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Studies on the Analysis of Human Serum Proteins by the Gel-based Microchip and SDS-PAGE

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ABSTRACT

Human serum proteins can be analyzed and evaluated by using either gel-based microchip (LabChip) or traditional SDS-PAGE. However, this study concludes that a sample under reducing conditions analyzed by SDS-PAGE is more time-consuming than when using a Bioanalyzer in combination with the LabChip to perform the same task. The assay software automatically calculated the size and concentrations of serum proteins and deglycosylated serum proteins. It displayed the results in real-time, thus eliminating lengthy procedures such as imaging and analyzing. Compared to the traditional reducing SDS-PAGE, LabChip also allowed the analysis of human serum proteins with more accuracy, and a faster turnaround time. This suggested that LabChip has the potential to be used in the clinical analysis of human serum proteins.

Key words: microfluidic technology, serum protein, LabChip, SDS-PAGE

INTRODUCTION

Serum contains a number of proteins, and is easily obtained in clinical diagnosis. Serum proteins have recently attracted more attention in biomedical field[(1)]. Proteins in serum could be analyzed by RP-HPLC[(2)] and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[(3,4)], which traditionally is a technique employed by biochemists to detect and characterize proteins[(5)]. It is also used to resolve proteins in a mixture based on their molecular size. Negatively charged SDS binds with proteins in the sample buffer. The proteins then migrate toward the positive pole in an electrical gradient, impeded by the polymerized and cross-linked polyacrylamide, which is a gel stained with Coomassie brilliant blue G-250, Sypro® Ruby or silver nitrite[(6,7)]. The analytical detection limits of conventional Coomassie Blue and colloidal Coomassie Blue stains are 10 and 50 ng of protein, respectively[(8)]. In addition, Novex® Tris-Glycine polyacrylamide gel can be used as an alternative to analyze proteins[(9,10)]. This gel is a kind of polyacrylamide gel which is based on the Laemmli system with minor modifications for maximum performance in the pre-cast format. Proteins get separated in this gel with the presence of SDS, and then get stained with the dyes.

To characterize proteins, the approach based on microfluidics technology (Agilent 2100 Bioanalyzer, Agilent Technologies) and the Bioanalyzer (with LabChip) has been introduced[(11-13)]. The Bioanalyzer contains 16 individually programmable high-voltage power supplies. It is a compact system for rapid and automated analysis of proteins that integrates multiple experimental procedures, such as sample handling, detecting and analysis, into a single process. The LabChip utilizes a network of channels and wells that are etched onto glass chips to build mini-laboratories[(14,15)]. The electro-kinetic force can move proteins in a finely controlled manner through the channels and detection is done through the Bioanalyzer's laser-induced fluorescence. Its red laser has a maximum excitation at approximately 630 nm (maximum detection at 680 nm), whereas the maximum excitation of the blue LED is at 470 nm (maximum detection at 525 nm)[(16,17)]. LabChip can analyze the DNA, RNA and protein samples. It automatically calculates the size and concentration of each separated peak and displays the results in real-time. According to our previous studies[(18,19)], glycoproteins, lipopolysaccharides and peptides under reducing conditions could be analyzed by SDS-PAGE and microchip.

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Therefore, a comparative study on the analysis of human serum proteins was conducted. The primary objective of this study is to evaluate the efficiency of both human serum proteins analytical methods – the gel-based microchip and SDS-PAGE.

MATERIALS AND METHODS

I. *Chemical and Reagents*

The Protein 200 Plus kit was obtained from Agilent Technologies (Palo Alto, CA, USA), while the Novex® Tris-glycine gels (4 - 20%, 1.0 mm × 10 well) and XCell *SureLock*™ Mini-Cell were the products of Invitrogen Co. (Carlsbad, CA, USA). HF cleavage apparatus (model FC2002S) was obtained from Toho Titanium Co. (Chigasaki, Kanagawa, Japan). Protein standards were obtained from Molecular Probes, Inc. (Eugene, OR, USA).

II. *Preparations of Human Serum Samples*

In this study, blood samples were obtained from Lampang Regional Cancer Center, Lampang, Thailand. The blood samples were centrifuged at 2,000 × g for 20 min at 4°C, and the supernatants (serum) were collected. Serum samples (N1, N2 and N3: normal human serum; L1, L2 and L3: lung cancer serum samples) were stored at -80°C until use. The human serum proteins and hydrogen fluoride treated serum proteins were also prepared, which was done by treating human serum proteins (HS) with hydrogen fluoride (HF) in the HF cleavage apparatus. The anhydrous HF (10 mL) was added to human serum samples (1 mg) and incubated at 0°C for 1 h with gentle agitation. After that the deglycosylated protein sample (HS-HF) was extinguished by using liquid nitrogen and then HF was removed by vacuum pump. A buffer (10 mM Tris-HCl, pH 7.5) was added to re-suspend the deglycosylated serum proteins, and then desalted by using the PD-10 desalting column (Sigma, St. Louis, MO, USA).

III. *Protein Quantification*

A modified Bradford protein quantification assay was utilized to quantify the proteins[20]. Standard curve was prepared using commercially available ovalbumin (Sigma, St. Louis, MO, USA). Each sample (100 µL) and Bradford reagents (1 mL) were added to the 1.5 mL microcentrifuge tube. Absorbance was determined at 595 nm by a UV spectrophotometer (Beckman DU640; Beckman Instruments, Palo Alto, CA, USA).

IV. *Gel-based Capillary Electrophoresis (LabChip)*

The LabChip analysis was performed by using the Protein 200 Plus LabChip kit. All chips were prepared according to the protocol provided with the kit. Samples (4 µL) were denatured by mixing with a sample buffer (2 µL) and then left in boiling water for 3 minutes. The sample buffer contained 4% SDS, fluorescent dye and two protein markers. After the denaturing step, samples were diluted with deionized water (84 µL) and the samples were loaded (6 µL) onto a primed chip (LabChip), which was prepped according to the manual provided with the kit. Samples were then separated in this LabChip. Detection was based on laser-induced fluorescence of the fluorescent dye, which was added to the polymer, and non-covalently bound to protein-SDS micelles.

V. *Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)*

The SDS-PAGE analysis was performed by using the precast Novex® Tris-glycine gels. Samples (6.5 µL), reducing agent (1.0 µL) and sample buffer (2.5 µL) were denatured in boiling water for 3 minutes. The sample buffer contains 10% glycerol, 70 mM Tris-HCl (pH 6.8), 2% SDS and 0.02% bromophenol blue. Then the protein ladder and samples (10 µL) were loaded into separate wells. After running an electrophoretical step, gels were stained with Coomassie brilliant blue G-250 and Sypro® Ruby[21,22]. The stained gels were digitized by using the EPSON perfection 1270 image scanner and the Typhoon 9200 image scanner (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

I. *LabChip and Protein 200 Plus Ladder*

The LabChip was used to conduct analysis on proteins in this study. The separation, detection and quantification of protein samples were performed by Bioanalyzer and its assay software[23]. The Protein 200 Plus Ladder and ten samples can be analyzed by Protein 200 Plus LabChip, and it can complete an analysis within 30 minutes, from beginning to the end of the analysis. The size, concentration and protein content of a Protein 200 Plus Ladder were shown in Figures 1 and 2. The sample buffer provided lower marker (6.0 kDa) and upper marker

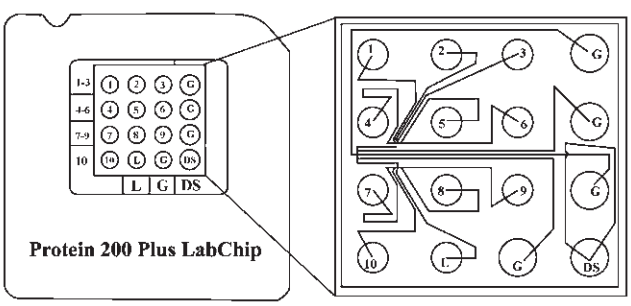


Figure 1. Protein 200 Plus LabChip used in the Agilent 2100 Bioanalyzer.

(200.0 kDa) serving as internal controls and the amount of protein in a sample are expressed as percentage of the total proteins. The molecular weights (MW) of 8 protein peaks from this Ladder were 14.4, 21.0, 29.0, 32.0, 53.0, 66.0, 97.4 and 117.0 kDa respectively. The concentrations of these proteins were 83.3, 68.3, 75.8, 165.1, 141.5, 80.4, 92.9 and 65.1 μg/mL, while protein contents were 10.2, 8.3, 9.3, 20.2, 17.3, 9.8, 11.4 and 8.0% respectively. These results of the size and concentration of each individual peak were automatically displayed in real-time by the assay software.

II. *Comparison of Human Serum Proteins by LabChip and SDS-PAGE*

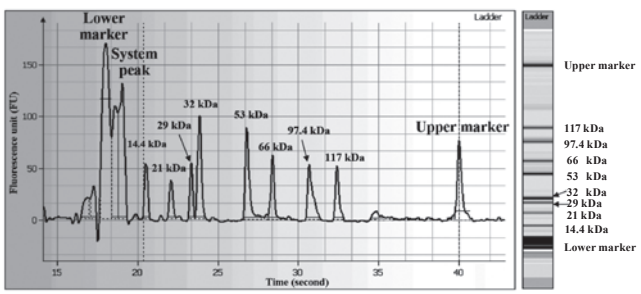
Human serum samples (N1, N2 and N3) were separated and analyzed by LabChip (Figures 3A and 4). Six major peaks were analyzed and found, and the molecular weights (MW) of these peaks are 27, 35, 57, 76, 112 and 200 kDa. Among all peaks, concentration of the major peak (MW = 57 kDa) was higher than 900 μg/mL in serum samples. According to our previous study, the peak (MW = 57 kDa) contains several proteins such as albumin, alpha-1-antitrypsin and alpha-1-B-glycoprotein[24]. Furthermore, the same peaks were found in lung cancer serum samples (L1, L2 and L3) and the concentration of major peak (MW = 57 kDa) was also higher than 900 μg/mL. The same samples were also separated by SDS-PAGE and stained with Sypro® Ruby (Figure 3B) and these results were similar with the data obtained from LabChip. Although serum samples could be analyzed by both LabChip and SDS-PAGE, LabChip is more suitable for sample quantification.

III. *Comparison of Serum and Hydrogen Fluoride Treated Serum Proteins by LabChip*

Serum proteins often retain carbohydrate chains which are connected by either O- or N-glycosidic linkages to proteins[25]. Chemical methods for deglycosylating glycoprotiens with anhydrous hydrogen fluoride (HF) effectively remove peripheral sugars from the oligosaccharide side chains of glycoproteins[26,27]. In this study, the serum proteins (HS) and deglycosylated serum proteins (HS-HF) were also prepared and analyzed by LabChip (Figure 5). Seven major peaks were found in the HS-HF sample, and the molecular weights (MW) of these peaks are 24, 31, 46, 53, 60, 74 and 136 kDa (Figure 5B). Compared to the results of HS sample (Figure 5A), the peaks (MW = 31 and 53 kDa) were increased. This indicated that the peaks (MW = 31 and 53 kDa) may contain the deglycosylated proteins. The results showed the different protein patterns between HS and HS-HF samples.

IV . *Limitations of LabChip*

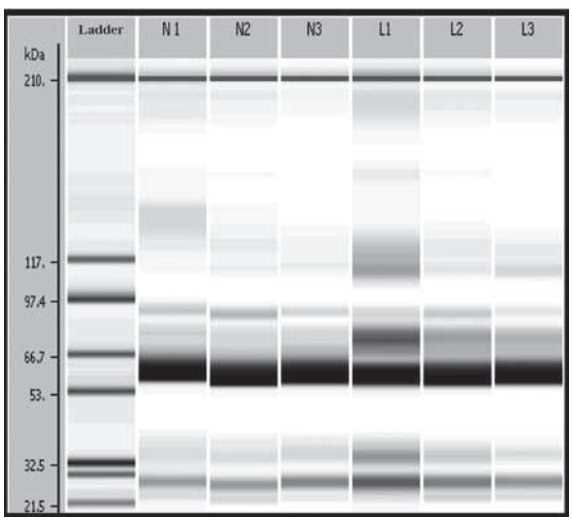
According to the results shown above, LabChip has the potential to be used in clinical analyses of human



Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
14.4	83.3	10.2	53.0	141.5	17.3
21.0	68.3	8.3	66.0	80.4	9.8
29.0	75.8	9.3	97.4	92.9	9.8
29.0	75.8	9.3	97.4	92.9	11.4
32.0	165.1	20.2	117.0	65.1	8.0

Figure 2. Analysis of the Protein 200 Plus Ladder. This ladder consists of 8 protein peaks.

(A)



(B)

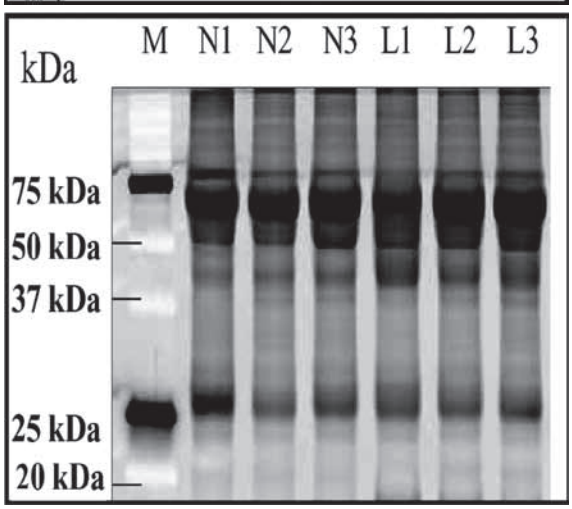
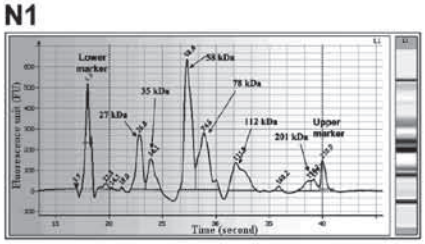


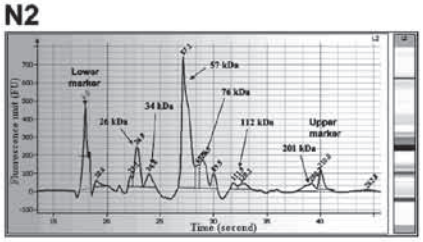
Figure 3. Comparison of human serum proteins by Protein 200 Plus LabChip (A) and SDS-PAGE (B). Ladder: protein markers; M: protein markers; N1, N2 and N3: normal human serum samples; L1, L2 and L3: lung cancer serum samples.

N1



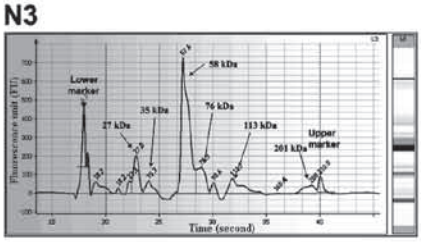
Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
27.0	224.4	10.3	78.3	79.6	3.7
35.1	107.3	4.9	112.1	6.9	0.3
58.8	1,295.2	59.5	201.4	41.5	1.9

N2



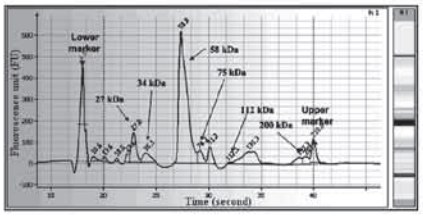
Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
26.3	227.0	11.2	76.6	76.1	3.8
34.3	87.4	4.3	112.3	22.3	1.1
56.9	1,324.8	65.4	200.8	50.1	2.5

N3



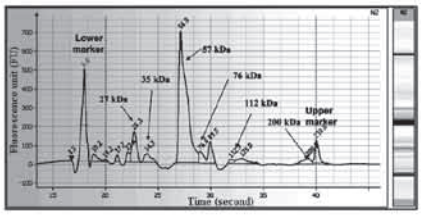
Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
27.1	389.6	14.4	75.9	74.6	2.8
35.0	184.8	6.9	113.8	111.2	4.1
57.8	1,820.6	67.5	201.5	50.8	1.9

L1



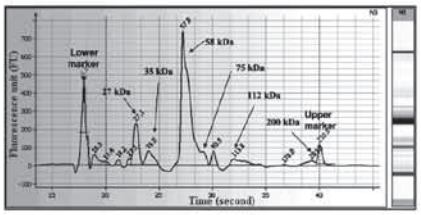
Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
26.8	415.8	15.6	74.6	579.9	21.8
34.1	253.7	9.5	111.9	327.5	12.3
58.4	956.4	35.9	199.7	47.0	1.8

L2



Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
26.8	319.4	13.8	75.9	230.9	10.0
34.4	116.1	5.0	111.9	36.3	1.6
57.1	1,253.30	54.2	200.3	71.1	3.1

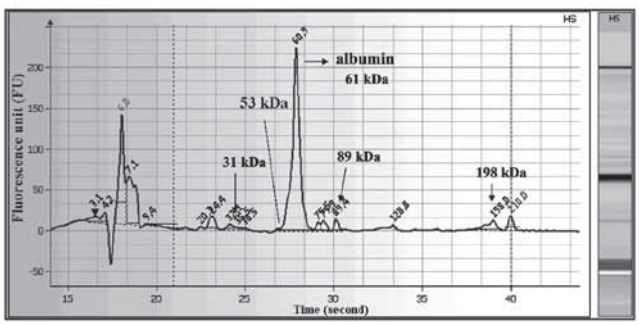
L3



Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
27.0	422.1	12.5	75.9	377.4	11.2
35.3	172.1	5.1	111.7	294.4	8.7
57.6	1,781.3	52.7	200.3	124.8	3.7

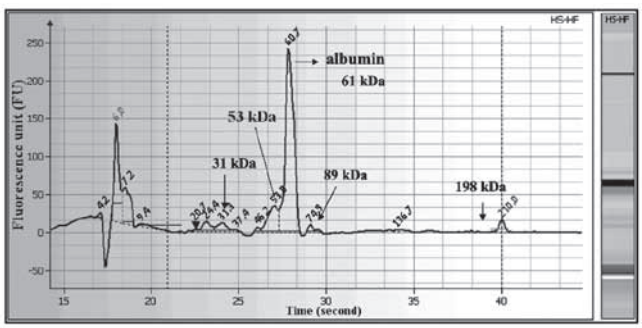
Figure 4. Analysis of the human serum proteins by using the Protein 200 Plus LabChip. N1, N2 and N3: normal human serum samples; L1, L2 and L3: lung cancer serum samples.

(A)



Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
24.4	185.3	5.1	79.9	102.8	2.8
32.1	84.3	2.3	89.4	90.4	2.5
60.9	2,844.6	78.6	198.0	152.5	4.2
75.5	77.9	2.2			

(B)



Size (kDa)	Concentration (μg/mL)	Protein content (%)	Size (kDa)	Concentration (μg/mL)	Protein content (%)
24.4	209.0	4.7	60.7	3,184.9	72.2
31.3	207.4	4.7	74.9	100.4	2.3
46.2	45.1	1.0	136.7	13.0	0.3
53.0	608.4	13.8			

Figure 5. Analysis of the serum proteins and hydrogen fluoride treated serum proteins by using the Protein 200 Plus LabChip. HS: human serum proteins; HS-HF: hydrogen fluoride treated serum proteins.

serum proteins, however, limitations of this approach were also discovered. For example, LabChip can be used to quantify human serum proteins but is only effective in the range between 5-200 kDa. When the molecular weight of a serum sample is above 200 kDa or below 5 kDa, it might not be detected by LabChip. In addition, LabChip can analyze serums with high levels of proteins. For those serums with low levels of proteins, an enrichment procedure may be needed. Furthermore, LabChip can be used to screen candidate proteins in serums but it can not identify those for which positive identification is possible, if a western blotting assay was performed

after SDS-PAGE. Therefore, the clinical application of LabChip still has many limitations yet to overcome.

CONCLUSIONS

We had evaluated the efficiency of 2 methods used to analyze human serum proteins in this study (LabChip and traditional SDS-PAGE). According to the results, both LabChip and SDS-PAGE can be used to analyze serum samples, however, LabChip is actually more suited to quantify serum proteins and can complete the task within 30 minutes, a much faster turnaround than using SDS-PAGE. In addition, we also found several limitations of clinical application of LabChip in this study.

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