[Full Paper]**Comparative 2-DE proteomic analysis clarified that the stability of ITIH-4 is decreased under the storage at 4°C****Xiulian Zhang, Yasuhiro Kuramitsu, Masanori Fujimoto and Kazuyuki Nakamura****SUMMARY**

Studies on blood plasma proteome were performed by two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) using samples supplied from China and the United States by collaborations of Human Plasma Proteome Project of HUPO. The plasma proteins were separated by isoelectric focusing using capillary type polyacrylamide gel (PAG) with carrier ampholytes pH 3.5–10 in the first dimension and with SDS-PAGE using 15%, 12.5% and 8% gel in the second dimension. The protein spots were identified by peptide mass fingerprinting using electrospray ionization MS/MS (LC/MSD Trap XCT, Agilent). More than 145 protein spots were identified by LC-MS/MS. The 2-DE patterns showed that the samples stored at 4°C for 3 days lost inter-alpha-trypsin inhibitor heavy chain 4 (ITIH-4).

Key words: ITIH-4, 2-DE, LC/MSD.

INTRODUCTION

After completion of human genome project we started one of the most important project of comprehensive analysis on proteins in human blood plasma and serum. The samples of plasma and serum are accessible sources of specimen that reflect dynamic organ functions in health and disease linked to clinical data. The aims for the project are to develop a database structure and repository for HUPO Human Plasma Proteome Project (HPPP) and to find biomarkers in diseases that can be used for diagnostic, prognostic or therapeutic purposes. This time we mainly develop the database and examine the effect of storage condition to plasma protein. We evaluated a technology of two-dimensional gel electrophoresis (2-DE) combined with liquid chromatography tandem mass spectrometry (LC/MS/MS) and a specimen handing through international collaboration by BD Bioscience (BD), National Institute for Biological Standards & Control, UK and Chinese Academy of Medical Sciences (CAMS).

We show here data of 145 major protein spots in Caucasian and Mongolian plasma samples and discuss the conditions for the technology and the storage of samples.

MATERIALS AND METHODS**1. Sample preparation**

Human plasma pooled samples (treated with Heparin) were gifted from China and the United States through BD Bioscience (Caucasian samples) and Chinese Academy of Medical Science (Mongolian samples) by the international collaboration of the HUPO plasma proteome project. The samples, which stored at –80°C, were thawed once to disperse into aliquots of 10 µl, and then incubated the 10 µl samples at 4°C for one day and 3 days respectively. The reproducibility was confirmed in four independent experiments.

2. Analytical 2-DE

Analytical 2-DE was carried out with a slight modification of O'Farrell's method¹⁾. Briefly, polyacrylamide(4% T, 5.4% C, 2% Ampholine pH 3.5–10 Amersham Biosciences, UK) capillary gels, 62 mm in length and 1.2 mm in diameter, were used for IEF in the first dimension. A sample containing 50 µg protein was loaded on each IEF gel and run at 16°C with a stepwise increase of voltage (15 min at 200 V, 15 min at 400 V, 30 min at 800 V and 288 min at 2,500 V). In

Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan.
Correspondence address: Kazuyuki Nakamura; Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-kogushi, Ube, Yamaguchi 755-8505, Japan.

Abbreviations: ITIH-4, inter-alpha-trypsin inhibitor heavy chain 4; 2-DE, two dimensional gel electrophoresis; DTT, Dithiothreitol; IEF, isoelectric focusing; CBB R-250, Coomassie Brilliant Blue R-250.

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the second dimension, SDS-polyacrylamide gel electrophoresis (PAGE) was carried out with 85 mm (W)×60 mm ×1 mm (T) slab-type gels (15% T, 2.67% C), run at 15 mA per plate. After 2-DE, proteins in gel were visualized by Coomassie Brilliant Blue (CBB) R-250 staining.

3. In-gel trypsin digestion

The protein spots of interest were cut out from gels with sterile single-use scalpel. The gel pieces were washed once with MilliQ (100 µl), and CBB dye was removed by three rinses in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM dithiothreitol (DTT) for 15 min and twice in 50% acetonitrile, 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel pieces were dehydrated twice in 100% acetonitrile for 30 min, and then re-swollen with an in-gel digestion reagent containing 10 mg/ml sequencing grade trypsin (Promega, Madison, WI) overnight at 30°C. The tryptic peptides were extracted from the gel pieces. The extracted peptides were dried with a lyophilizer for 18h. The peptides were eluted with 0.1% formic acid into a microfuge tube for the LC-MS/MS analysis.

4. Amino acid sequencing by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Lyophilized peptide was dissolved in a solution of 0.1% formic acid and then centrifuged at 15,000 *g* for 5 min, finally took 20 µl of the supernatant into 100 µl insert polypropylene for the LC-MS/MS analysis. Samples were analyzed by using an Agilent 1100 LC/MSD Trap XCT sys-

tem (Agilent Technologies. Inc. Palo Alto, CA). Results were analyzed with a Spectrum Mill MS Proteomics Workbench, which matched observed peptide masses and product ion masses with the theoretical values for all proteins in the SWISSPROT database.

RESULTS AND DISCUSSION

In order to develop the database and examine the effect of storage condition to plasma protein, the normal human plasma supplied from Caucasian and Mongolian were analyzed proteomically by means of 2-DE and LC-MS/MS. Data are representative of four independent experiments.

The protein spots were cut out from the gels, digested and lyophilized for MS analysis. More than 145 protein spots were identified by Agilent 1100 LC-MS/MS Trap XCT (Fig. 1 and Table 1). For the storage condition, we incubated samples at 4°C for one day and three days, as shown in Fig. 2, ITIH-4 decreased at 4°C for one day and disappeared after three days of Caucasian sample, whereas ITIH-4 of sample Mongolian did not disappear even after 7 days (data not shown) under the same conditions. Although there are some protein spots are different between Caucasian and Mongolian according to the 2-DE image, such as, spots 1, 2, 3, 4 and 5 (Fig. 3 arrows), it is still unclear whether the racial differences exist or not between Caucasian and Mongolian. The inter-alpha-trypsin inhibitor family is composed of protease inhibitors that are assembled from two precursor proteins: a light chain and different heavy chains. So far, the family of heavy chains

Fig. 1. Two-DE gel pattern of the normal human plasma supplied from BD. Numbers of spots are given.

Proteins were separated on a 3.5–10 linear pH gradient as the first dimension, and then on a 12.5% acrylamide gel as the second dimension. The gel was stained with Coomassie Brilliant Blue R-250 and the spots were identified by LC-MS/MS.

Table 1. Summary of the identification, pI, Mr, Accession number and content of the proteins numbered in Fig. 1.

No.	Protein name	Accession No.	pI	Mr	No.	Protein name	Accession No.	pI	Mr
1	Inter-alpha-trypsin inhibitor heavy chain H2 (Precursor)	P19823	6.4	106436	74	Haptoglobin-1 precursor	P00737	6.13	45205
2	Fibronectin (Precursor)	P02751	5.45	262606	75	Alpha-actin 1	P02568	5.23	42051
3	Fibronectin (Precursor)	P02751	5.45	262606	76	CD5 antigen-like precursor	O43866	5.28	38088
4	Alpha-2-macroglobulin (Precursor)	P01023	6	163277	77	Haptoglobin-1 precursor	P00737	6.13	45205
5	Complement factor H (Precursor)	P08603	6.28	139125	78	Haptoglobin-1 precursor	P00737	6.13	45205
6	Complement factor H (Precursor)	P08603	6.28	139125	79	Haptoglobin-1 precursor	P00737	6.13	45205
7	Ceruloplasmin precursor	P00450	5.44	122205	80	Haptoglobin-1 precursor	P00737	6.13	45205
8	Ceruloplasmin precursor	P00450	5.44	122205	81	Haptoglobin-1 precursor	P00737	6.13	38452
9	Ceruloplasmin precursor	P00450	5.44	122205	82	Haptoglobin-1 precursor	P00737	6.13	38452
10	Ceruloplasmin precursor	P00450	5.44	122205	83	Haptoglobin-1 precursor	P00737	6.13	38452
11	Ceruloplasmin precursor	P00450	5.44	122205	84	Serum albumin precursor	P02768	5.92	69366
12	Ceruloplasmin precursor	P00450	5.44	122205	85	Clusterin precursor	P10909	5.89	52494
13	Inter-alpha-trypsin inhibitor heavy chain H4 (Precursor)	Q14624	6.51	103358	86	Clusterin precursor	P10909	5.89	52494
14	Inter-alpha-trypsin inhibitor heavy chain H4 (Precursor)	Q14624	6.51	103358	87	Clusterin precursor	P10909	5.89	52494
15	Inter-alpha-trypsin inhibitor heavy chain H4 (Precursor)	Q14624	6.51	103358	88	Transthyretin precursor	P02766	5.52	15887
16	Inter-alpha-trypsin inhibitor heavy chain H4 (Precursor)	Q14624	6.51	103358	89	Apolipoprotein E precursor	P02649	5.65	36154
17	Ceruloplasmin precursor	P00450	5.44	122205	90	Apolipoprotein E precursor	P02649	5.65	36154
18	Inter-alpha-trypsin inhibitor heavy chain H4 (Precursor)	Q14624	6.51	103358	91	Ig lambda chain V-IV region	P01717	6.04	11516
19	Serum albumin precursor	P02768	5.92	69366	92	Ig lambda chain V-IV region	P01717	6.04	11516
20	Serum albumin precursor	P02768	5.92	69366	93	Ig lambda chain V-IV region	P01717	6.04	11516
21	Complement C1s component precursor	P09871	4.86	76684	94	Ig kappa chain V-I region SIE	P01605	7.96	11834
22	Complement C1s component precursor	P09871	4.86	76684	95	Ig kappa chain V-III region SIE	P01620	8.7	11775
23	Complement C4 (Precursor)	P01028	6.66	192771	96	Ig kappa chain V-III region SIE	P01620	8.7	11775
24	Complement C4 (Precursor)	P01028	6.66	192771	97	Immunoglobulin J chain	P01591	4.52	15594
25	Complement C4 (Precursor)	P01028	6.66	192771	98	Immunoglobulin J chain	P01591	4.52	15594
26	Complement C4 (Precursor)	P01028	6.66	192771	99	Apolipoprotein A-I precursor	P02647	5.56	30778
27	Complement C1r component precursor	P00736	5.89	80174	100	Apolipoprotein A-I precursor	P02647	5.56	30778
28	Complement C1r component precursor	P00736	5.89	80174	101	Apolipoprotein A-I precursor	P02647	5.56	30778
29	Serum albumin precursor	P02768	5.92	69366	102	Apolipoprotein A-I precursor	P02647	5.56	30778
30	Prothrombin precursor	P00734	5.64	70036	103	Haptoglobin-1 precursor	P00737	6.13	38452
31	Prothrombin precursor	P00734	5.64	70036	104	Haptoglobin-1 precursor	P00737	6.13	38452
32	Vitronectin (Precursor)	P04004	5.55	54305	105	Haptoglobin-1 precursor	P00737	6.13	38452
33	Vitronectin (Precursor)	P04004	5.55	54305	106	Transthyretin precursor (Prealbumin)	P02766	5.52	15887
34	Alpha-1B-glycoprotein (Precursor)	P04217	5.45	54209	107	Haptoglobin-1 precursor	P00737	6.13	38452
35	Alpha-1B-glycoprotein (Precursor)	P04217	5.45	54209	108	Alpha-2-macroglobulin (Precursor)	P01023	6	163277
36	Alpha-1B-glycoprotein (Precursor)	P04217	5.45	54209	109	Plasminogen precursor	P00747	7.04	90569
37	Ig mu chain C region	P01871	6.35	49557	110	Plasminogen precursor	P00747	7.04	90569
38	Ig mu chain C region	P01871	6.35	49557	111	Complement factor B precursor	P00751	6.67	85533
39	Ig heavy chain V-III region BRO	P01766	6.44	13226	112	Plasminogen precursor	P00747	7.04	90569
40	Complement component C9 precursor	P02748	5.43	63173	113	Plasminogen precursor	P00747	7.04	90569
41	Keratin (ck9)	P35527	5.14	61987	114	Plasminogen precursor	P00747	7.04	90569
42	Heparin cofactor II precursor	P05546	6.41	57070	115	Plasminogen precursor	P00747	7.04	90569
43	Heparin cofactor II precursor	P05546	6.41	57070	116	Serotransferrin precursor (Transferrin)	P02787	6.81	77050
44	Serum albumin precursor	P02768	5.92	69366	117	Serotransferrin precursor (Transferrin)	P02787	6.81	77050
45	Alpha-1-antichymotrypsin precursor	P01011	5.33	47651	118	Histidine-rich glycoprotein precursor	P04196	7.09	59578
46	Alpha-1-antichymotrypsin precursor	P01011	5.33	47651	119	Serotransferrin precursor (Transferrin)	P02787	6.81	77050
47	Alpha-1-antichymotrypsin precursor	P01011	5.33	47651	120	Complement C3 precursor	P01024	6.02	187165
48	Ig alpha-1 chain C region	P01876	6.08	37654	121	Fibrinogen alpha/alpha-E chain precursor	P02671	5.7	94973
49	Ig alpha-1 chain C region	P01876	6.08	37654	122	Fibrinogen alpha/alpha-E chain precursor	P02671	5.7	94973
50	Ig alpha-1 chain C region	P01876	6.08	37654	123	Fibrinogen alpha/alpha-E chain precursor	P02671	5.7	94973
51	Ig alpha-1 chain C region	P01876	6.08	37654	124	Serum albumin precursor	P02768	5.92	69367
52	Ig alpha-1 chain C region	P01876	6.08	37654	125	Serum albumin precursor	P02768	5.92	69367
53	Ig alpha-1 chain C region	P01876	6.08	37654	126	Fibrinogen alpha/alpha-E chain precursor	P02671	5.7	94973
54	Ig alpha-1 chain C region	P01876	6.08	37654	127	Fibrinogen beta chain precursor	P02675	8.54	55928
55	Ig alpha-1 chain C region	P01876	6.08	37654	128	Fibrinogen beta chain precursor	P02675	8.54	55928
56	Alpha-2-HS-glycoprotein precursor	P02765	5.43	39324	129	Fibrinogen beta chain precursor	P02675	8.54	55928
57	Alpha-2-HS-glycoprotein precursor	P02765	5.43	39324	130	Fibrinogen beta chain precursor	P02675	8.54	55928
58	Alpha-2-HS-glycoprotein precursor	P02765	5.43	39324	131	Fibrinogen beta chain precursor	P02675	8.54	55928
59	Alpha-2-HS-glycoprotein precursor	P02765	5.43	39324	132	Fibrinogen gamma chain precursor	P02679	5.37	51511
60	Alpha-1-antitrypsin precursor	P01009	5.37	46736	133	Fibrinogen gamma chain precursor	P02679	5.37	51511
61	Alpha-1-antitrypsin precursor	P01009	5.37	46736	134	Ig heavy chain V-III region BRO	P01766	6.44	13226
62	Vitamin D-binding protein precursor	P02774	5.4	52963	135	Ig heavy chain V-III region BRO	P01766	6.44	13226
63	Antithrombin-III precursor	P01008	6.32	52602	136	Ig heavy chain V-III region BRO	P01766	6.44	13226
64	Antithrombin-III precursor	P01008	6.32	52602	137	Ig heavy chain V-III region BRO	P01766	6.44	13226
65	Vitamin D-binding protein precursor	P02774	5.4	52963	138	Complement C4 precursor	P01028	6.66	192772
66	Vitamin D-binding protein precursor	P02774	5.4	52963	139	Ig kappa chain V-III region SIE	P01620	8.7	11775
67	Alpha-1-antitrypsin precursor	P01009	5.37	46736	140	Ig kappa chain V-III region SIE	P01620	8.7	11775
68	Alpha-1-antitrypsin precursor	P01009	5.37	46736	141	Ig kappa chain V-III region SIE	P01620	8.7	11775
69	Fibrinogen gamma chain precursor	P02679	5.37	51511	142	Ig kappa chain V-III region SIE	P01620	8.7	11775
70	Fibrinogen gamma chain precursor	P02679	5.37	51511	143	Ig kappa chain V-III region SIE	P01620	8.7	11775
71	Haptoglobin-1 precursor	P00737	6.13	45205	144	Serum albumin precursor	P02768	5.92	69367
72	Haptoglobin-1 precursor	P00737	6.13	45205	145	Serum albumin precursor	P02768	5.92	69367
73	Haptoglobin-1 precursor	P00737	6.13	45205

Fig. 2. Comparison of the 2-DE pattern of ITIH-4 in Caucasian and Mongolian samples that stored at 4°C for one day or three days detected by CBB R-250 staining.

Caucasian sample stored at 4°C for 3 days lost ITIH-4 (arrowhead). However Mongolian sample did not lose ITIH-4 (arrowhead), only with a little decline in it. Data are representative of four independent experiments.

Fig. 3. Two-dimensional gel electrophoresis protein profiles of normal Caucasian and Mongolian human plasma from BD (panel A) and CAMS (panel B), respectively.

The pH gradient of the first-dimensional electrophoresis is shown on top of the gels, and migration of high molecular weight (MW) markers for SDS-PAGE in the second dimension is shown on the right side of the gel. Arrows show the difference between Caucasian and Mongolian. Data are representative of four independent experiments.

consists of five members (ITIH-1, ITIH-2, ITIH-3, ITIH-4 and ITIH-5), encoded by five different genes located on two different chromosomes^{2,3}. ITIH-4 is a liver-restricted member of the serine protease inhibitor family⁴ found in high concentration in human plasma with an apparent Mr of 120,000, belongs to the type-II acute-phase-protein⁵ with diverse functions as an anti-apoptotic and matrix-stabilizing molecule important throughout the development⁶. ITIH-4

contains the vault protein inter-alpha-trypsin (VIT) domain and a von Willebrand type A (vWA) domain. ITIH-4 related to modulation of cell migration and proliferation during the development of the acute-phase response has been discussed⁷. Zhang Z, et al. reported that ITIH-4 is the biomarker of ovarian cancer⁸. Bhanumathy, et al. noted a disparity in the levels of ITIH-4 mRNA and protein levels. The disparity between protein and RNA levels is possibly

due to posttranslational modifications or to alter protein stability has been discussed⁴⁾. These modifications include proteolytic truncation by an aminopeptidase, cysteinylolation and glutathionylation⁹⁾. The cleavage fragment of ITIH-4 (up-regulated) independent validation to detect early stage invasive epithelial ovarian cancer from healthy controls⁸⁾ has been reported. So one possibility may be certain enzyme cleaved ITIH-4 during storage at 4°C. Although we do not know the real reason why the amount of ITIH-4 changed after the storage, it had better to store the sample at -80°C as soon as possible, especially for clinical diagnosis, because the storage condition may affect the plasma protein. Among the identified proteins, there were some proteins in which the theory and observed molecular weight is different, this probably might result from the post-translation, degradation and so on.

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