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.

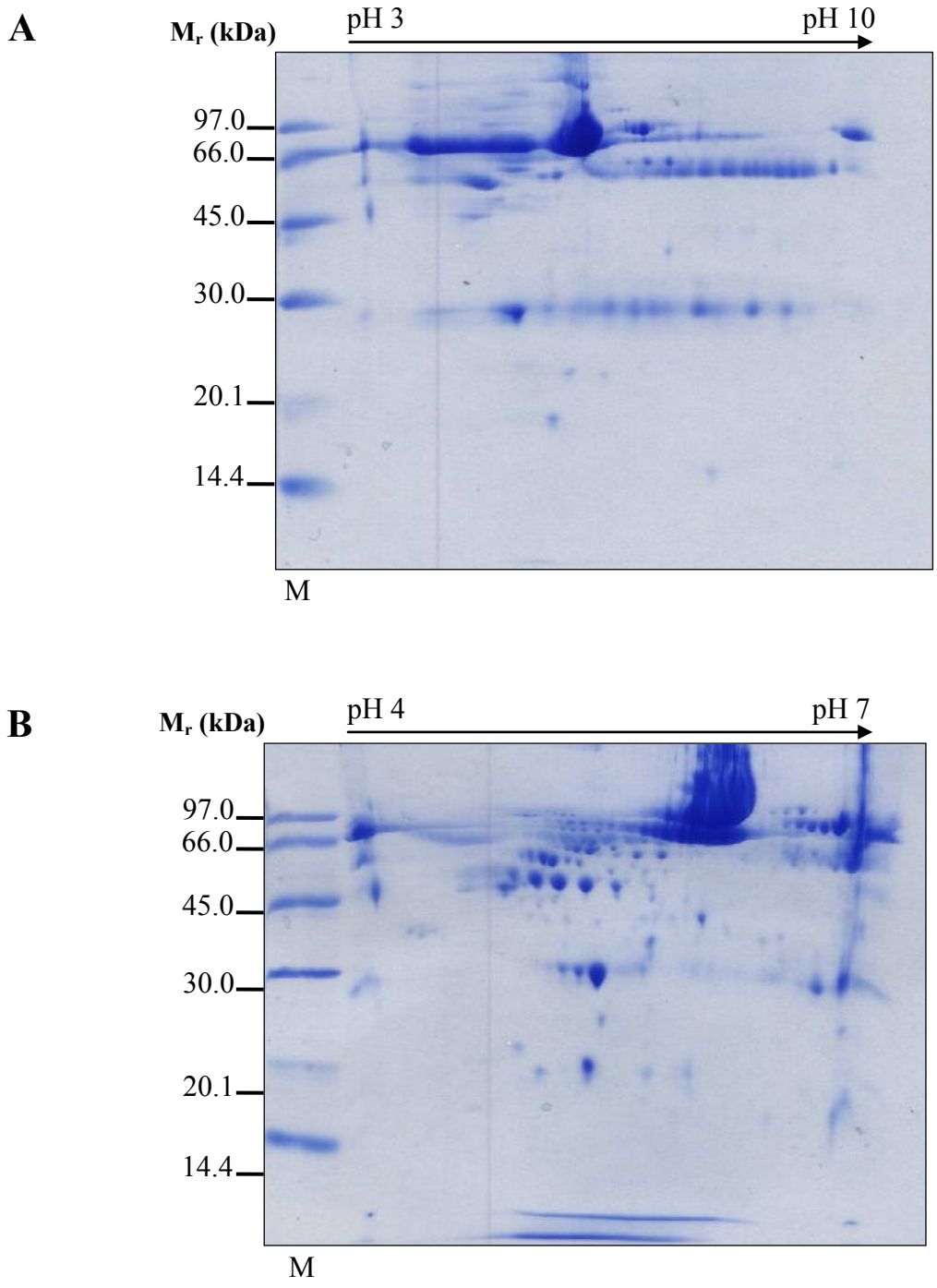


Figure 1 The plasma protein spot patterns separated in the first dimension by using immobilized pH gradient (IPG) strip pH 3-10 (A) and 4-7 (B)**.** 180 μg of plasma proteins were subjected to the analysis. The protein spots were visualized by staining with Coomassie blue G-250. M, low molecular weight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

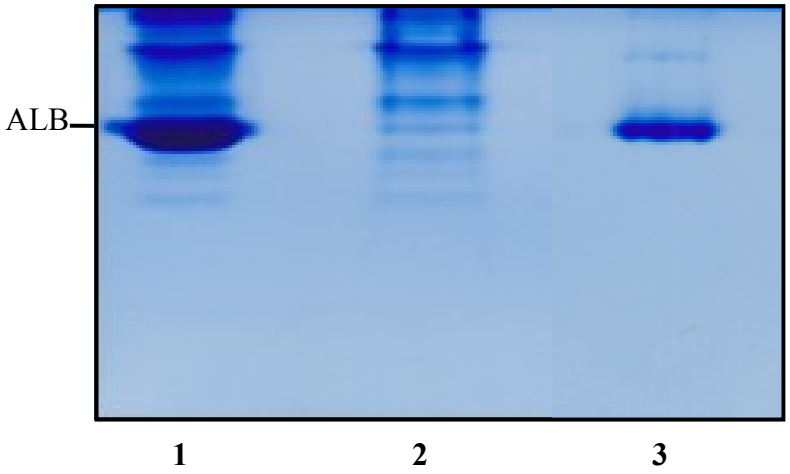


Figure 2 The native PAGE (10 % resolving gel) analysis of proteins from individual plasma samples before and after removal of proteins with Albumin and IgG Depletion Spin Trap (GE Healthcare). The proteins were detected by colloidal Coomassie brilliant blue (CBB) G-250 staining. 1, human plasma; 2, unbound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

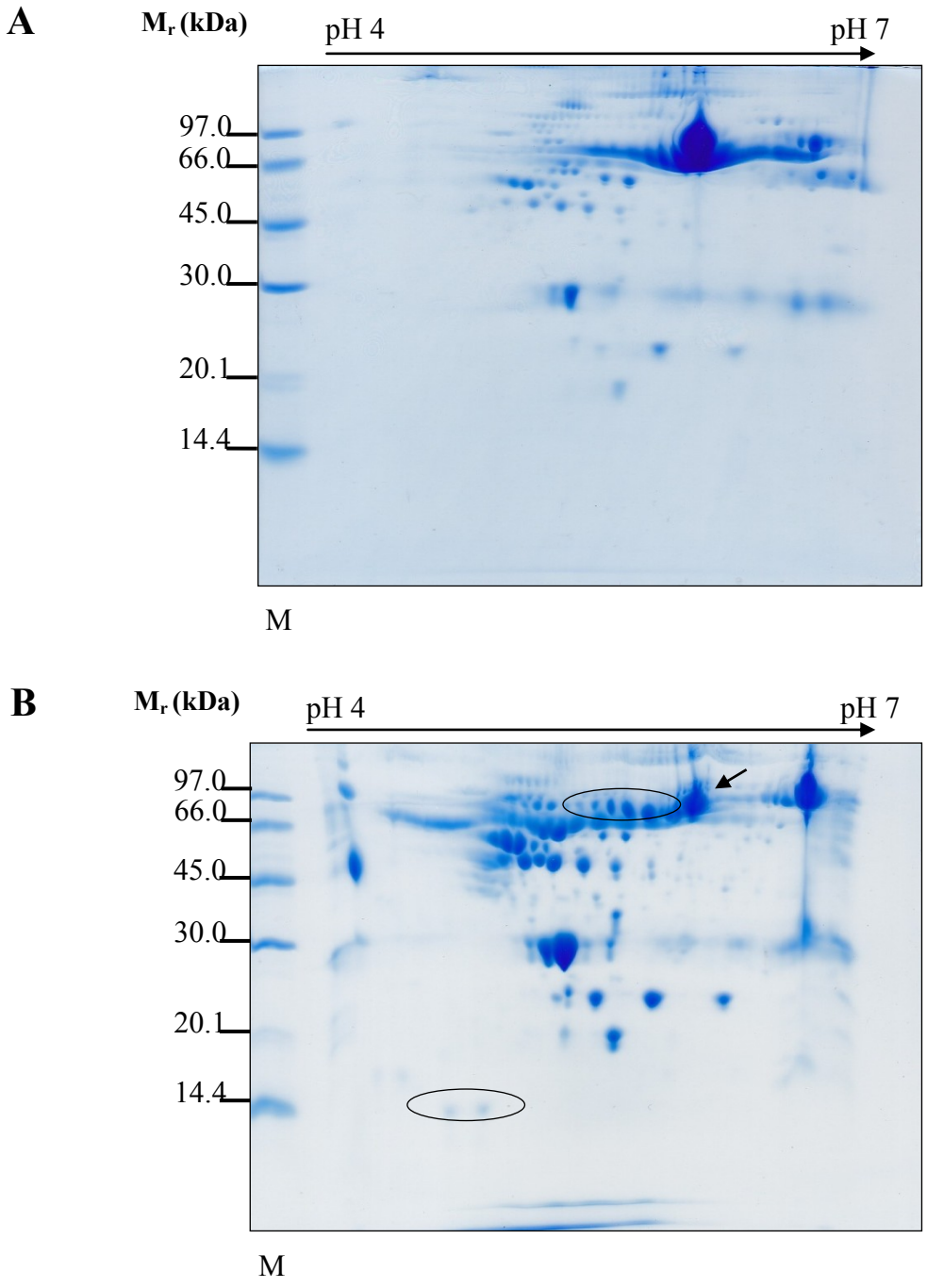
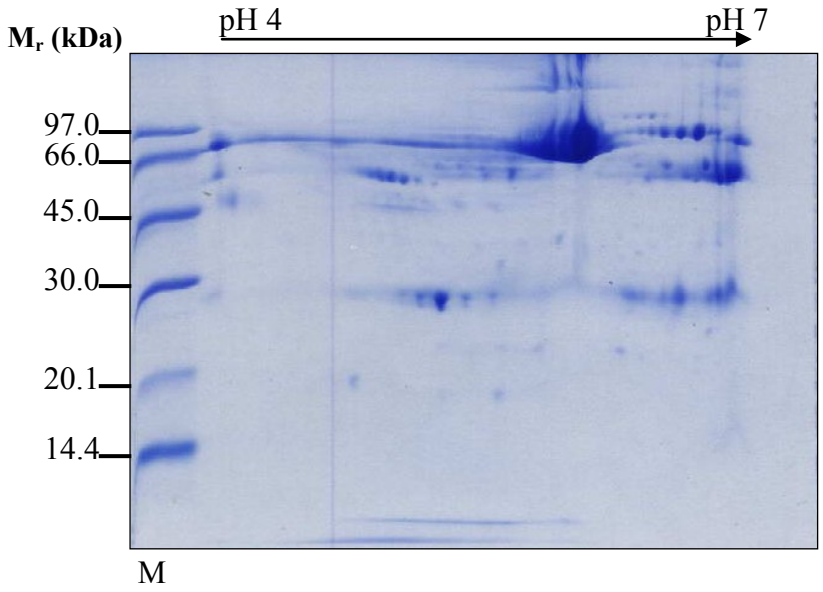


Figure 3 The protein patterns on 2DE gel of plasma before (A) and after (B) removal of major proteins with Albumin & IgG Depletion Spin Trap (GE Healthcare). Aliquots of sample (180 µg and 180 µg of total protein in plasma before and after depletion, respectively). M, low molecular weight protein markers; 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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pH 7
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66.0
45.0
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20.1
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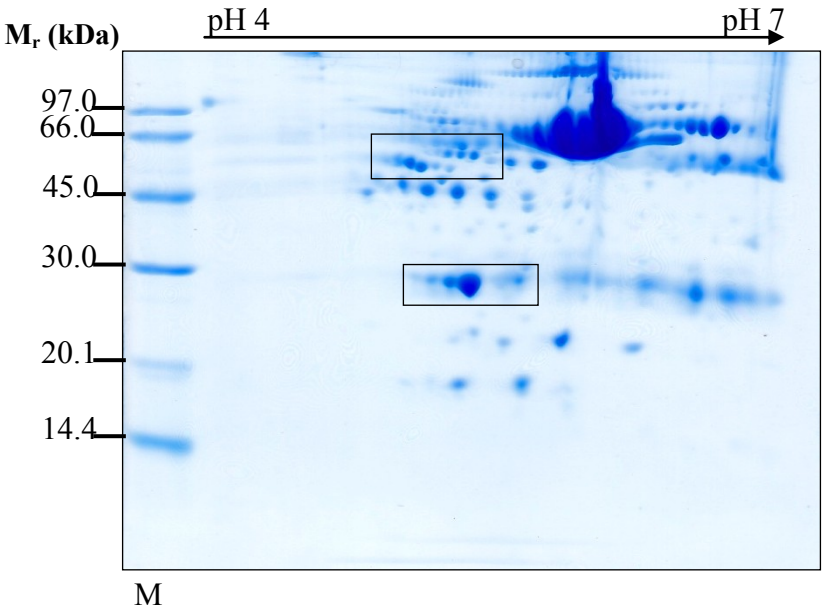
M

B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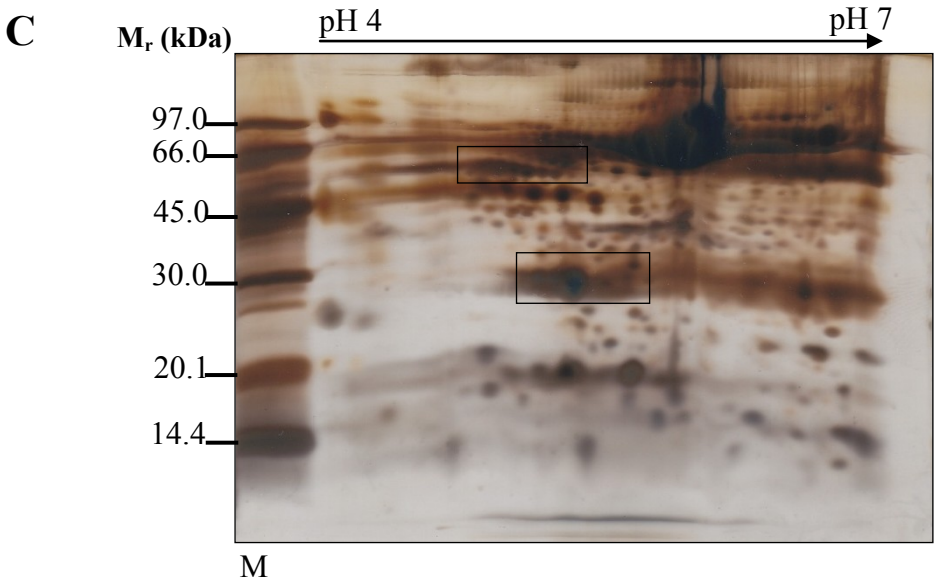


Figure 4 The patterns of protein spots on 2DE gel detected by staining with Coomassie blue R-250 (A), colloidal Coomassie brilliant blue G-250 (B) and silver nitrate (C). M is low molecular weight protein markers; rectangles, examples of spots that resolution of individual spots was decreased when stained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

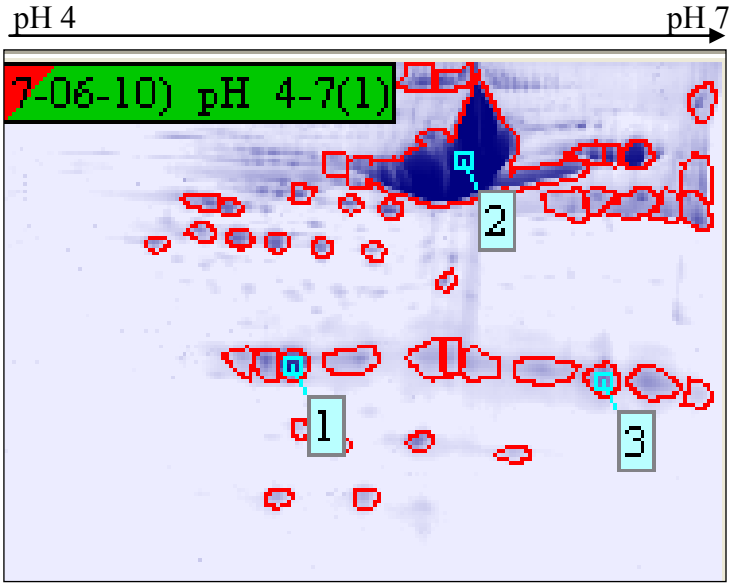
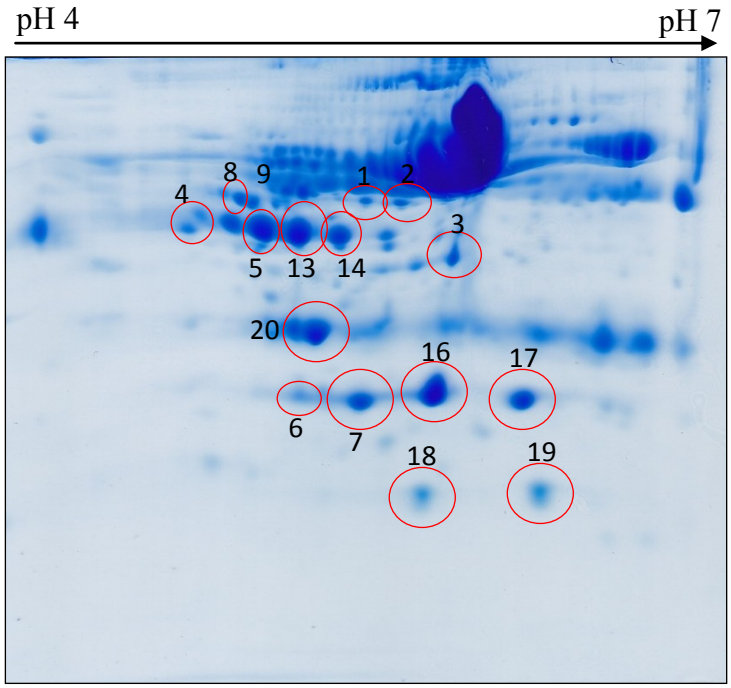
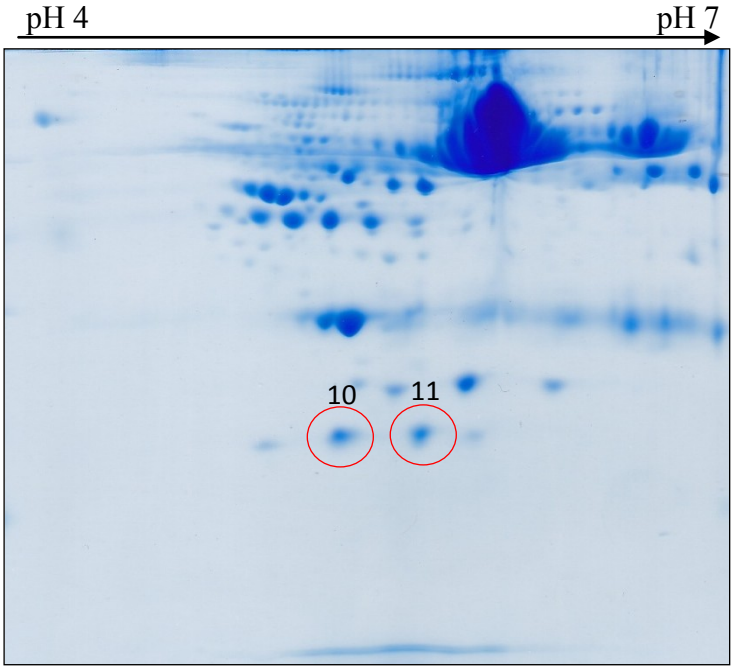


Figure 5 Positions of protein spots that used as landmarks for gel analysis and spot match by 2DE platinum software.

A



B



C

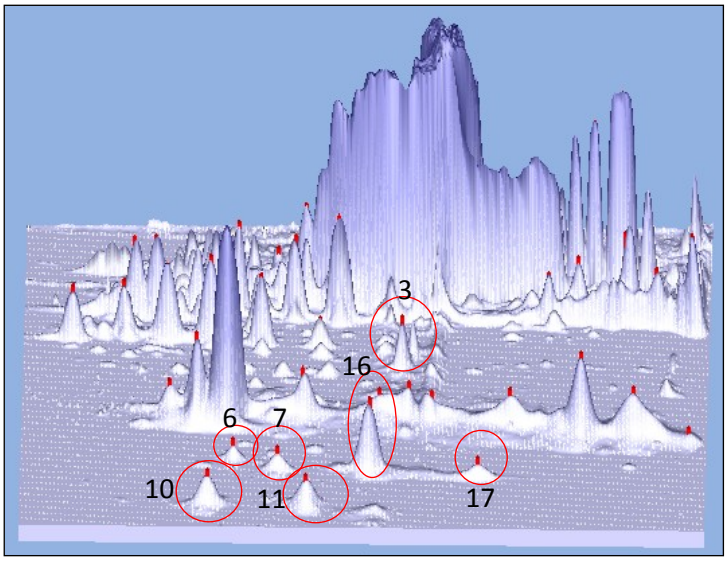


Figure 6 The 2DE protein patterns of whole plasma. Aliquot (180 µg) of plasma was separated in the first dimension on IPG strip pH 4-7, followed by in the second dimension on 12.5% (v/v) SDS-PAGE. The protein detection was performed by colloidal Coomassie blue G-250 staining. The spots that significantly 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