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**Combinatorial peptide ligand library plasma treatment:
advantages for accessing low-abundance proteins**

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5 **Combinatorial peptide ligand library plasma treatment: advantages for accessing**
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8 **low-abundance proteins**
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45 **Abbreviations**
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47 **CPLL: combinatorial peptide ligand library ; SELDI-TOF: surface enhanced laser desorption**
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49 **ionisation-time of flight**
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53 **Keywords : 2D-electrophoresis, deep proteome, MALDI-TOF, plasma**
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Abstract

Depletion of major blood proteins is one of the most promising approaches to accessing low abundance biomarkers. This study compared the use of combinatorial peptide ligand library (CPLL) and albumin and immunoglobulins (IgG) depletion technology for accessing these low abundance proteins in plasma using 2D-electrophoresis in an acidic restricted pH range (pH 4-7). Compared to native plasma, both techniques enlarge the visibility of other proteins than albumin and IgG, but there were marked differences in their composition. An increase of the number of spots was detected compared to native plasma (157 spots) with respectively, 427 and 557 spots detected with albumin and IgG depletion, and CPLL treatment. We selected 70 spots to be identified by MALDI-TOF related to their absence in the 2D gels from native or albumin and IgG depleted plasma. The 42 spots identified corresponded to 24 different proteins, with more than half of the proteins which did not belong to the major plasma proteins. CPLL treatment allowed the accessibility to proteolytic fragments obtained from major plasma proteins. We found a large superiority of the CPLL approach over the albumin and IgG depletion process. These findings show the utility of depleting major blood proteins to be able to access low-abundance proteins and the potential of CPLL to select and identify candidate biomarkers in clinical studies.

1. Introduction

From a clinical perspective, the easy availability of plasma and serum samples makes analysis of their proteomes the most interesting method of looking for potential biomarkers that can be used in daily practice. However, the analysis of blood fractions is rendered difficult by the complexity of these proteomes, with their overall vast number of proteins and is challenging due to the high dynamic concentration range (over 10 orders of magnitude) of constituent protein/peptides species. Serum and plasma are fluids with very high concentration of proteins, range 50-80 g/L. About 20 proteins account for 99% of the plasma proteome [1]. The abundant proteins, like albumin, produce in most proteomic approaches large signals which mask or interfere with the detection of low amount protein components. The challenge is therefore to reach the remaining 1%, known as the "deep proteome", which includes thousands of different proteins that may be masked by the high-abundance proteins [2].

Ideally, a depletion technique should be selective and remove 100% of the targeted protein (or proteins) without retaining non targeted proteins. An alternative approach, the combinatorial peptide ligand library (CPLL), seeks to compress the protein concentration range of plasma or serum, through the simultaneous one-step dilution of high-abundance and concentration of low-abundance proteins [3]. This technology uses a large, highly diverse library of combinatorial hexapeptide ligands bound to a chromatographic support [4]. The population of beads has such diversity that a binding partner should exist for most proteins in a sample, and each bead has an equivalent binding capacity. The high-abundance proteins saturate their affinity ligands, and the excess proteins are washed away. In contrast the medium- and low-abundance proteins are concentrated on their specific affinity ligands allowing the CPLL-treatment to enhance proteins of low- and very low-abundance. Recently, CPLL-treatment revealed a clear evidence of normalization of the protein repertoire with reduced dynamic range [5] and also a selective enrichment of species in several molecular weight ranges that were less apparent in the starting serum samples [6]. We also found that the use of CPLL increased resolution, with loss of most abundant plasma proteins, reproducibly and improved the intensity of low-abundance proteins [7].

The aim of the present study was to evaluate the combination of CPLL with two-dimension gel electrophoresis in a restricted acidic pH range regarding the efficiency, sensitivity and compatibility and investigate the accessibility of low abundance plasma proteins to improve plasma proteomic profiling in

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5 proteomic analysis. We also compared 2D profile of CPLL-treated plasma with the albumin and IgG depletion
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7 method.
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2. Materials and methods

2.1 Plasma sample

Human plasma was obtained from healthy human donors at Regional Blood Transfusion Center (Lille, France) using EDTA as anticoagulant.

2.2 Sample processing with albumin and IgG depletion kit

Plasma sample was depleted for albumin and IgG using the ProteoPrep Blue Albumin and IgG Depletion kit (Sigma-Aldrich) according to the manufacturer's protocol. After column equilibration, 25 μ L of plasma sample was added to the top of the packed medium bed and incubated at room temperature for 20 min. The spin column was centrifuged and the eluate was reapplied for 10 min. After centrifugation, the remaining unbound protein was washed by adding 100 μ L of Equilibration Buffer to the top of the medium bed, then centrifuged and pooled. Protein concentration of the pooled eluates (125 μ L) was determined with the Bio-Rad Protein Assay.

2.3 Sample processing with combinatorial peptide ligand library

Plasma samples were pretreated with the ProteoMiner protein enrichment kit (Bio-Rad Laboratories) as previously described [7-8]. After removing the storage solution (20% beads, 20% v/v aqueous EtOH, 0.5% v/v acetonitrile) by centrifugation at 1000 g for 2 min. the beads were washed with 1 mL deionized water followed by a 1 mL wash buffer (PBS buffer, 150 mM NaCl, 10 mM NaH₂PO₄, pH 7.4). One mL of plasma sample was then added to columns containing 500 μ L of washed beads and rotated end-to-end for 2 h at room temperature. Columns were then centrifuged to eliminate unbound proteins. Beads were then washed three times with 1 mL wash buffer and one time with 1 mL deionized water. Hundred μ L elution reagent (610 μ L of 5% acid acetic in lyophilised elution reagent constituted of 4 mol/L urea, 1% CHAPS) was then added to each column at room temperature over a period of 15 min and elution was performed by centrifugation; entire step was repeated twice. Elution samples obtained from the 3 centrifugations were mixed together and divided into 5 aliquots of 50 μ L and stored at -20 °C. Protein concentration was determined with the Bio-Rad Protein Assay.

2.4 2-DE

Two DE was performed as previously described in detail [9]. The first dimension (IEF) was performed on a Protean IEF Cell System (Biorad, Hercules, CA, USA) as follows : 100 µg (for analytical gels) or 500 µg (for preparative gels) of plasma proteins were mixed in 250 µl urea solubilization/rehydration buffer (8 mol/L Urea, CHAPS 2%, 97 mmol/L DTT and 5% ampholytes) for IPG strips (Genomic Solutions, Ann Arbor, USA), and loading buffer (8 mol/L Urea, CHAPS 4%, 65 mmol/L DTT and 40 mmol/L Tris) for IPG strips (Genomic Solutions) for a final volume of 450 µl. The mixture was applied on a dry IPG strip (length 24 cm, pH 4-7 linear gradient, GE Healthcare, Orsay, France). Complete sample uptake onto strip was achieved after 9 hours at 20°C without any current. Focusing was carried out at 20°C under a current limit of 50 µA per strip and performed at 50 V for 9 h (active rehydration step), 200 V for 1 h (linear progression), 1000 V for 1 h (linear progression) followed by a slow ramping to 10 000 V for 6 h (linear progression), and was completed at 10000 V for 4h30 (fast progression).

After IEF, the IPG strip gels were equilibrated by two consecutive incubations for 15 min each at room temperature with buffer containing 6 mol/L urea, 37.5 mmol/L Tris-HCl, pH 8.8, 30% glycerol (v/v) and 2 % SDS (w/v) with 2% DTT (w/v) for the first incubation, and 2.5% IAA (w/v) for the second one. The equilibrated IPG gels were applied to the top of a 12.5% Duracryl™ (Genomic Solutions) gel and sealed with low melting temperature agarose (GE Healthcare). Electrophoresis was carried out at 10°C with the Ettan Dalt^{six} large vertical system (GE Healthcare) in running buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS (w/v) at 70 V overnight.

The analytical gels were silver-stained according to the protocol previously described by Shevchenko *et al* [10], with minor modifications. Briefly, gels were fixed overnight in 30% ethanol (v/v) and 5% acetic acid (v/v) followed by 4 washes of 18 MΩ-H₂O. Gels were sensitized for 1 min in 0.02% sodium thiosulfate (w/v), followed by two 1 min washes in 18 MΩ-H₂O, and then incubated in 0.2% silver nitrate (w/v) for 30 min. Proteins were then visualized using developing solution (0.028% formalin (v/v), 0.0125 sodium thiosulfate (w/v), 2.4% sodium carbonate (w/v) until an appropriate level of staining was achieved, after which development was stopped by adding 10% acetic acid (v/v).

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5 Gels were scanned with an Imagescanner® scanner (GE Healthcare) to yield images at 200 density per
6 inch. Image analysis was performed with ImageMaster 2D Platinum® 6.0 software (GE Healthcare). Spots
7 were detected automatically according to three parameters: smooth 5, min area 4 and saliency 2.
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10 11 12 13 **2.5 Mass spectrometry**

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15 Spots corresponding to plasma proteins detected after CPPL treatment were manually excised from a
16 preparative gel prepared with at least 500 µg proteins and colloidal blue stained as previously described [11],
17 and washed for 15 min with 100 µL of 50 mM ammonium bicarbonate solution and then twice for 15 min with
18 50 mM ammonium bicarbonate/50% ACN. They were then dried after adding 100 µL of ACN for 10 min. After
19 the supernatant was discarded, tubes were left open for 10 min to complete solvent evaporation. Trypsin
20 digestion was performed as previously described [12]. The spots were rehydrated with a solution containing
21 0.025% ProteasMAX™ Surfactant, Trypsin Enhancer (Promega) in 50 mM ammonium bicarbonate and 3 µL
22 of 40 µg/mL Trypsin Gold (Promega) in 50 mM acetic acid. After overnight digestion at 37°C, peptide
23 extraction was carried out in two steps according to the manufacturer's protocol for ProteasMAX™ Surfactant,
24 Trypsin Enhancer. For acquisition of the mass spectra of the extracted and desalted peptides, we mixed 0.5
25 µL of the peptide solution with 0.5 µL of matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid
26 dissolved in 0.1% TFA / 50% ACN) on the MALDI-TOF MS target. External calibration was performed with a
27 peptide mixture resulting from the tryptic digest of BSA (0.5 µg/mL). MALDI-TOF MS was then performed
28 with a Voyager DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a
29 337.1 nm nitrogen laser and a delayed extraction facility (125 msec). All spectra were acquired in a positive
30 ion reflector mode under 20 kV voltage, 61% grid. Typically, 300 laser shots were recorded per sample. The
31 mass spectra were then calibrated before protein identification by peptide mass fingerprinting, conducted by
32 running the MASCOT web searcher (<http://www.matrixscience.com/>, Matrix Science, UK) against the NCBI
33 20100312 (10570301 sequences; 3602205473 residues) with the following parameters: Fixed modifications:
34 Carbamidomethyl (C) and variable modifications: Oxidation (M); Peptide Mass Tolerance: ± 50 ppm; Peptide
35 Charge State: 1+; Max Missed Cleavages: 1.
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3. Results and discussion

The first aim of this work was to demonstrate the utility of accessing low-abundance blood proteins. For that purpose, we tested the feasibility of using the CPLL technology to reduce the concentration of the major proteins from plasma so that we could analyze the CPLL-treated plasma samples with the 2D technology. The reproducibility of the CPLL technology was tested on plasma and serum using two types of array (CM10 and Q10) on surface-enhanced laser desorption ionisation-time of flight (SELDI-TOF), showing a good qualitative and quantitative reproducibility [7]. And recently, the coefficient of variation of combination of bead technology and 2D-electrophoresis was estimated at 14.2% based on six independent experiments [13]. Our choice was also to perform 2D-electrophoresis using a narrow acidic range of pH (4 to 7) as a majority of plasma proteins has acidic pH value. The other reasons is to have a better resolution for detecting more polypeptidic spots and in our case corresponding to low abundance proteins.

2D-gel analysis of plasma depleted for albumin and IgG or CPLL-treated

All the experiments were performed using plasma obtained using EDTA as anticoagulant as HUPO recommended to limit the variability. Plasma was chosen to avoid additional time ex vivo for clotting, and EDTA gave more consistent results with less proteolytic cleavage than heparin or citrate-anticoagulated plasma [14].

We have chosen to separate native, depleted for albumin and IgG and CPLL-treated plasma proteins in an acidic restricted pH range to increase the resolution of 2D-gel. We compared 2D profiles of these three plasma-treated samples as well the flow-through of CPLL column by loading for each 100 μg of proteins (Fig. 1). Interestingly, we observed the same profile for native plasma (Fig. 1A) and the flow-through CPLL column (Fig. 1C) in accordance with previous data of Fasoli *et al.*, [15] showing the same SDS-PAGE profile for human serum proteins and the flow-through from peptide libraries. For both profiles, we detected the major plasma proteins, albumin and IgG. Depletion method for albumin and IgG (Fig. 1B) enlarged the visibility of other proteins, but its suffers from several limitations such as depletion of associated species and dilution of the collected depleted samples. CPLL-plasma treatment allows the detection of more and new spots (Fig. 1D) compared to native plasma (Fig. 1A) with a good resolution of the 2D-gel. A comparison of 2D-gel profiles of the plasma proteins following albumin and IgG depletion (Fig. 1B) or CPLL processing (Fig. 1D)

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5 clearly demonstrated that there were marked differences in the polypeptidic spots detected. Interestingly, if
6 we compared with the number of spots detected by Sihlbom *et al*, [13] we obtained 2-fold more spots
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8 detected using narrow range of pH.

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10 We then precisely quantified the number of spots detected in each gel (Fig. 1 online) using Platinum 6.0
11 software as the same amount of plasma (treated or not) proteins was loaded in the 2D gel. Table 1
12 summarized the number of spots detected. For native plasma, we detected 157 spots corresponding to the
13 major plasma proteins and in the flow-through 208 spots corresponding to an increase of 132% of the spots
14 detected. This is in accordance with the removal of only albumin tested using CM10 protein array and SELDI-
15 TOF showing an increase of 144% [16]. Albumin and IgG removal allows to increase the number of spots
16 detected to 427, corresponding to an increase of 271% compared to native plasma, confirming that depletion
17 method enlarged the visibility of other proteins. CPLL treatment allows to detect 557 spots showing an
18 increase of 355% compared to native plasma and of 130% compared to albumin and IgG depletion method.
19 The ability to detect more 2D-spots seemed related to the capacity to load more equivalent plasma proteins
20 on the gels, and to “unmasking” spots following the removal of major proteins. Indeed, protein concentration
21 of CPLL-treated plasma is 7 $\mu\text{g}/\text{mL}$ compared to 70 $\mu\text{g}/\text{mL}$ before loading on the CPLL-column. CPLL
22 technique allows the finding of low- to very low-abundance proteins. We observed a larger superiority of the
23 CPLL approach over the albumin and IgG depletion process. In fact, recently it was shown that removal of
24 the twenty major blood proteins by depletion column allowed to detect a lower number of peaks by SELDI-
25 TOF technology than with removal of 6 or 12 proteins [16]. This could explain why the depletion methods
26 suffered from several limitations, such as co-depletion of associated proteins, as we observed with albumin
27 and IgG removal column. Apolipoprotein-A1, Apolipoprotein-A IV, serotransferrin and α 1-acid glycoprotein
28 were shown to bind to the column, in addition to albumin and IgG (personal communications) and no
29 concentration of less abundant proteins but their dilution. The other major problem is that the proteins bound
30 will depend on the depletion column used as hypothesized by Roche *et al.*, [16], showing that the variability
31 observed depends on the type of columns, which are of different commercial origins, or to the removal not
32 only the selected proteins but also of peptides and proteins associated with them. We conclude to a large
33 superiority of the CPLL approach over the albumin and IgG depletion process, with no dilution of samples
34 and with more spots detected and an increased resolution of the 2D-gel.

2D-reference map of plasma deep proteome

The next step was to identify the proteins corresponding to the polypeptidic spots. The most abundant spots detected in the 2D gel of CPLL-treated plasma were not processed for mass spectrometry identification. As summarized in figure 2, we selected 70 spots to be identified related to their absence in the other 2D gels (Fig. 1A, B, C) and to their low abundance compared to major plasma proteins (Fig. 1D).

Twenty-eight spots over 70 were not identified by mass spectrometry corresponding to the 40% of the spots of low abundance selected for protein identification. The 42 polypeptidic spots identified corresponded to 24 different proteins (Table 2). Among these proteins, 13 have been already identified by the same CPLL approach [13]. Eleven new proteins were identified by mass spectrometry compared to previous work [13], probably due to the use of a narrow range of pH for the IEF step: cadherin-5, CD5 antigen, tropomodulin-3, FAST kinase domain-containing protein 1, cytokeratin-10, complement C4 protein, cytokeratin-2e, retinol-binding protein 4, glutathione peroxidase 3, isoform 2 of mannan-binding lectin serine protease 2 and phosphatidylinositol transfer protein beta isoform. Interestingly, most of these proteins (except complement C4 protein) did not belong to the twenty major plasma proteins, showing the interest of this technology to access low-abundance proteins.

Several of the 24 proteins identified are in the concentration range of $\mu\text{g/mL}$ to g/L in plasma and have been identified, which shows the ability of the CPLL-treatment to detect high, medium, and low abundance proteins. We were also able to identify different isoforms of fibrinogen γ (spots 14-18) and fibrinogen β (spots 7-9), complement factor H (spots 10-12), apolipoprotein E (spots 20-24) and cytokeratin-10 (spots 34-36) from 2D gel of CPLL-treated plasma.

Among the proteins identified, two have already been described [13] but in our study, by the apparent experimental M_r and P_i , we could precisely specify that it was the fibrinogen C-terminal for fibrinogen- α chain and the activation peptide fragment 2 for prothrombin. This shows another advantage of CPLL-treatment, the accessibility to proteolytic fragments obtained from major plasma proteins as we previously showed by SELDI approach [7].

Concluding remarks

We have chosen the CPLL technology to reduce the dynamic range of protein concentration in plasma and unmask previously undetected proteins. Release at once, almost quantitatively all proteins adsorbed by the CPLL-beads with no dilution showed the large superiority of the CPLL approach over the albumin and IgG depletion process. Our results show that the CPLL technology, in combination with 2D-gel performed in a narrow pH range, offered the possibility to reach low abundant proteins for studying plasma proteome with good efficiency. In fact, the method is relatively fast and readily automated but the need of 1 mL of serum or plasma for CPLL treatment could be a limiting factor for the analysis of some clinical samples because of restricted availability. For comparative proteomic studies involving plasma/serum samples, the CPLL strategy is attractive because the sample will retain all representative proteins within the sample, keeping in mind that the major proteins [1] could not be compared and quantified after CPLL-treatment. Several papers report phenomenon of losses for high/medium-abundance proteins [17-20]. We also should pay attention that the mechanism of reduction of protein concentration range depends on several parameters like environmental conditions (pH), temperature, diffusion time [15]. Comparison between patterns obtained from similar biological extracts make sense only if the environmental conditions are strictly the same. But we have also to keep in mind, that discovered biomarkers will be difficult to validate due to the lack of sensitive, specific, multiplex assays for most human proteins [21].

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All authors declare no financial / commercial conflicts of interest.

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5. Figure legends

Figure 1. 2D-gel performed with native, albumin and IgG depleted and CPLL-treated plasma. Plasma proteins (100 μg) were separated on a linear pH gradient (4-7) followed by a 12% SDS-PAGE. A representative 2D-gel image of each conditions was established and compared to native plasma (A). Plasma immunodepleted for albumin and IgG (B) was compared to CPLL-treated plasma (D). Flow-through CPLL plasma (C) showed the same profile as native plasma contrarily to CPLL-treated plasma (D). The positions of M_r are indicated on the left and the pI on the bottom of the gels.

Figure 2. Representative 2-D map of plasma proteins captured by CPLL. Plasma proteins captured by CPLL (500 μg) were separated on a linear pH gradient (4-7) followed by a 12% SDS-PAGE. The 70 polypeptidic spots indicated by a number were selected for protein identification by mass spectrometry. The corresponding identifications are listed in Table 2. The positions of M_r are indicated on the left and the pI on the bottom of the gel.

Table 1. Number of spots detected before and after plasma treatment.

Samples	Number of spots detected
Native	157
Albumin and IgG removal	427
Flow-through CPLL	208
CPLL-elution	557

2D-gels from Fig. 1 were scanned at 200 dpi. Spots were detected automatically by Platinum®6.0 software according to three parameters (smooth: 5, min area: 5, saliency: 2) as indicated in online figure 1.

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elps.201000188-R1

Table 2. List of proteins identified from the spots detected by 2D-Silver stained gel from CPLL-treated plasma.

Spot Number	Accession number	Protein name	Mr (kDa)		Pi		Number of matched peptides/total peptides	Sequence coverage (%)	MASCOT Score
			Theo	Exp	Theo	Exp			
1	P33151	Cadherin-5	82.56	85	5.01	4.6	5/9	6.4	55
2		<i>NI</i>		55		4.45			
3		<i>NI</i>		50		4.8			
4		<i>NI</i>		50		4.85			
5	O43866	CD5-antigen	36.05	50	5.21	5.3	4/36	12	22
6	P06727	Apolipoprotein A-IV	43.40	48	5.18	5.15	15/30	32	134
7	P02675	Fibrinogen-β chain	50.76	58	7.95	6.15	7/13	11	70
8				52		6.1	9/19	15	71
9				58		6.4	5/11	14	47
10	Q03591	Complement factor H-related protein 1	35.74	50	7.10	6.25	7/33	21	50
11				51		6.15	12/21	34	122
12				46		6.2	5/29	16	31
13		<i>NI</i>		52		6.5			
14	P02679	Fibrinogen-γ chain	48.48	48	5.24	6.1	7/14	16	99
15				34		5.6	4/7	8	48
16				33		5.7	7/14	15	92
17				35		5.4	5/28	12	36
18				22		5.2	5/16	10	44
19		<i>NI</i>		48		6.25			
20	P02649	Apolipoprotein E	34.23	44	5.52	5.8	12/17	29	134
21				37		5.6	12/20	25	115
22				37		5.8	6/17	14	36
23				39		5.4	13/25	36	118
24				39		5.5	14/28	47	146
25		<i>NI</i>		43.5		6.1			
26		<i>NI</i>		44		6.2			
27		<i>NI</i>		48		4.7			
28	Q9NYL9	Tropomodulin- 3	39.59	44	5.08	4.6	4/10	15	48
29		<i>NI</i>		46		4.5			
30		<i>NI</i>		37		4.85			
31		<i>NI</i>		37		4.9			
32	Q53R41	FAST kinase domain-containing protein 1	97.41	39	7.8	5.3	13/68	18	56
33		<i>NI</i>		37		5.5			
34	P13645	Cytokeratin- 10	58.83	36	5.13	5.55	5/23	12	39
35				33		4.7	8/53	22	50
36				22		4.6	6/33	15	294
37	P02760	Alpha-1-microglobulin	20.85	35	6.13	5	4/28	19	48
38	Q6U2M5	C4B1	47.55	33	5.92	5.45	6/17	17	64
39		<i>NI</i>		33		5.55			
40	P35908	Cytokeratin-2e	65.43	31	8.07	4.9	10/60	23	45
41		<i>NI</i>		31		5.1			
42		<i>NI</i>		32		5.2			
43	P0COL4	Complement C4-A	33.07	32	6.37	5.4	9/25	4	35

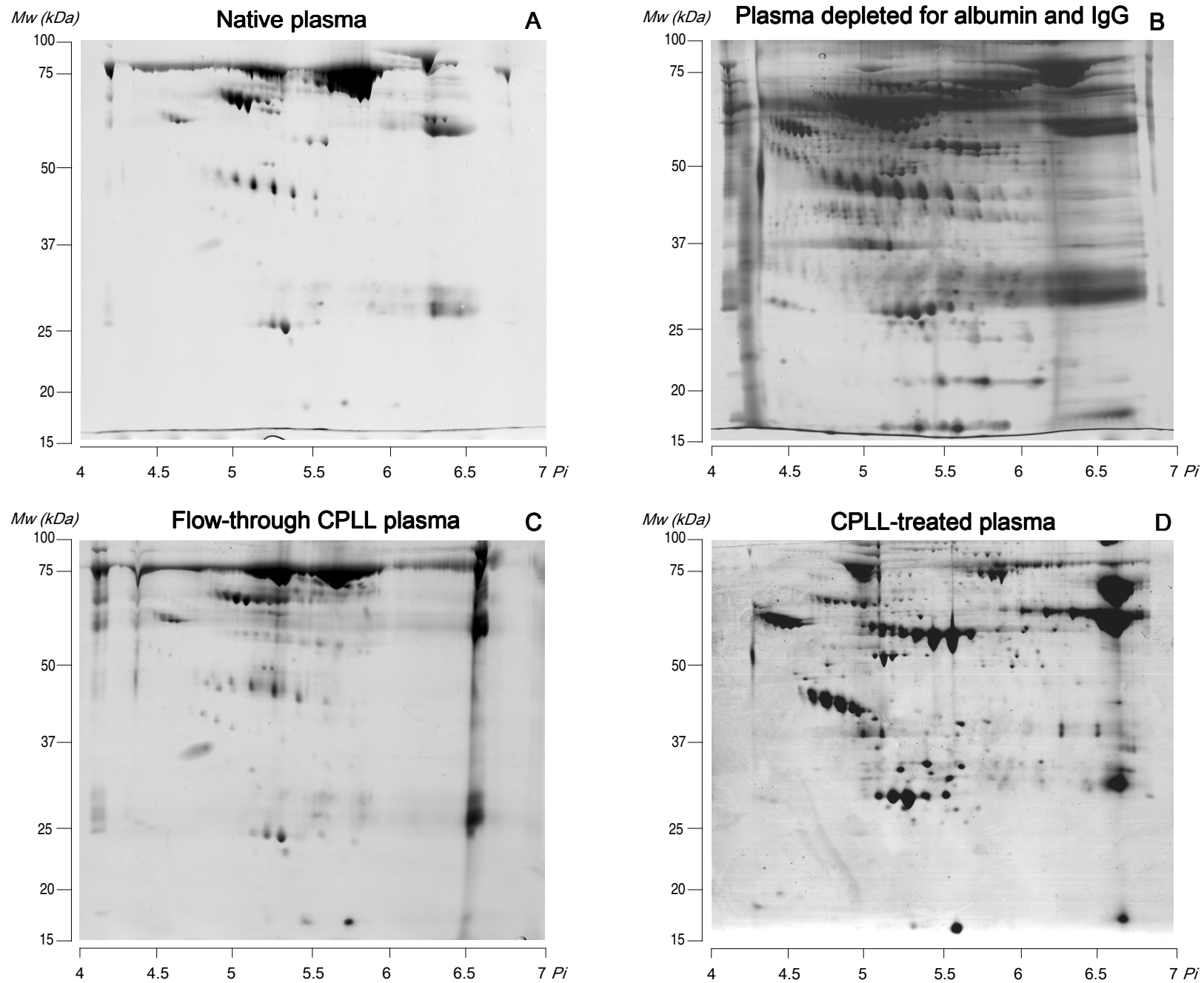
		precursor (C4 - γ chain)							
44	P02743	Serum amyloid-P component	23.26	29	6.12	5.55	5/19	26	56
45				31		5.8	4/21	22	28
46				31		6.1	3/21	17	30
47	Q15485	Ficolin-2	31.41	32	6.65	5.6	3/9	10	33
48		NI		31		6.25			
49		NI		32		6.3			
50		NI		29		6.4			
51		NI		27		6.25			
52	P02747	Complement C1q subcomponent subunit C	22.81	24	8.33	6.2	5/19	22	52
53	P02753	Retinol-binding protein 4	21.07	24	5.27	5.3	5/21	26	57
54	P02647	Apolipoprotein A-I	28.08	23	5.27	5.55	10/29	35	97
55				24		5.65	10/26	29	98
56	P22352	Glutathione peroxidase 3	23.31	23	7.85	5.7	7/23	29	71
57		NI		22.5		5.55			
58	O00187-2	Mannan-binding lectin serine protease 2 (isoform 2)	20.63	22	6.2	5.4	5/8	7	53
59				22		5.6	4/14	7	46
60	P48739	Phosphatidylinositol transfer protein beta isoform	31.41	20	6.44	5.7	4/17	22	43
61	P02671	Fibrinogen- α chain (Fibrinogen C- terminal)	27.31	22	4.63	6.25	5/13	7	36
62		NI		22		5.1			
63	P00734	Prothrombin (activation peptide fragment 2)	14.16	16	4.25	4.2	3/16	53	47
64		NI		17		4.25			
65		NI		17		4.3			
66		NI		16.5		4.45			
67		NI		15.5		4.7			
68		NI		15		5.3			
69		NI		15		5.6			
70		NI		15.5		6.7			

Spot number corresponds to those indicated in Fig. 2

Theo : theoretical ; Exp : experimental

MS. Monoisotopic peptides masses were searched for NCBI protein databases using MASCOT search engine (<http://www.matrixscience.com/>). Assignments were made according to UniProtKB/SwissProt Release 15.13 / 57.13 of 19-Jan-10 (514 112 entries).

NI: not identified by MALDI-TOF spectrometry analysis



ELECTROPHORESIS

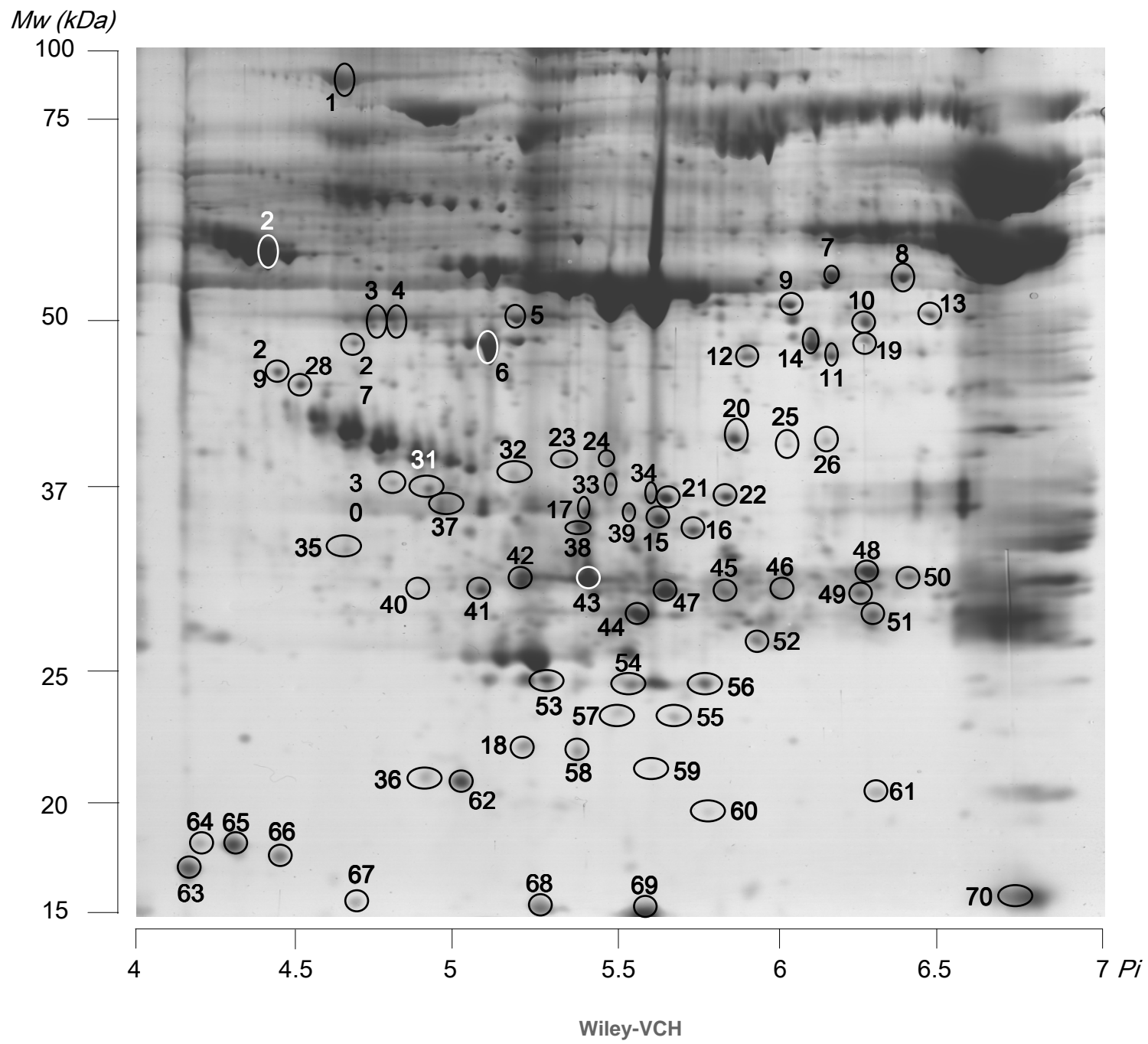


Figure 2

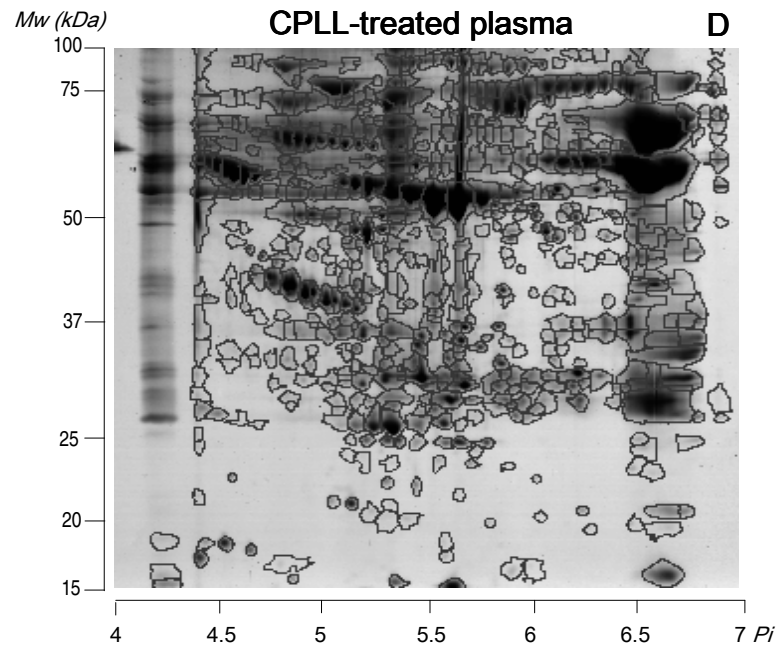
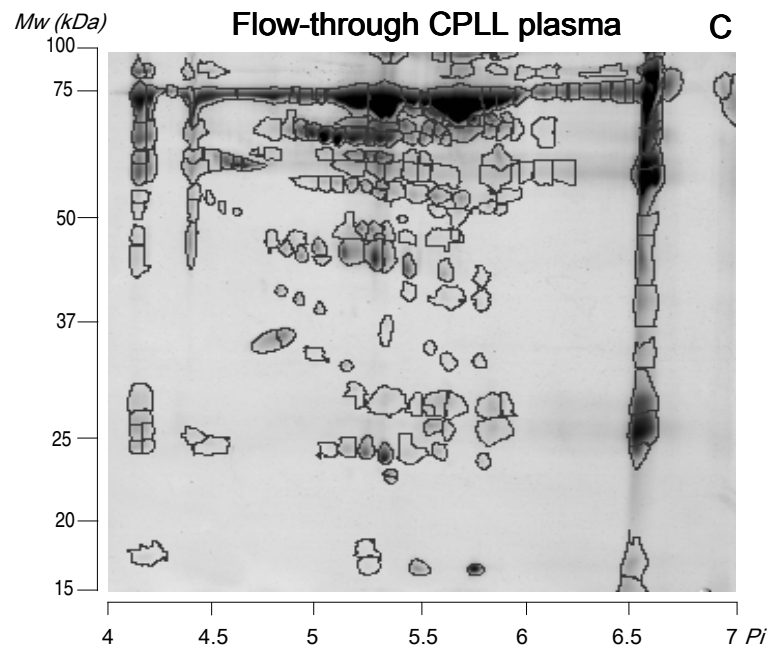
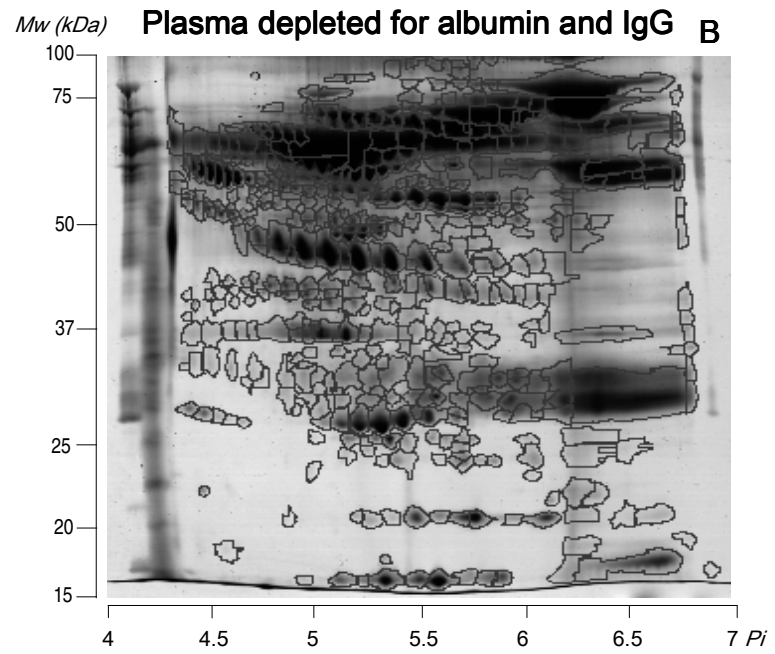
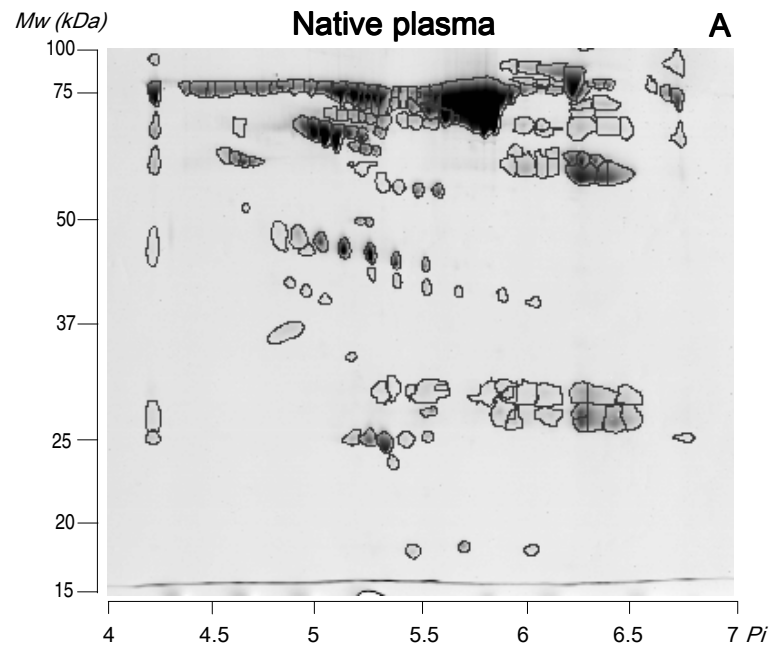


Figure 1 online