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Short communication

Analysis of the endogenous human serum peptides by on-line extraction with restricted-access material and HPLC-MS/MS identification

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ABSTRACT

The selective extraction of endogenous serum peptides has been a challenge due to the high abundant proteins present in serum. Here a simple on-line extraction of peptides from human serum using strong cation-exchange diol silica restricted-access materials (SCX-RAM) coupled with two-dimensional RP–RP liquid chromatography mass spectrometry was developed. The operation of the on-line extraction system is simple to use and does not need complex equipments. The two-dimensional RP–RP was proved to be orthogonal and efficient to separate peptides extracted from human serum.

1. Introduction

The low molecular weight (LMW) of proteome was dismissed as the biological trash for it is a product of high level proteases and too small to be biologically relevant [1,2]. Recent reports using mass spectrometry-based profiling has indicated that the peptidome may reflect biological events and contain diagnostic biomarkers [3,4]. The endogenous peptides have obtained more and more attention in recent years for the potential biomarker in the body fluid [5,6]. The serum peptidome contains high dimension of information that may reflect the physiological and pathological state of the individual [2]. Since the peptidome is the low molecular weight (LMW) of proteome, it should be separated from the high concentration of proteins in serum before analysis. Up to now different sample pretreatment methods such as solid phase extraction (SPE) and ultrafiltration have been developed to capture the LMW portion of proteome [7–9]. However, these methods either extracted peptides according to their physicochemical properties or molecular weight, which does not provide the selectivity for the extraction of peptides. Several nanomaterials, which have the refined pore size to exclude the high abundant proteins, have been developed to enrich the low molecular weight peptides from serum samples [10–12]. However, most of these methods were performed with off-line mode.

Restrict access materials (RAMs) were specially designed to have the pore size of 6–8 nm with hydrophobic or other functional groups in the internal surface and hydrophilic moiety on the outer surface [13,14]. Macromolecules such as proteins are excluded from the internal surface by a size exclusion mechanism and only the LMW components can be retained on the stationary phase. Therefore, molecules were retained on RAMs based on both size exclusion and other physicochemical mechanisms. Restricted access materials were mostly used for drug and metabolite analysis in body fluids by direct injection [15–17]. Recently with the need of proteomic analysis, it has become an emerging opportunity for RAMs to provide selective extraction of peptides in complex body fluids [18–23]. For example Unger et al. [23], developed an automated multidimensional system by coupling RAMs with ion exchange and reversed phase chromatography to map peptides in human hemofiltrate. However, the system was complex with four switching valves, three gradient pumps, one isocratic pump, six chromatographic columns and two UV detectors. Besides, fractions were collected for MALDI-TOF MS analysis and only the molecular weight of peptides can be obtained.

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In this report, we developed a simple system by combing the SCX-RAM extraction on-line with reversed phase chromatography to desalt and fractionate with the UV detection. This system was constructed with only one switching valve, two gradient pumps and one UV detector, which was easy to build up and operate. After peptides were extracted, two-dimensional RP–RP separation using different pH elution was used for separation and identification of peptides, which was found to be good orthogonality for separation of the serum peptides.

2. Experimental

2.1. Chemical and reagents

Formic acid, triethylamine, alpha-cyano-4-hydroxycinnamic acid (CHCA) and trifluoroacetic acid were purchased from Sigma (St. Louis, MO, USA). Acetonitrile was chromatographic grade from Merck (Darmstadt, Germany). Water used in all procedures was prepared using a Milli-Q system (Milford, MA, USA). Human serum was provided by Dalian Red Cross Blood Center and was stored at −80 °C for further analysis.

2.2. Operation of the on-line extraction system

The scheme of the on-line extraction system is shown in Fig. 1. It consists of an LC-10ATvp (Shimadu, Kyoto, Japan), an LC-10ADvp pump (Shimadu, Kyoto, Japan), a two-position, four-port switching valve (Valco Instruments, Houston, TX) and an SPD-10Avp UV detector (Shimadzu, Kyoto, Japan). The SCX-RAM column was 20 mm × 4 mm i.d. with packing materials of Lichroprep^R 60 XDS (SO_3-Diol, 25–40 μm). The C18 analytical column was prepared in-house by packing 5 μm, 300 Å Kromasil-ODS (Kromasil, Sweden) materials into a stainless steel column (150 mm × 4.6 mm i.d.). The chromatographic data were collected with a data acquisition board (National Chromatographic R&A Center, Dalian, China). The UV detection wavelength was set at 214 nm. The mobile phases for four pumps were as follows: A was 20 mM ammonium acetate buffer (pH 3.0), B was 1 M ammonium acetate buffer (pH 3.0), C was water with 0.01% (v/v) triethylamine and D was acetonitrile with 0.01% (v/v) triethylamine, respectively. The extraction and prefractionation processes consisted of the following four steps:

1. Loading of the serum sample onto the SCX-RAM column: 400 μL of serum sample was injected onto the SCX-RAM column. Switching valve was set at position I, and pump A eluted at a flow rate of 0.2 mL/min to load the peptide onto the SCX-RAM column with the buffer of 20 mM ammonium acetate buffer (pH 3.0) for 15 min to equilibrate.
2. Eluting of peptides from the SCX-RAM column onto the C18 column: Switching valve was turned to position II, pump B began to elute the peptide from the SCX-RAM onto the C18 column with 1 M ammonium acetate buffer (pH 3.0) at a flow rate of 0.5 mL/min for 10 min.
3. Desalting of peptides on the C18 column: Switching valve was turned back to position I, pump C began to elute for desalting of peptides sample with water with 0.01% (v/v) triethylamine at a flow rate of 0.5 mL/min for 10 min.
4. Separation of the peptides on the C18 column: Switching valve was keep at position I, pumps C and D began to separate peptides on the C18 analytical column at a flow rate of 0.8 mL/min with a gradient elution as shown in Fig. 2. The fractions were collected every 3 min automatically. The collection fractions were then lyophilized and redissolved in 5 μL 0.1% FA when analyzed.

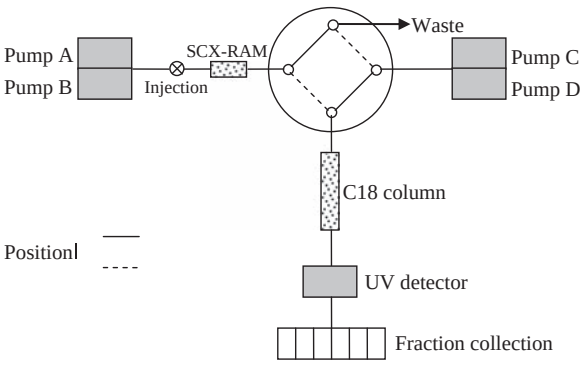

Fig. 1. Schematic representation of the on-line extraction and separation system.

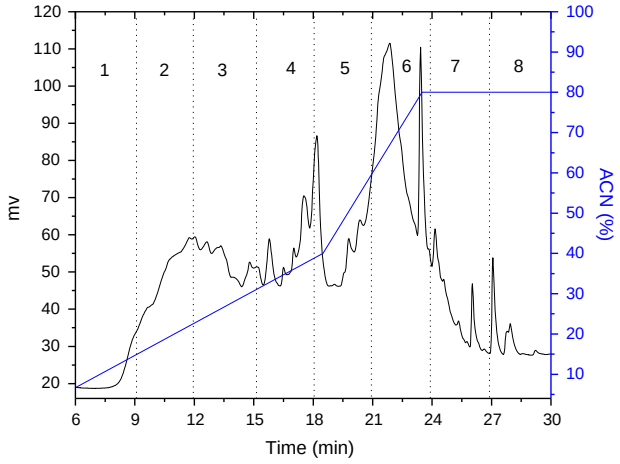

Fig. 2. The chromatogram and gradient elution profile for the first dimensional separation using a C18 analytical column at high pH.

2.3. NanoLC-MS/MS

A Finnigan surveyor MS pump (ThermoFinnigan, San Jose, CA) was used to deliver mobile phase. The pump flow rate was split by a micro-splitter valve to achieve a column flow rate of about 200 nL/min. For the capillary separation column, one end of the fused-silica capillary (75 μm i.d. × 120 mm length) was manually pulled to a fine point of ~5 μm with a flame torch. The column was in-house packed with C18 AQ particles (5 μm, 120 Å) from Michrom BioResources (Auburn, CA, USA) using a pneumatic pump. The μRPLC column was directly coupled to a LTQ linear ion trap mass spectrometer from ThermoFinnigan (San Jose, CA, USA) with a nanospray source. The LTQ instrument was operated at a positive ion detection mode. A voltage of 1.8 kV was applied to the cross. The temperature to heat capillary was set at 170 °C. Normalized collision energy was 35.0. The number of ions stored in the ion trap was regulated by the automatic gain control. Voltages across the capillary and the quadrupole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. The mass spectrometer was set at one full MS scan followed by ten MS/MS scans on the 10 most intense ions from the MS spectrum. The mobile phase consisted of A, 0.1% formic acid in water, and B, 0.1% formic acid in acetonitrile. The gradient elution program was set as follows: 98%A–90% A (0–3 min), 90%A–65%A (3–36 min), 65%A–20%A (36–38 min), 20%A–20%A (38–48 min), 20%–98% (48–51 min), 98%A–98%A (51–60 min).

2.4. Data processing and analysis

Protein/peptide identifications were searched against database IPI human (v3.04) using the SEQUEST algorithm from Thermo Electron (San Jose, CA). Search parameters used were as follows:

no enzyme, no static modification were set; variable modification was set for oxidation on Met, the mass type of peptide is set at an average. False positive rates (FPR), which were used to evaluate the reliability of the identified peptides, were calculated by using the following equation: FPR=2*n(rev)/(n(rev)+n(forw)), where n (forw) and n(rev) are the number of peptides identified in proteins with forward (normal) and reversed sequence, respectively. To have a high confident identification of peptides the search results were filtrated by a critical parameter to achieve a false positive rate (FPR) of less than 5%. Briefly, the search results were first filtrated by setting lowest X_{corr} as 1.9, 2.2 and 3.75 corresponding to 1+, 2+ and 3+ charge states, respectively, and a minimum delta correlation (ΔC_n) was set at 0.2.

3. Results and discussion

3.1. Extraction and prefractionation of peptides from human serum

Different types of RAMs (C8, C18, SCX) have been developed for the direct injection of body fluids [24]. RAMs were mostly used for direct injection of body fluids in drug and metabolite analysis. Reversed phase (RP) RAM was the most used one in the previous reports because the small molecules are mostly hydrophobic compounds. However, peptides were charged molecules in solution, and the use of RP-RAM will extract the hydrophobic compounds simultaneously with peptides, which may interfere the following mass spectrometry analysis. Therefore, for peptide extraction SCX-RAM should provide much better performance than RP-RAM. The use of SCX-RAM will extract peptides and simultaneously discard the hydrophobic components which will interfere with the MS signal of peptides. In this report we used SCX-RAM for the extraction of peptide from serum for peptidome analysis. The extraction procedure consisted of four steps: namely loading, elution, desalting and separation as described in the experimental section. During the loading process the SCX-RAM can exclude proteins out of the pore and the low molecular weight peptides were efficiently retained on the inter pore with sulfonic groups. After that, the retained peptides were then eluted down by different salt gradients.

Though the SCX-RAM can extract peptides according to the ion exchange mechanism, peptides are eluted with salt gradient and cannot be analyzed by mass spectrometry directly. RP chromatography can efficiently desalt the peptide sample and is compatible to mass spectrometry. In this system we connected the SCX-RAM with the RP column via a switching valve. By simply switching the valve, the effluent from the SCX-RAM can be turned from the waste to the C18 column as shown in Fig. 1. With the salt eluted from the SCX-RAM to RP column peptides can be concentrated on the top of the C18 analytical column, where the column efficiency will not be affected. In the next step, the C18 analytical column will desalt peptides sample being eluted with water and separated the peptide according to their hrdrophobicities with gradient elution. The chromatogram and gradient elution profile are shown in Fig. 2. The effluents from the C18 analytical column were then collected automatically every 3 min for further analysis. The combination of SCX-RAM and RP mode was complementary to each other. Here, the C18 analytical column played the role of both desalting and prefractionation of peptides extracted by SCX-RAM.

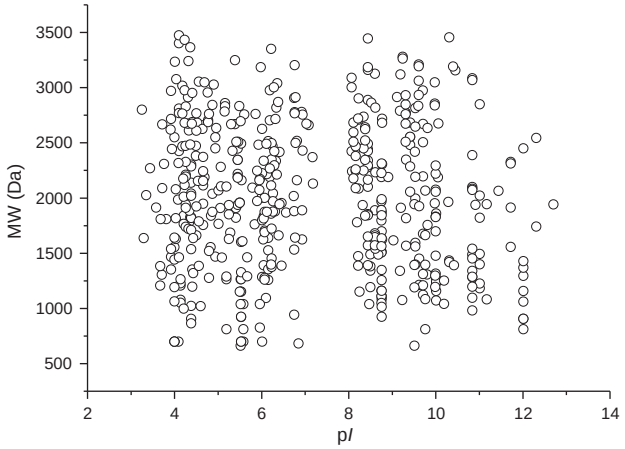

Fig. 3. Distribution of the pIs and MW of the identified peptides.

3.2. Separation and identification of peptides by nanoLC-MS/MS

NanoLC-MS/MS is the most commonly used platform for the identification in shotgun proteomics due to its high sensitivity and powerful ability for sequence identification. To have a further separation and identification of peptides fractions from the RP column were collected and submitted to nanoLC-MS/MS analysis. The p*I* and MW distribution of peptides identified are shown in Fig. 3. We can see that the majority of the identified peptides have the molecular weight between 500 Da and 3500 Da and p*I* between 3 and 12. For current most commonly used mass spectrometry, peptides in this molecular range are most easily identified. Peptides with higher molecular weight will need additional dissociation conditions and better resolutions for mass spectrometry analysis. The identified peptide number in each fraction is shown in Fig. 4(a). From it we can see that most peptides were identified in fractions 2–5, which were eluted with ACN% of 15–60%, which is consistent with the molecular weight distribution of the identified peptides. There were also peptides eluted with higher ACN proportion according to the chromatogram in Fig. 2. However, they were not identified under current MS conditions due to the high molecular weight of these peptides. The accumulative peptide number can be seen in Fig. 4(b). We can see that the number of identified peptides increased steadily with the number of fractions analyzed according to the number of peptides identified in each fraction, which indicated peptides have low overlapping between fractions.

3.3. Evaluation of the orthogonality of the two-dimensional RP–RP system

Single dimensional separation always lacks sufficient resolution capability for the separation of the large number of peptides in proteomic research. There are number of modes for two dimensional separations. The most widespread used mode was SCX-RP using a salt gradient due to the high resolution and orthogonality of SCX with RP. Although the widespread use of SCX-RP mode, it has been reported recently that SCX-RP separation modes are not completely orthogonal [25]. In principle, the retention in SCX mode is driven by the solute charge. Since the tryptic peptides are mostly 2+ (arginine/lysine at C-terminal plus primary amine at N-terminal), or 3+ charged (containing histidine or missed cleavage site in the sequence) at the acidic pH for SCX separation, the majority clustered within a relatively narrow elution window. The remaining parts of the chromatogram are only sparsely populated by the 1+, 4+, and 5+ charged peptides. Gilar et al. [25] investigated the orthogonality of SEC-RP, SCX-RP, HILIC-RP, and RP–RP two-dimensional systems and found that RP–RP system with different pH had the highest practical peak capacity. Since peptides are charged molecules comprised of ionizable basic and acidic functional groups, the change of mobile phase pH should have a pronounced effect on their retention behavior [26]. The isoelectric constant (p*I*) of peptides varies from 3 to 12,

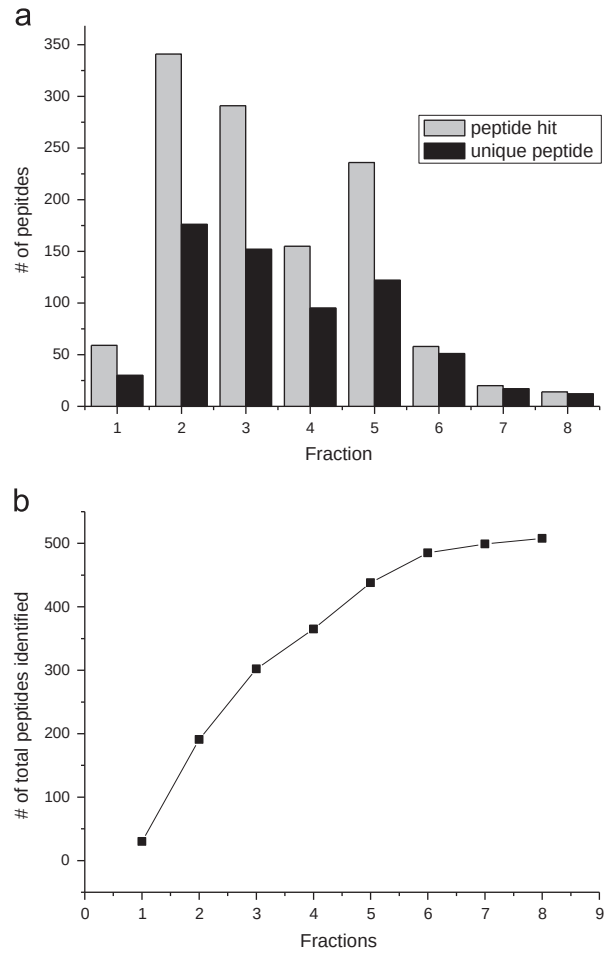

Fig. 4. (a) The identified peptide number in each fraction from the first dimension and (b) the cumulative curve of peptides identified in fractions.

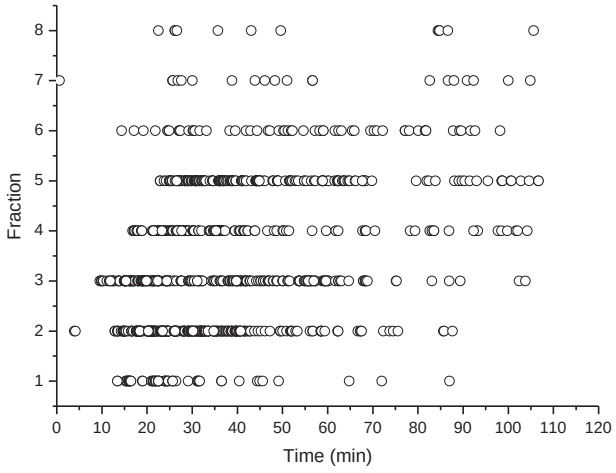

Fig. 5. 2D plot for the retention time of the identified peptides in the second dimension.

based on the amino acid composition. At a high pH, the basic amino acids are neutral, and the acidic amino acids are negatively charged. At the low pH, the basic amino acids are positively charged, and the acidic amino acids are neutral. This change in selectivity produces orthogonality between the first and second RP separations, resulting in an effective two-dimensional separation [27,28]. Besides, RP exhibits high resolution, good peak capacity, and MS compatibility. Therefore, orthogonal separation could be obtained when the first and second dimensions were carried out using different pH elutions.

Here we adopted two-dimensional separations using RP–RP mode with different pH elutions to separate the serum peptides. To make a pH of 8 in the first dimension, which is within the tolerance of most reversed stationary phase, water/ACN contained 0.01% (v/v) triethylamine was employed. Water/ACN contained 0.1% FA was used as the second dimension, which will show different selectivity of a higher pH. To check whether this two-dimensional system was orthogonal for the separation of the serum peptides we characterized the retention time distribution of the second dimension which is shown in Fig. 5. We can clearly see that the retention time of peptides distributed orthogonally among fractions eluted from the first dimension, which indicated that the two-dimensional RP–RP separation using different pH had good orthogonality.

4. Conclusion

An on-line serum peptide extraction coupled with two-dimensional RP–RP separation system was constructed with simple equipments, which was easy to operate and demonstrated to be efficient for both extraction and separation of peptides in serum. The two-dimensional RP–RP system was proved to be orthogonal for the separation of endogenous peptides.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.011.

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