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Short Communication

Selective extraction of peptides in acidic human plasma by porous silica nanoparticles for peptidome analysis with 2-D LC-MS/MS

In this study, an improved method for human plasma peptidome analysis including selective porous silica nanoparticles (MCM-41) extraction and subsequent online 2-D nano-LC-MS/MS analysis was established. Enhanced enrichment efficiency for the MCM-41 extraction was obtained by adjusting the pH of the plasma sample to 2.5. A total of 1680 unique peptides were identified in the plasma sample obtained from one healthy donor, which is nearly twice the amount identified from the native state of the plasma sample. The hydrophobic property, molecular weight (MW), and p*I* distribution of the identified peptides at pH 2.5 and native state of the plasma sample were systematically investigated and compared. Furthermore, many unusual cleaved peptides from plasma proteins (*e.g.*, HSA) were observed at pH 2.5, which clearly show a ladder pattern. The cleavage patterns for all of the identified peptides at pH 2.5 were summarized, and chymosin and cathepsin D were confirmed as the possible peptidases responsible for the change of cleavage pattern in peptide profiling.

Keywords: Human plasma peptidome / Mass spectrometry / Mesoporous silica / 2-D Nano-LC / Peptidases

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1 Introduction

Peptidomics, which is defined as the comprehensively qualitative and quantitative analysis of peptides in biological samples, is an area of increasing interest for the biomarker discovery [1–3]. For its easy accessibility and recording of the current physiological states of the body, human plasma becomes one of the rich sources for the peptidome study [4]. Recently, the advancement in MS techniques and various separation methods has resulted in great progress on different levels of human plasma peptidome study [5–7].

Although the robust approaches based on MS have been established for peptide analysis, the sample pretreatment of the complex human plasma becomes a key issue before systematic analysis. Conventionally, various materials carrying charged or hydrophobic groups are applied widely for peptide enrichment [8, 9]. A key problem of this method is that both peptides and proteins will be equally enriched resulting in low specificity for peptides. Centrifugal ultrafiltration with accurate MW cutoff is another method most widely used for peptide enrichment based on a size-exclusion mechanism [10, 11]. Unfortunately, because of the tremendously high-dynamitic range of the human plasma, the operation time will be prolonged sharply if a large amount of plasma sample is applied. At the same time, some small molecular contaminants (*e.g.*, salts) will also be concentrated. Highly ordered mesoporous silica particles, especially MCM-41 with pore size of 2 nm, are a useful complement for selective peptide enrichment based on the combination effect of size-exclusion and adsorption mechanism [12]. The unique properties of these kinds of materials are their high surface area, extremely narrow pore size distribution, and perfectly adjustable pore size. Another obvious property is their extremely high in-pore surface area (nearly 95% of the total surface area of the material), which is the key point for ensuring enough capacity for enriching peptides, but repelling other high MW proteins outside [13].

In this study, highly ordered porous silica nanoparticles, MCM-41, were used for selective extraction of peptides from human plasma at the adjusted pH 2.5. After

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Abbreviations: GRAVY, grand average hydrophobicity; **MW,** molecular weight

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that, a 2-D nano-LC-MS/MS system was used for fully resolving and analyzing the enriched peptides. Based on this comprehensive characterization and systematic comparison with results in native state human plasma previously reported [12], the peptide composition influenced by the pH adjustment was systematically investigated, and the possible proteases involving these changes were discussed.

2 Experimental

2.1 Materials and reagents

Formic acid, acetic acid, and TFA were purchased from Sigma (St. Louis, MO, USA). ACN was of chromatographic grade and purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical or HPLC grade. Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Milford, MA, USA).

2.2 Sample preparation

Fresh human plasma from one healthy female donor (O type) was purchased from Zhuanghe Blood Center (Dalian, China), which was obtained from fresh human blood by centrifugation at 4°C. An initial protein concentration of 61 mg/mL was determined in plasma using Bradford method.

The slurry of MCM-41 was prepared by mixing and sonicating 10 mg of silica with 1 mL of 100 mM glycine (pH 2.5), and then the slurry was immediately mixed with 4 mL of diluted human plasma (1 mL of plasma in 3 mL of 100 mM glycine, pH 2.5) and shaken at 25°C for 2 h. The suspension was centrifuged and washed with deionized water three times (1 mL for each time). The peptides retained on silica surface were firstly eluted with 1 mL of 1 M NaCl solution, and then with aqueous CH_3CN. After that, the eluted peptides were analyzed by either 1-D or 2-D nano-LC-MS/MS.

2.3 Sample analysis

1-D nano-LC-MS/MS was performed on the μRPLC-MS/MS system. The system consisted of a quaternary pump, an autosampler, and an LTQ mass spectrometer equipped with a nanospray source. The inner diameter for the separation columns was 75 μm and the packed length is about 12 cm. The column was directly coupled to the mass spectrometer. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in ACN. Gradient elution from 5 to 35% of ACN was applied to each sample. The LTQ instrument was operated in the positive ion mode. A voltage of 1.8 kV was applied to the cross. The mass spectrometer was set as one full MS scan followed by 11 MS/MS scans.

The configuration of 2-D nano-LC-MS/MS was identical to our previous report [12]. The peptide samples were loaded onto the SCX column. After that, a series of salt elution steps with salt concentrations of 25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 mM NH_4Ac were used to elute peptides from the SCX column onto the analytical column. After each step of salt elution, a subsequent RPLC-MS/MS was executed.

2.4 Database searching

The acquired MS/MS spectra were searched with the SEQUEST algorithm as reported before [12]. Peptide identification should meet the following criteria: $X_{corr} \geq 1.9$ with charge state of +1, $X_{corr} \geq 2.2$ with charge state of +2, and $X_{corr} \geq 3.75$ with charge state of +3. In addition, ΔCn cutoff values were ≥0.17. By using the above parameters, a false positive rate of the peptide identification less than 5% can be obtained for two randomly selected samples, demonstrating the reliability of the identified results in this study.

3 Results and discussion

It is generally accepted that the hydrophobic property of peptides can be improved by reducing the sample solution pH to the acidic range. At the same time, the ionization of silanol groups of silica particles will be largely suppressed at low pH, which results in suppression of electrostatic adsorption of plasma peptides. Thus, peptide enrichment efficiency by hydrophobic interaction can be further improved. Based on these considerations, we initially attempt to improve the peptide enrichment efficiency of the MCM-41 by adjusting the sample solution pH to 2.5. The adsorbed peptides were eluted with 50% ACN and analyzed by 1-D nano-LC-MS/MS. Finally, 369 peptides were identified from human plasma by this approach which is improved greatly when compared with 284 peptides identified in the native state human plasma, as summarized in Table 1. It is observed that only 17 peptides were identified by elution of peptides adsorbed on MCM-41 with 1 M NaCl at pH 2.5, which indicated that the electrostatic interaction of plasma peptides with silica surface is very weak.

For further reducing the complexity of the peptide sample before MS analysis, a 2-D chromatography system with combination of SCX trap column and RP analytical column as described previously [14] was used. Due to the powerful separation efficiency, the peptides enriched by MCM-41 were directly eluted with 80% ACN for the 2-D analysis. A total of 1680 unique peptides were finally identified. On comparing with our previous results for

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Table 1. Comparison analysis of the identified peptides by two elutions at different pH conditions

	50% ACN	1 M NaCl	Total
Native state[a)]	284	41	301
pH 2.5	369	17	369

a) The data for the native state of human plasma were cited from ref. [10].

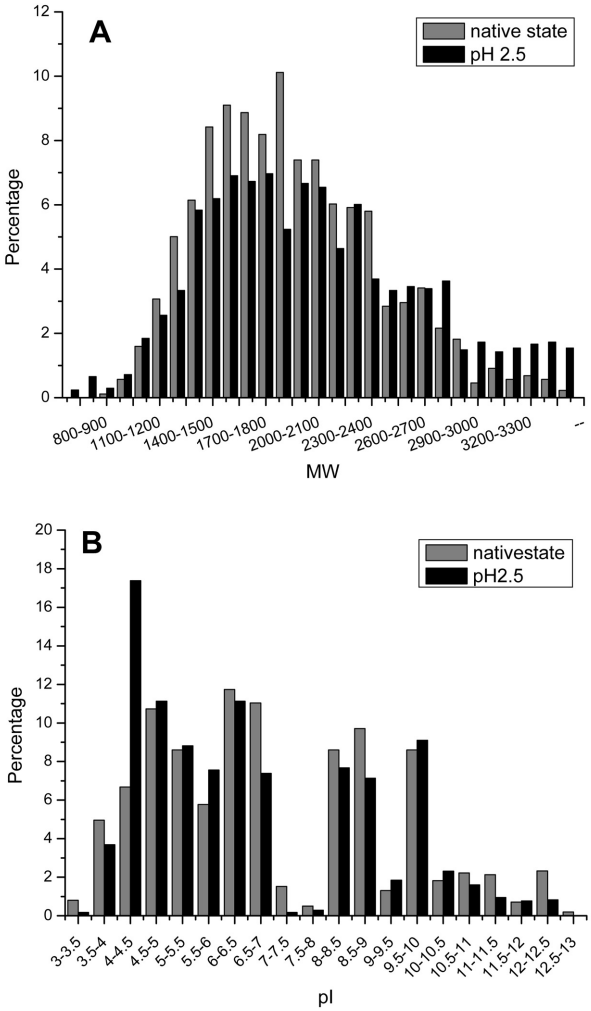

Figure 1. Distribution of molecular weight (MW) (A) and p*I* (B) of peptides identified from human plasma at pH 2.5 and native state. The data for the native state of human plasma were cited from ref. [10].

analyzing the human plasma peptidome in its native state with the same approach [12], totally 692 more peptides were identified in this study. It can be concluded from this result that the MCM-41 nanoparticle really has high capacity for peptide enrichment from a limited amount of human plasma based on hydrophobic property, and the pH adjustment of human plasma to 2.5

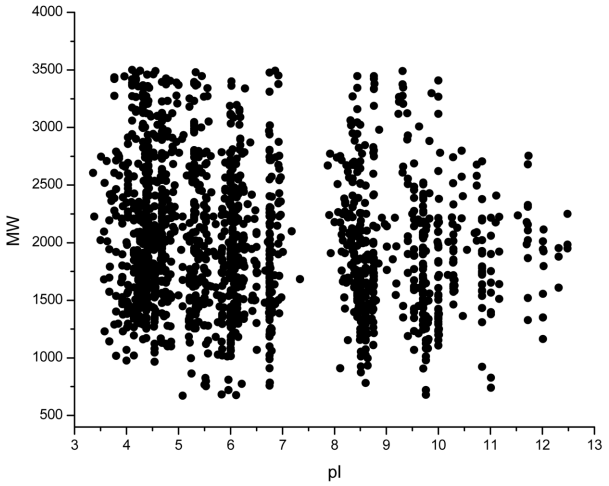

Figure 2. Scatter plot of MW *versus* p*I* distribution of the peptides enriched with MCM-41 at pH 2.5 and identified by 2-D LC-MS/MS.

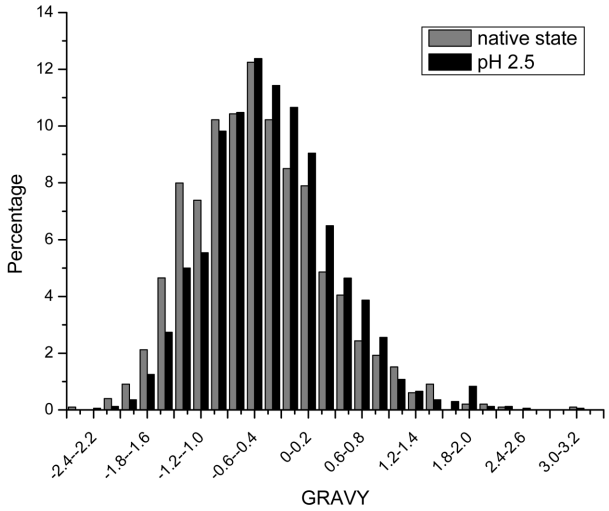

Figure 3. Distribution of GRAVY of peptides identified at pH 2.5 and native state. The data for the native state of human plasma were cited from ref. [10].

obviously improves the hydrophobic property of the human plasma peptidome.

For fully investigating the properties of the identified peptides at pH 2.5, the MW and p*I* distribution of the identified peptides at pH 2.5 were depicted and compared with native state results as described previously [12]. Peptides identified from human plasma at pH 2.5 in native state, all spread over a broad range of MW and p*I*, as shown in Fig. 1, and some distribution differences can be observed. In addition, the MW *versus* p*I* distribution of the identified peptides in human plasma at pH 2.5 is depicted in Fig. 2. It is obvious that the points of the identified peptides clustered into two groups in the acidic and basic pH range, which is in agreement with our pre-

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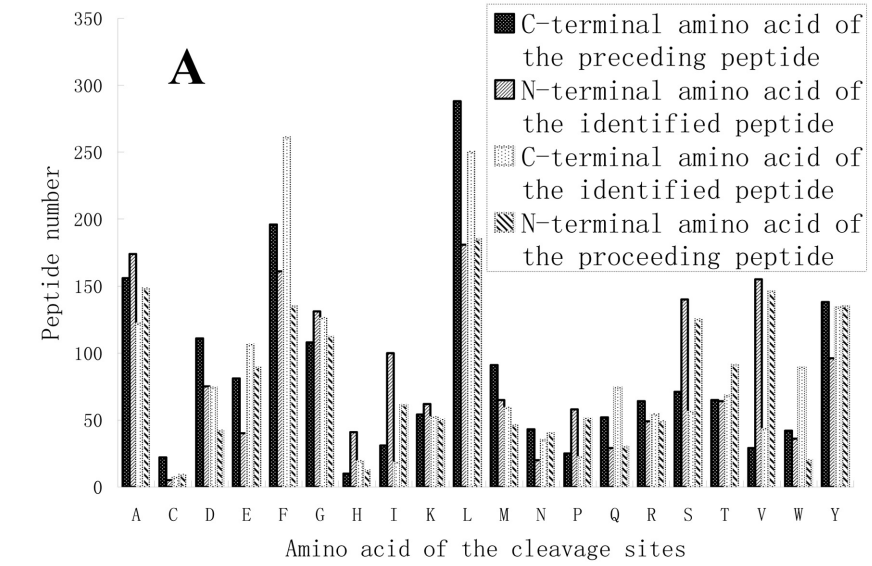
R.DAHKSEVAHRFKDL.G

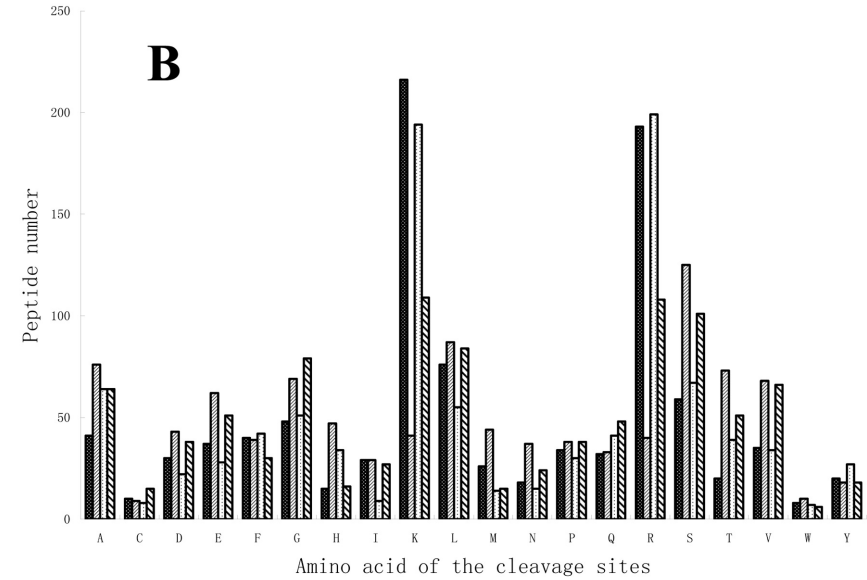
.....

R.DAHKSEVAHRFKDLGEENFKALVL.I

Figure 4. A selected peptide ladder sequence identified in HSA.

vious results from native state human plasma [12] and human urine (data not shown). The hydrophobicity of the identified peptides at pH 2.5 and native state of human plasma were also investigated as illustrated by the grand average hydrophobicity (GRAVY) values [15]. The higher the GRAVY value is, the more hydrophobic the peptide is. As shown in Fig. 3, clearly more peptides





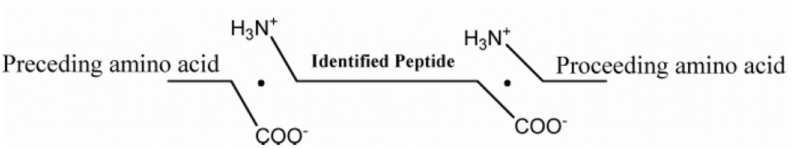


Figure 5. Peptide cleavage patterns for the peptides identified at pH 2.5 (A) and native state (B). The data for the native state of human plasma were cited from ref. [10].

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were identified at pH 2.5 in the range of GRAVY value −0.8 to 0.8. The average GRAVY values for the peptides identified at pH 2.5 and native state were −0.311 and −0.458, respectively. These results indicate that more hydrophobic peptides are enriched and identified at pH 2.5.

Interestingly, in this preliminary result for identifying the human plasma peptidome at pH 2.5, some highly abundant proteins, which are normally not likely to be cleaved in the native state of human plasma, show obvious ladders of their cleaved peptides. For example, 753 peptides belonging to HSA were generated at pH 2.5, and these peptides show an obvious ladder pattern as seen in Fig. 4. On comparison, only 12 peptides were identified from the native state of human plasma. However, for the second highest abundant proteins of human plasma, immunoglobulin group, only 36 peptides were observed at pH 2.5. Based on this observation, we believe that the cleavage of HSA at pH 2.5 is realized by certain proteases with some specificity. To confirm this deduction, human plasma was directly digested by trypsin without denaturing and cleavage of disulfide bonds, and only a limited number of peptides belonging to HSA were identified (data not shown). As we know, HSA is a global protein which makes the digestion difficult without the denaturing procedure. However, the activated peptidases at pH 2.5 can cleave HSA more easily. This means that some kind of structure specificity of the related proteases may be existing to ensure this high cleavage efficiency. As another example, apolipoprotein A-I, serotransferrin, and alpha 2 macroglobulin which are generally stable in the native state of the human plasma are also cleaved greatly at pH 2.5 with the identified peptide numbers of 1566, 634, and 246, respectively. This result also supports the existence of specificity of these peptidases which are different from the cases in the native state of human plasma.

To further elucidate these unusual cleavages at pH 2.5, the cleavage patterns of the four cleavage sites for all of the identified peptides at pH 2.5 were shown in Fig. 5A. It can be seen that leucine (L) and phenylalanine (F) dominate the cleavage site of the C-terminal amino acid of the preceding peptide and the C-terminal amino acid of the identified peptide; leucine (L) is the most cleavaged site of N-terminal amino acid of the identified peptide and N-terminal amino acid of the preceding peptide. By searching an online database of peptidases for cleavage of these bonds (http://merops.sanger.ac.uk/), we found that chymosin and cathepsin D specifically cleaved the leucine–leucine and phenylalanine–leucine bonds. Both chymosin and cathepsin D are endopeptidases which are generally active at acidic pH. Hence, it is potentially possible that the newly generated peptides at pH 2.5 are cleaved by these two peptidases and other related acidic peptidases. On comparison, the cleavage patterns of the four cleavage sites for the identified peptides at the native state of human plasma represent obviously different cases. As shown in Fig 5B, lysine (K) and arginine (R) dominate the cleavage site of the C-terminal amino acid of the preceding peptide and C-terminal amino acid of the identified peptide. A similar result was reported by Zheng *et al.* [6] by analyzing the low MW serum peptidome: lysine (K) and arginine (R) dominate the cleavage site of the C-terminal amino acid of the preceding peptide. These two cleavage sites are the characteristic cleavage sites for trypsin. As we know, many trypsin-like serine proteases, such as prothrombin, plasminogen, and coagulation factors, exist in the human plasma in large amounts [16]. Hence, it is possible that some peptides in the human plasma in their native state are cleaved by this type of proteases.

4 Concluding remarks

An improved method for efficiently capturing and globally identifying low MW peptides in human plasma has been demonstrated by adjusting the pH of human plasma sample to pH 2.5. A large amount of unusually cleaved peptides were generated at this pH, and possible peptidases responsible for these unusual cleavages were determined as chymosin and cathepsin D. These results show the peptidase influence by artificial methods and the importance of quality control of blood samples before peptidome analysis. It will be helpful to control effectively these unusual cleavages by deactivating the related peptidases, such as by adding the corresponding inhibitors and denaturing or precipitating the proteins of the human plasma before peptidome analysis.

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