



Research Article

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A novel extraction method of plasma peptides for peptidomics analysis

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ABSTRACT

Different methods for plasma peptidome preparation in peptidomics analysis had been compared in this study. Plasma proteins were either precipitated with acetonitrile or subjected to thermal denaturation. The eluent was subjected to analyze by gel filtration high pressure liquid chromatography. The results indicated that acetonitrile could effectively precipitated plasma proteins, and had the best extracting effect. 2.5 fold acetonitrile was optimal. The results showed that the method was satisfactory and would be applied in peptidomics analysis of preparation.

Key words: plasma; peptidome; acetonitrile precipitation

INTRODUCTION

Lung cancer is a serious and lethal disease without a benchmark test. The clinical diagnosis of lung cancer mainly relies on traditional diagnostics such as the imaging, cytopathology and histology, which are all lack of sensitivity and specificity high enough for an early diagnostics. The current detection methods are not suitable for a large-scale screening of the lung cancer. Therefore, it is particularly important to search for new markers for early diagnosis of lung cancer. With the development of proteomics technology, plasma proteomic research has been become possible. The connective tissue-activating peptide III was reported as a new lung cancer early detection marker [1], and alphavbeta 6 peptide was a potential prediction marker for non-small cell lung cancer [2]. However, these markers are not used for clinical testing due to the potential instability of biomarkers. For example, the half-life of GLP-1 and Ghrelin peptide are 1-10 minutes and 10-30 minutes, respectively [3,4]. Samples of hydrolysis in the body are the primary cause of the change. The hydrolysis is starting from the protein which is splitted into polypeptide chains by hydrolase [5]. Further breakage of the amino acids on peptide edge cut by polypeptidase happened and the smaller polypeptide was obtained. Therefore, it is necessary to detect these markers degradation of peptide [6]. A variety of peptide group of extraction method is established, such as ultrafiltration [7] and solid phase extraction [8], was covered limited. Some studies utilizing magnetic beads extraction of peptidome were expensive and too much residual proteins [9]. In this study, Plasma peptidome extraction method was established which will prepare for lung cancer plasma peptidome study.

EXPERIMENTAL SECTION

1.1 Plasma samples

Human blood plasma samples were collected with informed consent from the lung squamous carcinoma (stage I to IV) at the Second Yueyang Hospital. The control plasma samples were also obtained with informed consent from 35 healthy volunteers who received medical checkup at the Second Yueyang Hospital. To circumvent undesirable degradation of proteins and peptides, the same strict laboratory protocol was used for each sample. Briefly, all venous blood specimens were collected into BD P100 blood collection tubes (Becton Dichinson Company, NJ, USA). Plasma fractions were separated with centrifugation at 1500 x g for 15 min at 4 °C, pipeted out and saved immediately stored at -80 °C. One freeze-and-thaw procedure was permitted for any plasma samples used in the present study. This study was approved by the Ethical Committee of the Second Hospital of Yueyang, and the

Ethical Committee of Hunan Institute of Science and Technology.

1.2 Comparison of thermal denaturation and Acetonitrile degeneration method

Five samples were thawed, vortexed and heated at 100 °C for 30 min. The samples were spun with centrifugation at 10000 x g for 30 min at 4 °C to remove the pellets following filtration with Spin-X 0.45 µm spin filters (Corning Incorporated, Corning, NY, USA). The samples were loaded into Zorbax psm 60 column (6.2 x 250 mm, Agilent, USA) coupled with 1525 HPLC system (Waters, USA). The peptidome fraction was collected from 12 to 36 minutes in the constant flow of 5% acetonitrile at 0.5 ml/min flow rate. The collected fractions were dried-up with Vacuum freeze drying (Scientz Corporation, Ningbo, China). For comparison, the same samples were denatured with addition of up to 3 x volume of 100% acetonitrile (ACN). The samples were incubated at room temperature for 30 min, followed by centrifugation as described above. The best denaturation method was selected for follow-up study.

1.3 Comparison of different proportion of acetonitrile precipitation method to extract polypeptide group

Five samples were thawed and mixed with 100% ACN at the ratios of 1: 1, 1.5: 1, 2: 1, 2.5: 1, 3: 1, 10: 1 (ACN: plasma). After incubation at room temperature for 30 min, the mixtures were centrifuged for 10 min at 12000 rpm. The supernatant was collected and passed through 0.45 µm membrane filter. The samples were purified by high pressure liquid chromatography (Waters 1525 HPLC, Waters Corporation, Milford) using a 45-min elution of 5 % eluent B (acetonitrile) in eluent A (double distilled H₂O) over 45 min on a gel filtration column (Zorbax PSM 60 column, 6.2 x 250 mm, Agilent, USA) at 0.5 mL/min flow rate. The fractions were collected from 12-36 min. The freeze-dried samples were measured by MALDI-TOF mass spectrometer.

1.4 Mass spectrometry

All mass spectrometry (MS) experiments were performed using an Applied Biosystems (Framingham, MA01701, USA) Voyager DE-STR time-of-flight mass spectrometer equipped with a N₂ laser (377 nm, 3ns pulse width, 20 Hz repetition rate). All mass spectra were acquired in the reflectron mode with delayed extraction. External calibration was performed with a mixture of peptide standards (angiotensin I, m/z 1296.6853; ACTH fragment 1-17, m/z 2093.0867; ACTH fragment 18-39, m/z 2465.1989), and measured mass accuracy was typically within ± 0.3 Da. The matrix used for all mass spectra was *o*-cyano-4-hydroxycinnamic acid (CHCA) at a saturated solution in 50% acetonitrile solution with 0.1 % TFA. Samples for mass spectra were prepared using 2 µL of the peptide solution mixed with 5µL of the matrix solution and applied to the stainless-steel sample plate. The mixture was air dried before insertion into the mass spectrometer.

RESULTS AND DISCUSSION

2.1 Compared thermal denaturation method with acetonitrile degeneration method

Reproducible and effective separation of the peptidome fractions from plasma proteins was essential for biomarker discovery. We first optimized a gel filtration chromatography method and evaluated the peptide recovery. To avoid proteolytic degradation of plasma proteins resulted from the proteases and peptidases intrinsic to plasma samples, all samples were collected using the BD P100 tube with inclusion of protein stabilizers (BD, USA). Proteins were either precipitated with acetonitrile or by thermal denaturation. The collected samples were supplied to analysis by Gel filtration high pressure liquid chromatography (Fig. 1). The chromatographic peak at the retention time of 11.5 min was detected as protein peak. The higher the protein peak area ratio, the less the protein by denaturation. As shown in Fig.1 that protein peak area ratio was 91% from thermal denaturation and 84% from acetonitrile degeneration, it was obvious that the acetonitrile denaturation method is more effective than thermal method. In addition, after thermal denaturation, the peak at retention time of 39.5 min was proven to be the molecular weight of 500 Da. It indicated that Proteins or peptides have degraded into smaller molecules in the process of degeneration. Such degradation is not helpful for subsequent analysis. Therefore, acetonitrile degeneration method without degradation was selected.

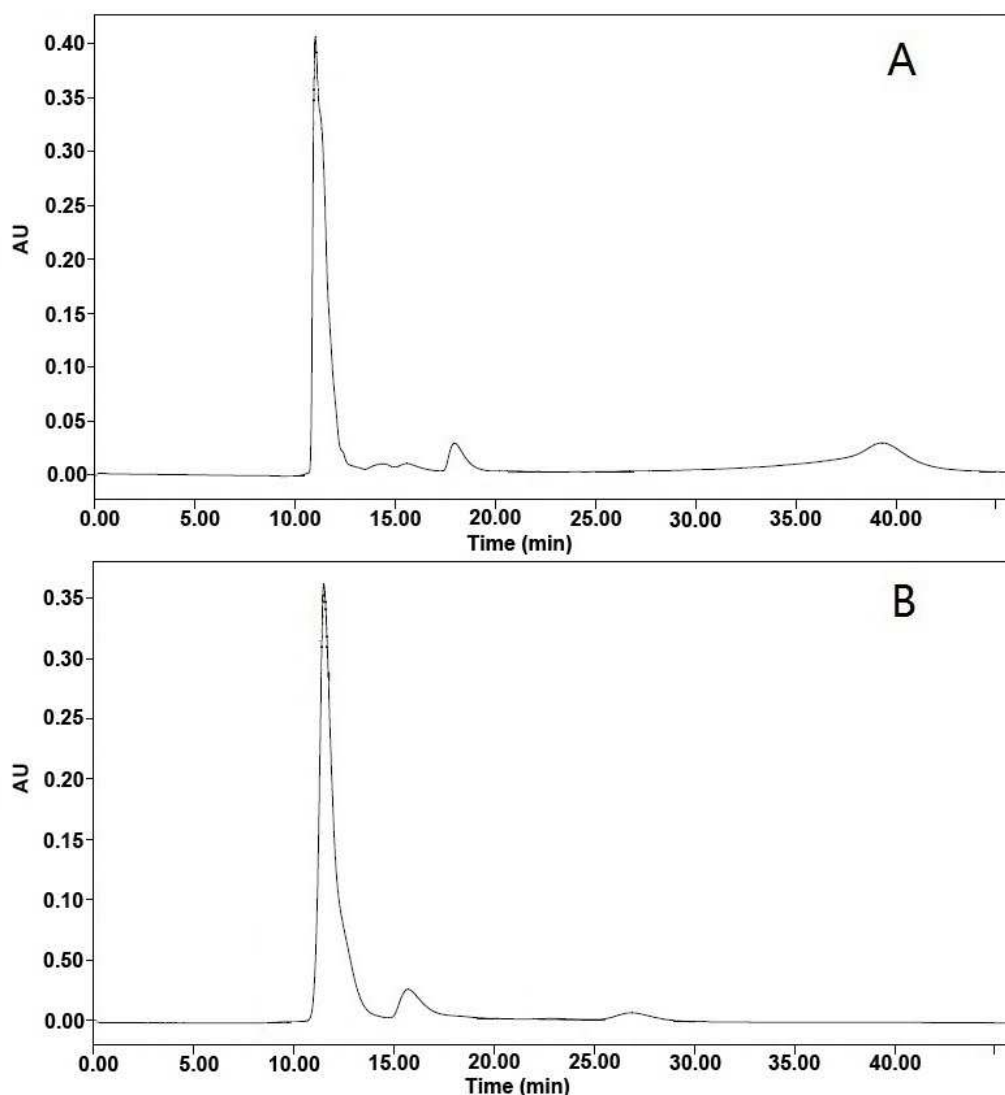


Fig.1. Gel-filtration high pressure liquid chromatography

(A) The supernatant of the plasma by using thermal denaturation; (B) The supernatant of the plasma by using acetonitrile precipitation

2.2 Comparison of different proportion of acetonitrile precipitation method to extract peptidome

According to the 2 and 10 x volumes of acetonitrile to plasma for protein removal, the extracted peptidome by MALDI-TOF MS was shown on Fig.2. While 10 x volume of acetonitrile used for denaturing could not be able to obtain enough peptides 2 x volume of acetonitrile preparation displayed a peptide profile with many more peaks (Fig 2).

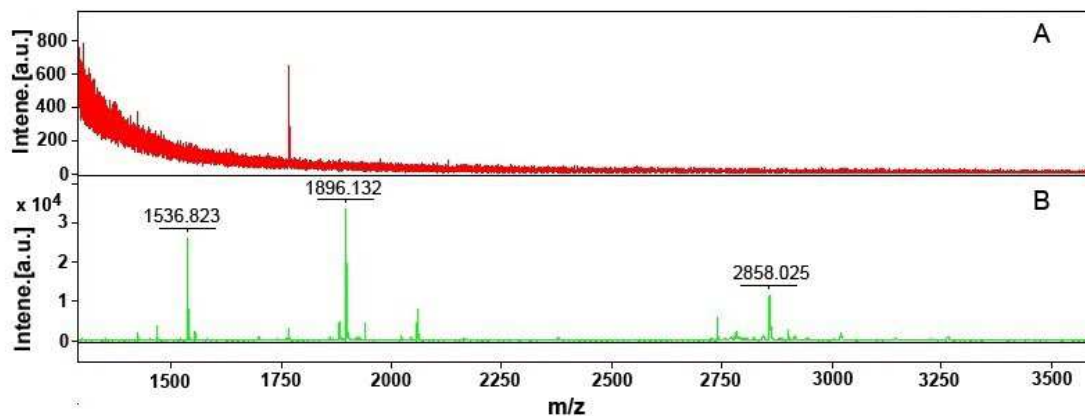


Fig.2. MALDI-TOF mass spectra of the peptidome

(A) The peptidome was collected from 10 times volume of the acetonitrile to plasma in acetonitrile precipitation and analyzed using MALDI-TOF mass spectra; (B) The peptidome was collected from 2 times volume of the acetonitrile to plasma in acetonitrile precipitation and analyzed using MALDI-TOF mass spectra

In order to optimize the extraction, we compared five ratio of acetonitrile:plasma sample (v/v) and the results were shown in Fig.3. In the range of 1000 Da-3000 Da the peaks at 1896 was detected only at 2x, 2.5x and 3x volume of acetonitrile to plasma. For further comparison, the range 1900-2400 Da was selected for spectrum acquisition (Fig.4). The obtained results of five different ratios showed that there was no peptide peak when the 1x and 1.5 x were used in extraction. The results were shown five peaks using 2 x and 3 x volume of acetonitrile to plasma in extraction with no obvious difference in terms of the peak intensity. It was suggested to select 2.5:1 ratio in all ACN-based protein removal.

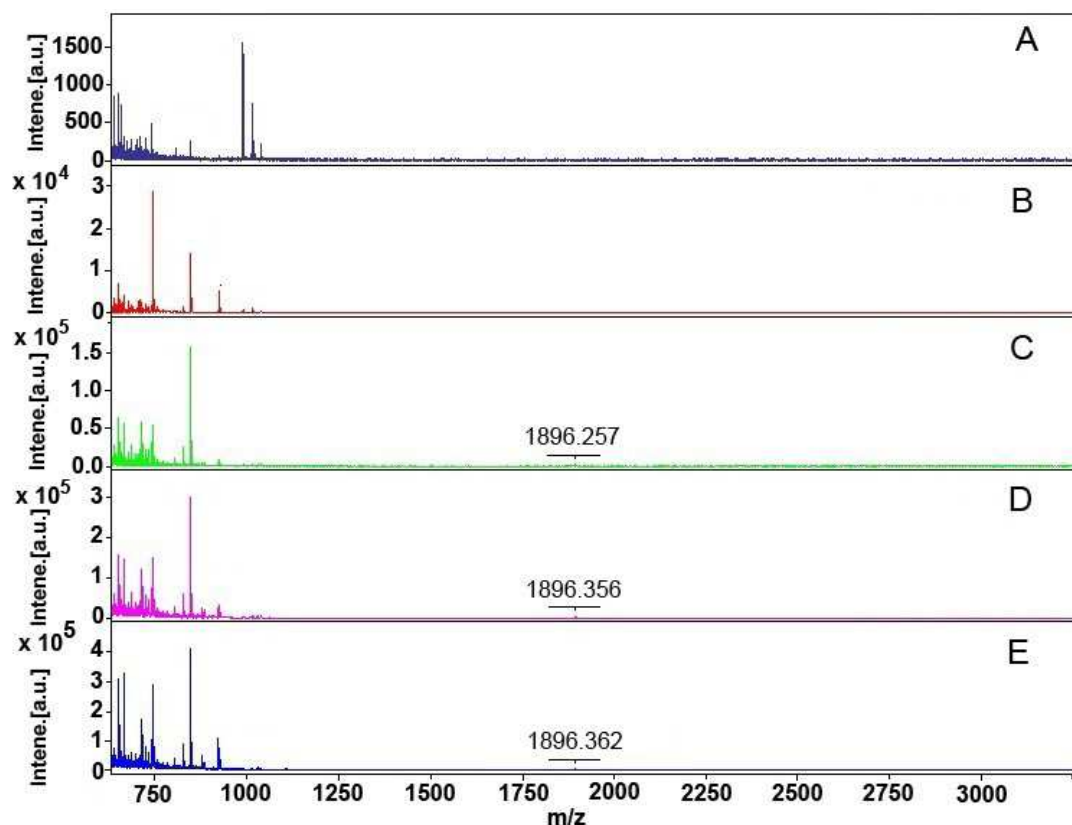


Fig.3. MALDI-TOF mass spectra of the peptidome (1000 -3000 Da)

(A) The peptidome was collected from 1 times volume of the acetonitrile to plasma in acetonitrile precipitation; (B) The peptidome was collected from 1.5 times volume of the acetonitrile to plasma in acetonitrile precipitation; (C) The peptidome was collected from 2 times volume of the acetonitrile to plasma in acetonitrile precipitation; (D) The peptidome was collected from 2.5 times volume of the acetonitrile to plasma in acetonitrile precipitation; (E) The peptidome was collected from 3 times volume of the acetonitrile to plasma in acetonitrile precipitation

The results showed that the method was satisfactory and would be applied for peptidomics analysis of preparation. Therefore, screening of the markers from plasma sample for lung cancer detection should be carried out by employing 2.5:1 ratio (v/v) of ACN: collect plasma sample. Specifically, ten samples collected from non-small cell lung cancer were thawed and mixed, and then incubated for 30 min at room temperature by addition of ACN of 2.5 x of volume of plasma sample. After that, they were centrifuged for 10 min at 12,000 rpm. The supernatant was collected and filtrated. The sample was followed by high pressure gel filtration chromatography eluted with 5 % ACN solution for 45 min. Samples fractions were collected from 12 to 36 min, pooled and freeze-dried. For comparison, normal plasma polypeptide extract group was analyzed. The establishment of the plasma polypeptide extract method is the basis for discovering stable peptides biomarker of lung cancer diseases.

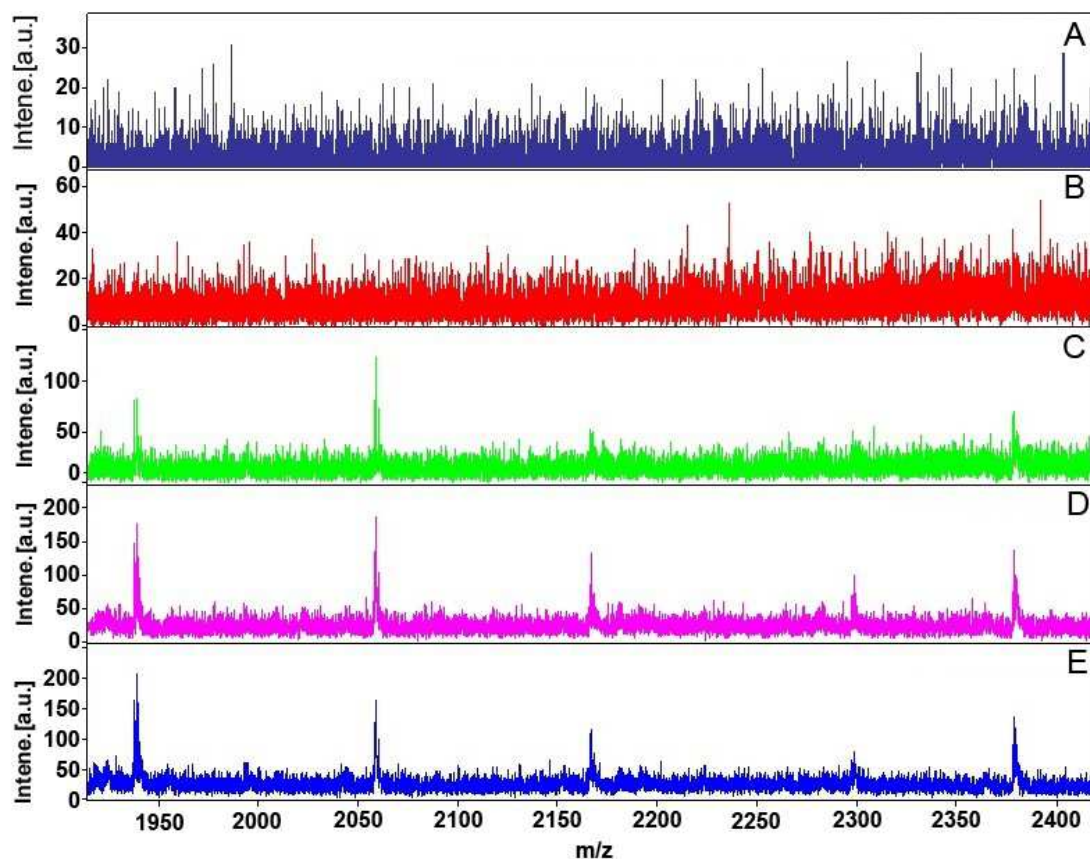


Fig.4. MALDI-TOF mass spectra of the peptidome (1900 -2400 Da)

(A) The peptidome was collected from 1 times volume of the acetonitrile to plasma in acetonitrile precipitation; (B) The peptidome was collected from 1.5 times volume of the acetonitrile to plasma in acetonitrile precipitation; (C) The peptidome was collected from 2 times volume of the acetonitrile to plasma in acetonitrile precipitation; (D) The peptidome was collected from 2.5 times volume of the acetonitrile to plasma in acetonitrile precipitation; (E) The peptidome was collected from 3 times volume of the acetonitrile to plasma in acetonitrile precipitation

CONCLUSION

In the present studies, we had established an effective and convenient method to extract peptides for biomarker discovery. The methodology to purify unabridged plasma samples without loss of targeted components is crucial. Hence our peptidome extraction technology provides the outstanding features of data comprehensiveness, which absolutely fit the indepth screening of novel biomarkers from disease samples such as serum and plasma compared to previous technologies described above.

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