA) signaling (for review sees Waltereit and Weller, 2003) participate in the synaptic networking. Dysfunctions in PKA and its substrates such as Rap1, small guanosine triphosphate (GTP)-binding protein

belonging to the ras family, are associated with depressive disorders (Perez et al., 2000; Odagaki et al., 2001; Perez et al., 2001; for reviews see Perez et al., 2000; Perez and Tardito, 2001). It reported that abnormalities in some components of cAMP signaling in patients were likely related to treatment rather than symptom of the disease (Lowther and Katona, 1997; Dowlatshahi et al., 1998). In addition, the knock-out (KO) of the rap1a gene showed impairment of synaptic plasticity (Pan et al., 2008). In this study, the expression level of Rap 1A was lower in MDD than in the normal group, in addition, lower in NR than in FR and SR. These findings should support the association of the cAMP-PKA signaling with depression and its relationship with the responsiveness to the treatment with antidepressant. Moreover, it implied to a possible use Rap 1A as one of biomarkers for the prediction of the treatment with antidepressant in particular fluoxetine in advanced.

CHAPTER 5

CONCLUSION

In this thesis, plasma samples from MDD (n = 48) and the normal (n = 20) groups were analyzed by 2DE, and followed by MALDI-TOF MS. The proteins in plasma, either before or after depletion of albumin and IgG, were successfully separated into spots by IEF and SDS-PAGE in the first and second dimensions, respectively. In comparison, the 2DE gel staining with colloidal Coomassie blue G-250 was the most informative, and this staining process was selected for the entire gels carried out in this thesis. According to %V comparison of the matched spots, the expression levels of α1-antitrypsin, fibrinogen, haptoglobin, transcritption factor, apo AI, apo AII, apo E, HDL associated protein, IgG light chain and C3 were significantly higher in MDD than in the normal. Therefore, these proteins are possible biomarker candidates in plasma for differentiating MDD from the normal, though the controversy of high expressions of apo AI, apo AII and C3 in MDD is needed to be validated. In comparison among NR, FR and SR, the expressions of α1-antitrypsin, apo AI, C3, haptoglobin and IgG light chain were significantly highest in NR, whereas, that of transcription factor and Rap 1A were significantly lowest in NR. These proteins in particular Rap 1A are biomarker candidates in plasma for early prediction of the responsiveness to fluoxetine and, may be, other SSIRs.

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VITAE

Name **Mr. Surasak Supuntee**

Student ID **5310220078**

Education Attainment

Degree	Name of Instutation	Year of Graduation
B.Sc.(Aquatic Science)	Prince of Songkla University	2009

Scholarship Awards during Enrolment

A scholarship awards from the Graduated Research Fund, Prince of Songkla University

A scholarship awards from the Graduated Research Fund, National Research Council of Thailand (NRCT).

University Academic Excellence Strengthening Program in Biochemistry, Prince of Songkla University

List of Publication and Proceeding

Supuntee, S., Leelawatwattana, L., Prapunpoj, V and Prapunpoj, P. 2012. Dynamic protein expression profiles in Thai major depressive disorder with different responses to Fluoxetine. Proceeding of the 4th Northeast Pharmacy Research Conference 2012 "Pharmacy Profession in Harmony. *Isan Journal of Pharmaceutical Sciences*. Konkean University, Vol 8(1) : ISSN : 1905-0852., January – April 2012. pp. 47-54.

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