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Proteomic study of serum using gel chromatography and MALDI-TOF MS reveals diagnostic biomarkers in male patients with liver-cancer

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Abstract

Human serum has been widely employed clinically for diagnosing various fatal diseases. However, the concentration of most proteins in human serum is too low to be directly measured using normal analytical methods. In order to obtain reliable analytical results from proteomic analysis of human serum, appropriate sample preparation is essential. A combined off-line analytical technique of gel chromatography and matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF MS) has been successfully established to separate proteins for MS analysis. Using these combined techniques, 176 mass signal peaks of proteins/peptides were found in 6 of 18 fractions from normal male serum (NMS) in the presence of buffer consisting of NH_4HCO_3 and acetonitrile. A simple gel chromatography column packed with Sephadex G-50 removed most signal-suppressing compounds such as salts and high abundance proteins (HAP). The molecular mass to charge (*m/z*) ratios of differential peptides revealed in serum of male patient with liver-cancer (LCMPS) compared to NMS were 5365, 5644 and 6462, and these peptides can be used as biomarkers to clinically diagnose liver-cancer. The simple and convenient chromatographic method described here is not only superior to recently described HPLC separation for MS analysis, but also reveals many novel and significant serum biomarkers for the clinical diagnosis of various diseases.
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Keywords: Male serum; Sephadex G-50; MALDI-TOF MS; Biomarker; Liver-cancer

1. Introduction

Recently, MALDI-TOF MS has become an important tool for proteomic research [1], showing high molecular mass accuracy, relatively high tolerance to salts, and excellent sensitivity. These characteristics make it an attractive analytical method by which to generate protein expression profiles of biological samples for proteomic research. Another important characteristic is that molecular ions formed by MALDI-TOF MS show fewer charges than those formed by liquid chromatography (LC)-TOF MS, resulting in the acquisition of more easily interpreted mass spectrum information [2,3].

Human serum is the liquid portion of blood after removal of the corpuscles and fibrin and it is a complex medium containing thousands of different types of proteins/peptides, originating throughout the body [4]. These may be potential biomarkers for the clinical diagnosis of various fatal diseases. Within this fluid lies information relating to the many processes taking place within the body [5]. Identification of the proteins/peptides in

Abbreviations: NMS, normal male serum; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; LCMPS, serum of male patient with liver-cancer; HAS, human serum albumin; HAP, high abundance proteins; LAP, low abundance proteins; HMW, high molecular weight; LMW, low molecular weight; TFAt, rifluoroacetic acid; DHB, 2,5-dihydroxycinnamic acid; SA, sinapinic acid; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; LC, liquid chromatography; SELDI, surface-enhanced laser desorption and ionization

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human serum can provide valuable information regarding how the body reacts to changes, particularly in diseases such as diabetes and cancers. Normally, the protein content of human serum is about 60–80 mg per ml plasma, which implies it is very protein-rich. It has been estimated that up to 10,000 proteins/peptides may be commonly present in serum, but most of these would be in low abundance [6,7], and are thus difficult to measure directly by current analytical methods. About 90% of the total protein content of serum consists of only 10 proteins of high abundance characteristics, such as human serum albumin (HAS) and IgG, etc. The other 10% also favors high abundance species with 12 proteins making up 9%. The remaining 1% belongs to the low abundance biologically interesting population, whose composition and function are very difficult to analyze and identify using normal analytical methods [4,8].

Human serum potentially carries an archive of important histological information determination of which could serve to improve early disease detection. It is for this reason that serum proteins are attracting increasing interest in proteomic research, which is currently striving to broadly characterize these proteins. The expectation is that characterization of the thousands of individual serum proteins/peptides present in serum will enable the discovery of increasing numbers of reliable disease biomarkers [9]. However, serum analysis is challenging, since the protein content of serum is dominated by a handful of proteins such as albumin, transferritin, haptoglobulin, immunglobulin and lipoproteins [7], while and the mass signals of low abundance proteins (LAP) in the serum are strongly suppressed by these HAP. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), a primary method for separating and comparing complex mixtures, has played a critical role in developing our understanding of the complexity and variety of proteins contained in cells and body fluids. Although impressive improvements in 2D-PAGE techniques have occurred in recent years, limitations remain [10,11]. Centrifugal ultrafiltration has been used to enrich the serum proteome, which contains low molecular weight (LMW) proteins, followed by analysis using novel MS technology in order to identify them [4]. Other protocols have been used to overcome the presence of the abundant (and masking) proteins, such as using chromatographic absorbents, immunoaffinity methods and ultrafiltration techniques. Nevertheless, there are still no methods available that can efficiently separate, detect, and quantify most of the proteins within a given proteome in human serum [12–15]. HPLC separation has always been considered to be a relatively effective treatment prior to proteome analysis, but it is not simple and uses organic solvents, which easily denature proteins. In addition, the complex HPLC separation process results in the loss of many proteins. It is for these reasons that an optimized experimental method with separation in aqueous solution is desirable both to obtain reliable results and to reduce protein loss in the proteomic analysis of human serum.

Here, we report a method aimed at obtaining high resolution, reducing loss of proteins and producing more molecular ions for MS analysis. This involves the use of a column separation technology to collect LAP and to remove HAP from human serum prior to determination of diagnostic cancer biomarkers by differential comparison of the proteomes both NMS and LCMPS. This technology also improves separation, removes some signal-suppressing factors such as salts, and increases the amount of molecular ions available for MS analysis in proteomic research.

2. Experimental

2.1. Materials

HPLC grade acetonitrile and trifluoroacetic acid (TFA) were obtained from the Merck Company; a Sephadex G-50 gel filtration resin from Pharmacia (Sweden); sinapinic acid (SA) and 2,5-dihydroxycinnamic acid (DHB) from Sigma (St. Louis, MO, USA) for MS analysis without further purification; other analytical grade inorganic and organic chemicals from commercial sources in China.

2.2. Serum sample preparation

Blood samples of 50 male volunteers were obtained from the Zhongshan Hospital, Xiamen, China. The control blood samples were obtained from 25 volunteers without obvious signs of disease, while carcinomatous blood samples were obtained from 25 patients with liver-cancer, diagnosed through various clinical pathways. In addition, 300 blood samples were obtained from unknown males (both NMS and LCMPS) in order to validate the biomarker data and allow statistical analysis. Once the blood samples were collected from the volunteers, each sample was immediately allowed to coagulate for 30 min at 4 °C and centrifuged for 10 min at $3500 \times g$ in a swinging bucket centrifuge. The supernatant fluid, the serum, was isolated and stored at room temperature for 2 h, then frozen at −70 °C. Before use, the samples were thawed and then refrozen in order to remove thawless precipitate by centrifugation for 10 min at $6000 \times g$ before use. In order to avoid discrepancy of protein/peptide types among these volunteer sera, the 25 NMS samples were pooled for protein separation before MS analysis. Similarly, the 25 LCMPS samples were also pooled before separation in the gel column. In addition, individual serum samples from both NMS and LCMPS were selected in order to identify typical biomarkers.

2.3. Sample preparation with column separation

Prior to separation with the Sephadex G-50 gel column, the pooled samples (either NMS or LCMPS), which had been stored at −70 °C, were thawed in ice-water, and then 0.35 ml mixed with 0.35 ml of buffer A [25 mM NH_4HCO_3, pH 8.2, containing 20% (vol./vol.) acetonitrile]. The mixture was suspended for an hour and then centrifuged for 10 min at $5000 \times g$ before loading onto the gel column for protein separation. Protein separation was performed using a 250 mm × 10 mm column packed with Sephadex G-50 gel at a flow rate of 0.1 ml/min. The column was equilibrated with 0.1% TFA solution and then 0.7 ml of sample plus buffer mixture (prepared as described above) was loaded on the column and eluted with 0.1% TFA solution at a flow rate of 0.1 ml/min. A collector equipped with a 280 nm monitor and a recorder were used to collect fractional samples at a controlled

sample volume. A total of 18 protein fractions were collected from each NMS or LCMPS sample for analysis by MALDI-TOF MS. Fraction volumes were 1.0 ml.

2.4. Analysis by MALDI-TOF MS

All MALDI-TOF MS mass spectra were obtained using a Bruker Reflex III (Breman, Germany) equipped with a 337 nm UV nitrogen laser producing 3 ns pulses. The mass spectra of proteins/peptides were obtained in the reflective mode. Mass assignments were performed with unmanipulated spectra for an optimal correction between observed and calculated mass. Protein samples were prepared in a solution containing SA (in acetone containing 2% TFA, 10 μl) and DHB saturated in water (pH 6.0). A portion (5 μl) of this solution was loaded onto the target, and the solvent was removed by air-drying. Finally, the loaded target was transferred to the mass spectrometer with optimal laser intensity for analysis. Mass spectra were collected in the positive-ion mode using an acceleration voltage of 25 kV and a delay of 475 ns. The grid voltage, guide wire voltage, and low mass gate were set to 94.0%, 0.15% and 1000.0 *m/z*, respectively. Each mass spectrum collected represents the sum of the data from 400 laser shots. For direct analysis, the serum sample for MALDI-TOF MS analysis was diluted 10-, 25-, 50- and 100-fold in 0.1% TFA solution. The samples, prepared as mixtures of 2 μl serum samples and 2 μl of SA matrix, were directly loaded onto the target for MS analysis.

2.5. Data processing and analysis

The data from each mass spectrum (i.e., the relative signal intensity versus *m/z*) were visualized using XACQ, XMASS and Biotools software provided by Bruker Co., Germany. All mass spectra were treated by a smoothing technology using a 19-point Guassian smoothing routine prior to subsequent peak picking analysis. Noting the *m/z* of the salient peaks in each mass spectrum generated peak tables. An analytical procedure for serum proteins, as described by Wang et al. and Huang et al. was employed to ascertain the numbers of unique ion signals measured by MALDI-TOF MS [16–18].

3. Results and discussion

3.1. Direct analysis of NMS proteins

An important characteristic of MALDI is that proteins/peptides are ionized using a relatively small charge [16,18]. In addition, the laser intensity of MALDI is able to decompose ferritin (consisting of 24 subunits) into its constituent subunits for MS analysis [17–19]. Even so, human serum is still a very complex system consisting of various compounds with salts, proteins, nucleic acids, lipids, and saccharides. These compounds strongly affect ionization and decomposition of the peptides/proteins to for MS analysis. Though several experimental strategies have been developed to reduce the suppression of mass signals in the serum proteome during MALDI-TOF MS analysis, satisfactory separation of proteome serum samples to reveal diagnostic biomarkers of various diseases with MS analysis is still difficult using the current separation methods. Fig. 1 shows an NMS mass spectrum measured by MALDI-TOF MS directly, without protein separation in advance. Only seven protein/peptide ion peaks with *m/z* ranging from 5000 to 90,000 were observed in the spectrum, indicating that the salts strongly suppressed peptide/protein ions, resulting in their ion signals being too weak to be measured by MALDI-TOF MS. An appropriate sample treatment by dialysis to remove salts resulted in improved protein/peptide mass signals in the serum (Fig. 2). Thus 19 different protein/peptide ion peaks with *m/z* ranging from 5000 to 70,000 were observed: that is 12 more than without dialysis. Furthermore, more than 7 different ion signals with *m/z* ranging from 5000 to 70,000 were observed compared to samples which were directly diluted 10-, 25- and 100-fold with

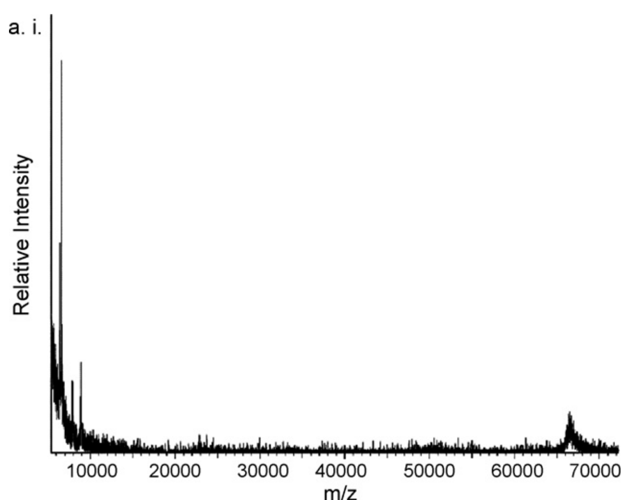

Fig. 1. MALDI-TOF mass spectrum of NMS with direct analysis before aqueous dialysis. The ion signals of peptides/proteins measured by MALDI-TOF MS are shown in the *m/z* range 5000–70,000. A total of seven ion signals of proteins from the NMS are observed.

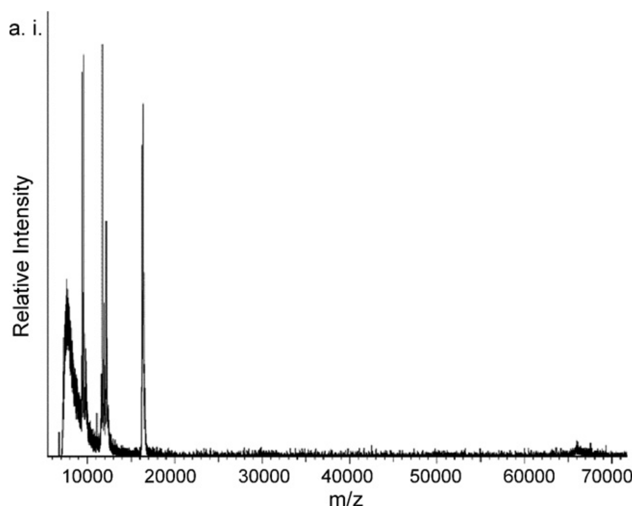

Fig. 2. MALDI-TOF mass spectrum of NMS with direct analysis after aqueous dialysis. The ion signals of peptides/proteins measured by MALDI-TOF-MS are shown in the *m/z* range 5000–70,000. A total of 19 ion signals of proteins from the NMS are observed.

0.1% TFA solution at pH 7.2. It is clear that high salt concentration plays an important role in signal suppression of protein molecular ions during MS analysis. Though dialysis and dilution are able to reduce the intensity of signal suppression during MS analysis, both still lack enough capacity to improve mass signal intensity, and so most of the peptides/proteins in the serum still cannot be observed using MALDI-TOF MS directly (Figs. 1 and 2).

3.2. Characteristics of NMS fraction mass spectra

In order to enhance the formation of molecular ions for MS analysis, HPLC and liquid phase isoelectric focusing have been developed to reduce signal suppression by the HAP in human serum [3,4]. These techniques produce more protein/peptide ions in the serum for MS analysis than does direct ionization without protein separation in advance. However, the fundamental aspects of signal suppression are not well understood. We suggest that signal suppression strongly depends on contaminants such as the matrix and HAP [16]. Though current combined approaches, such as HP LC-MALDI-TOF MS, have the ability to reduce signal suppression, they still have shortfalls. These shortfalls include the requirement for expensive analytical instruments and well-trained technicians to carry out protein separation and analysis, as well as loss and denaturation of serum proteins and cross contamination among serum samples during separation. It is for this reason that we have developed another combined off-line technology, using a gel column packed with Sephadex G-50 together with MALDI-TOF MS, as an important tool for proteome analysis of human serum. This method can eliminate the shortfalls of the current off-line combined methods of HPLC and MALDI-TOF MS. It is very difficult to find peptide biomarkers from human serum for diagnosing various fatal diseases using just HPLC separation without the aid of MS analysis. The current methods for finding biomarkers emphasize combined separation technology such as HPLC or other chromatographic methods for removing HAP from human serum and MS for proteome analysis, rather than a simple analysis with just HPLC. A simple gel column for removing HAP from serum has advantages over HPLC, and these advantages can be summarized as: simplicity, convenience, speed, cheapness, low cross pollution and high-throughput.

We have developed a home-made monitor of high sensitivity equipped with a recorder and a collector for recording a representative separation chromatogram of NMS. The NMS samples are separated into a total of 18 fractions by the gel column packed with Sephadex G-50 before MS analysis. Fig. 3 shows four major absorbance peaks of proteins/peptides from the NMS, and each peak (A–D) consists of several components, meaning that the gel column still lacks the ability to effectively separate the complex proteome in NMS into single purified proteins within the 18 fractions. This separation technique enables us to dilute, as well as remove from the serum, the HAP and the various salts having signal suppression characteristics.

Fig. 4 shows representative MALDI-TOF mass spectra from 6 of the 18 NMS protein fractions in the presence of buffer A, which consists of NH_4HCO_3 and acetonitrile [a 10% (vol./vol.) acetonitrile-total reactive volume]. Although peaks in the region of *m/z* 10,000–75,000 can be attributed to proteins with HMW, only a few protein peaks can be observed in Fig. 4A in the presence of buffer A. This means that most serum proteins with HMW can still not form molecular ions for MS analysis. Many of the peaks in the mass region *m/z* 4000–16,000 in Fig. 4B correspond to proteins/peptides of LMW. Among these peaks, about 176 have been measured by MALDI-TOF MS directly. It can be seen that the Sephadex G-50 column is able to remove signal-suppressing compounds effectively, enabling formation of protein/peptide molecular ions with characteristics of LMW in the mass region of 4000–16,000 compared to the results obtained without prior separation of the serum proteome (see Figs. 1 and 2).

In order to understand the role of buffer A in improving the capacity to form molecular ions for MS analysis, the pro-

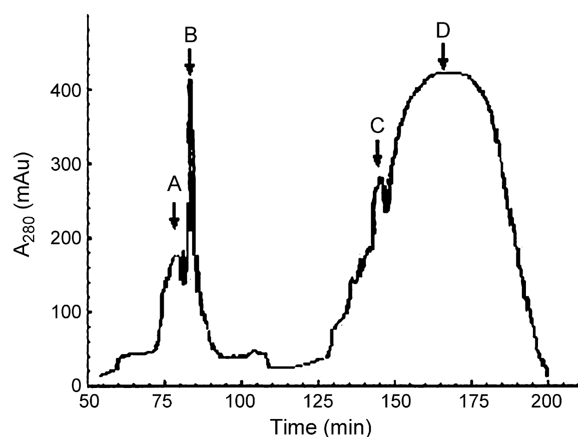

Fig. 3. Chromatogram of proteins/peptides from the NMS eluted by 0.1% TFA solution on the gel column packed with Sephadex G-50. Four major peaks of NMS proteins are recorded by a monitor equipped with a recorder and collector at 280 nm.

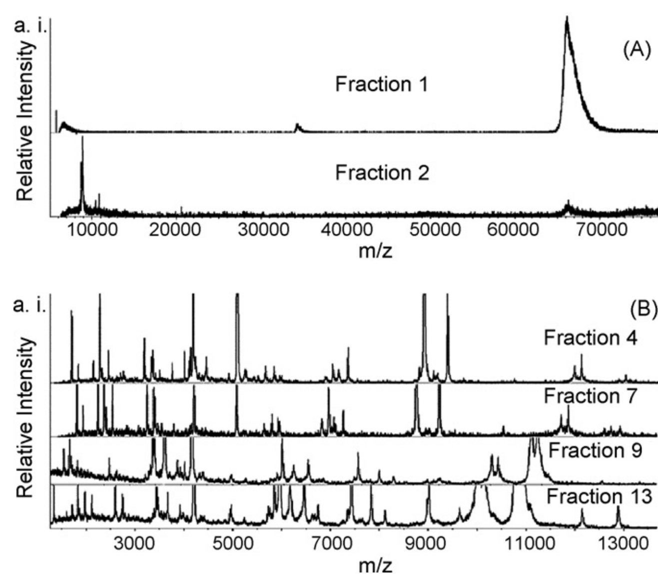

Fig. 4. MALDI-TOF mass spectra of 6 of the total 18 gel fractions from NMS separated by a gel column packed with Sephadex G-50 in the presence of buffer A. Buffer A consists of NH_4HCO_3 and acetonitrile at pH 8.2.

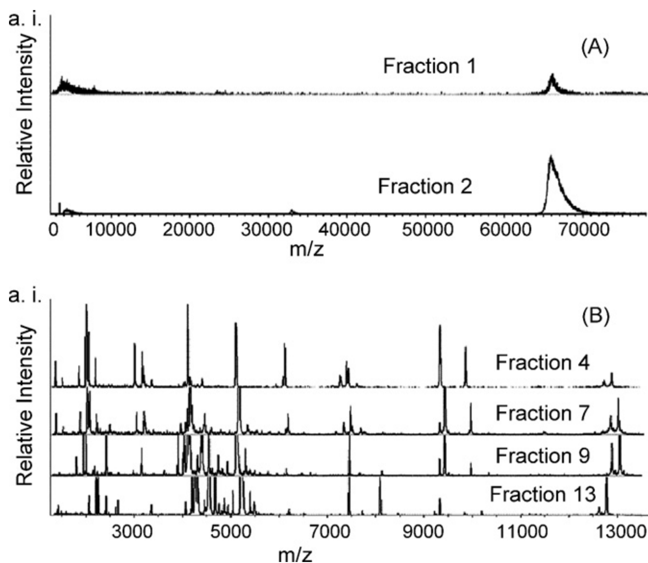

Fig. 5. MALDI-TOF mass spectra of six of the total 18 gel fractions from NMS separated by a gel column packed with Sephadex G-50 in the absence of buffer A.

tein/peptide samples of NMS were separated with a similar size gel column and measured by MALDI-TOF MS in the absence of buffer A. Fig. 5 illustrates representative peptide/protein spectra of 6 of the total 18 gel fractions of NMS. Comparing Fig. 5A with the peak numbers shown in Fig. 4A, similar numbers of mass signal peaks with HMW are observed. In this experiment, it can be seen that buffer A did not enhance the formation of molecular ions for MS analysis. This suggests that fractions 1 and 2 may still contain many HAP with HMW characteristics. These result in the signal suppression of molecular ions, and so only a few mass spectral peaks around *m/z* 10,000–75,000 are observed, with slightly different molecular masses. With reference to Fig. 5B, 143 mass peaks of molecular ions with *m/z* ranging from 4000 to 14,000 were observed by MALDI-TOF MS directly in the absence of buffer A. This is lower than the 176 mass signal peaks observed in the presence of buffer A (Fig. 4B). In summary, buffer A lacks the ability to enhance the mass signal intensity of HMW protein molecules, but it results in reduced interaction among peptides/proteins with LMW and, because it consists of 10% acetonitrile, it weakens signal suppression by salts, thus resulting in more mass peaks with LMW peptides/proteins. However, a gradient eluant for HPLC separation, consisting of one or two organic solvents covering concentration proportions from 30 to 100% results in most serum proteins being denatured easily during separation, a similar disadvantage to that posed by 2D-PAGE separation. Significantly, the gel column separation method with water eluant described here has the advantages of greatly reducing the level of denatured proteins in human serum and markedly improving the protein ionization for MS analysis.

3.3. Characteristics of LCMPS fraction mass spectra

Once more, in order to avoid having differential protein/peptide characteristics among serum samples, the 25 LCMPS samples were mixed prior to proteome separation. Based on similar separation conditions to those described above, the mixed LCMPS sample was also separated into a total of 18 fractions. Fig. 6 shows the MALDI-TOF mass spectra of the proteins/peptides in 6 out of these 18 fractions. The ion signals of the peptides/proteins measured by MALDI-TOF MS had *m/z* ranges from 1000 to 15,000 and from 15,000 to 80,000 as shown in Fig. 6B and A, respectively. Similar to the results shown in Fig. 5A, mass spectral peaks of only a few proteins are shown in Fig. 6A. It is clear that few differential proteins of HMW occurred in the 15,000 to 80,000 *m/z* range both in NMS and LCMPS, indicating that it is very difficult to look for biomarkers diagnosing liver-cancer within the HMW range. Fig. 6B shows four representative mass spectra of different LCMPS fractions separated by the Sephadex G-50 column, in which about 130 protein/peptide ion signals in the *m/z* range 1000 to 15,000 can be clearly observed. For better comparison of the NMS (Fig. 5B) and LCMPS (Fig. 6C) results, it is useful to show the serum peaks on the same scale. For this reason the lower end of the LCMPS spectra from Fig. 6B are enlarged in Fig. 6C. Based on the differential signal *m/z* of their molecular ions, several differential LMW peptides were noted at 5365, 5644, and 6426 *m/z* (a–c, respectively in Fig. 6C). It is suggested that these peaks can be used as reference biomarkers to diagnose liver-cancer. Further analysis of individual serum samples by MALDI-TOF MS revealed the same three differential *m/z* peptide peaks, indicating that each serum sample from the LCMPS rather than the NMS showed the same biomarker peptides with *m/z* of 5365, 5644, and 6426. Statistical analysis of the results obtained from the serum of 100 LCMPS volunteers revealed these differential peptides (with *m/z* of 5365, 5644, 6426) in 84%, 68% and 76% of the cases, respectively. Further experiments showed that these differential *m/z* values could not be found in the serum of male

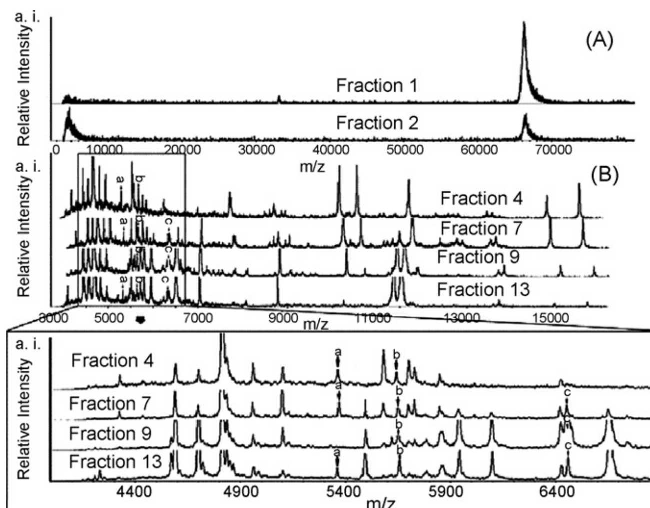

Fig. 6. MALDI-TOF mass spectra with 6 of the total 18 gel fractions from LCMPS separated by a gel column packed with Sephadex G-50 in the absence of buffer A. The mass peaks of differential peptides from the LCMPS were observed to have *m/z* of 5365, 5644, and 6426, respectively. In order to clearly see the differential peptides of both NMS and LCMPS, MALDI-TOF mass spectra of the LCMPS fractions with *m/z* range from 4300 to 6800 are further enlarged in (C).

patients with lung-cancer using similar separation and analytical methods. The diagnosic biomarkers of the patient serum with lung-cancer such as *m/z* 6139, 6544, 8417, 8480, 11109 can be found once the serum separated with Sephadex G-75 column [20]. Because of this, we suggest that those LCMPS volunteers who did not reveal the differential peptides may have had other cancers such as lung-cancer synchronously, resulting in the disappearance of, or shift in, these diagnosic biomarkers of liver-cancer.

Based on the premise that sera from cancer patients contain differential proteins and peptides, detectable by MS [21], serum proteomics and peptidomics can help oncologists in their quest for cancer biomarkers with high diagnostic accuracy. Comparisons of the results (Figs. 5 and 6) support this premise since these differential peptides from LCMPS sera were found in our laboratory using 2D-PAGE equipped with a high voltage device. With reference to proteomic analysis recently described by Chromy et al. [22], similar resolution and closeness of protein spots from LCMPS using the 2D-PAGE method have been obtained by our group after depletion of high abundance proteins with a gel column packed with Sephadex-50 as described here. Depleting serum of high abundance proteins in this way before proteomic analysis is an ideal method, that can be used to find diagnostic biomarkers of various fatal diseases in humans and other animals, based on their differential serum proteins/peptides.

4. Conclusion

Human serum contains various free salts and disturbing agents, which strongly suppress molecular ion formation of proteins/peptides during MS analysis. Appropriate sample preparation to remove the salts and HAP from the serum is thus essential for obtaining reliable results in proteome analysis [19]. Though HPLC has been widely employed to remove HAP and obtain LAP, our group found that it still exhibited many drawbacks in a combined off-line HPLC and MALDI-TOF MS technique for finding biomarkers. Based largely on our experimental results, we found that human serum separated by HPLC for subsequent proteome analysis by MALDI-TOF MS yielded differential mass signal peaks with different ions in repeated measurements. This indicates that there were decomposed peptides/proteins in the serum fraction particularly in terms of differential peptides, and that this resulted from the HPLC process leading to many of the serum proteins being easily decomposed by the MALDI-TOF MS laser. Furthermore, a set of proteins/peptides with LMW characteristics were shown to have at least a five-fold increase in intensity following HAP removal by the gel column. Such problems still pose an important limitation in serum proteomics.

The Cibracon Bule protein A/G chromatography method [23,24] has been used to deplete serum of albumin and immunoglobulins, and an increasing number of methods for the removal of other HAP from human sera are becoming commercially available (such as the Millipore Montage Albumin Depletion Kit), thus making serum analysis a more routine laboratory procedure [25]. In addition, a combined off-line method of MALDI-TOF MS and surface-enhanced laser desorption and ionization (SELDI) can profile protein intensity from human serum for MS analysis [25,26]. Based on our results, we suggest that the gel column method described here is simple, inexpensive and high reproducible, and is suitable for sample preparation and high-throughput screening in the clinic. We have shown that the differential *m/z* values of 5365, 5644, and 6426 from LCMPS can be employed as reference biomarkers for diagnosing liver-cancer. Using the same separation and analytical methods, we can also found the several diagnosic biomarkers such as *m/z* 6139, 6544, 8417, 8480, 11109 *m/z* from serum of male patients with lung-cancer. The *m/z* values of these latter biomarkers are larger than those of LCMPS and, for this reason, packing the gel column with Sephadex G-75 rather than Sepadex G-50 should be selected for separation of serum proteins/peptides and finding biomarkers, since the pore diameter of the former separation gel is larger.

To sum up, we have indicated that, compared to recently described HPLC separation and MALDI-TOF MS analysis, the simple and convenient chromatographic method described here is not only superior for MS analysis, but also reveals many novel and significant serum biomarkers for the clinical diagnosis of various diseases.

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