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# Analysis of the human urine endogenous peptides by nanoparticle extraction and mass spectrometry identification

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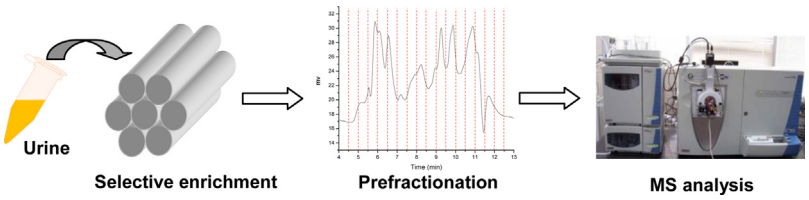
## HIGHLIGHTS

- Human urine peptides were selectively enriched by mesoporous nanoparticles.
- The extracted peptides were prefractionated by size exclusion chromatography.
- The pH of urine should be kept at its native state to avoid protein proteolysis.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Peptides in urine are excreted by kidney from the blood and tissues, which are composed of a large amount of hormones, cytokines, regulatory factors and the metabolized fragments of proteins. The peptide distribution in urine will reflect the physiological and pathophysiological processes in body. In past, limited information was reported about the composition of the peptides in urine. One possible reason is that the peptides in urine are fairly low abundant and there are high concentrations of salts and organic metabolites in the urine. In this report, we extracted the peptides from human urine by highly ordered mesoporous silica particles with the pore size of 2 nm, which will exclude the high molecular weight proteins over 12 kDa. The extracted peptides were then separated into fractions according to their molecular weight by size exclusion chromatography. Each of the fractions was further analyzed by MALDI-TOF MS and μRPLC–MS/MS. Totally, 193 peptides were identified by two-dimensional SEC/μRPLC–MS/MS analysis. By analyzing the progenitor protein of the peptides; we found that two-thirds of the proteins differed from the reported urine proteome database, and the high abundant proteins in urine proteome were less detected in the urine peptidome. The developed extraction and separation methods were efficient for the profiling of the endogenous peptides in human urine. The peptidome in human urine was complementary to the human urinary proteome and may provide an emerging field for biomarker discovery.

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## 1. Introduction

Urine is produced by the kidney via blood filtration to remove the low molecular weight proteins/peptides and other waste products such as salts and metabolites. The proteins/peptides in urine can reflect the physiological state of an individual and collection of urine is noninvasive [1]. Accordingly, urine could

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provide a rich source for cancer biomarkers and offer promise for clinical diagnosis [2]. However, urine is only used for diagnosis of a few diseases directly correlated with the function of kidney [3]. One of the reasons is probably that the protein/peptide concentration in urine is fairly low and there are too many contaminants. Therefore, efficient method needs to be developed for the in-depth study of urine sample.

Biomarker discovery by means of proteomic approach has progressed greatly in recent years since the human genome sequencing has been finished [4]. The proteome of human urine has been investigated increasingly in recent years. Various sample extraction [5–10] and separation methods [11,12] were explored for the urinary proteome analysis. For example, Pieper et al. [12] identified 150 proteins by using 2D-gel and MALDI-TOF MS and Sun et al. [6] identified 226 proteins by 1D-gel and multi-dimensional LC–MS/MS. Mann et al. [13] identified more than 1500 proteins in human urine by integrating one-dimensional sodium sulfate polyacrylamide gel electrophoresis and LC–MS/MS identification, which produced the most comprehensive database of human urine proteome established for high quality references in future proteome studies.

Peptides have a role in the inflammatory response, tumor biology, and endocrine processes, presenting them as appealing biomarker candidates. Peptidome, which is defined as the low molecular weight (LMW) portion of proteome (MW $<$ 15, 000 Da) [14–16], has attracted increasing attention in recent years [17–19]. Most reports are focused on the peptidome of serum/plasma because they are most commonly used in clinical diagnosis and contain high content of peptides/proteins [20–22]. Cancer diagnosis has been successfully achieved by the profiling of peptides in serum [17,23–25]. Petricoin et al. [26] first reported the diagnosis of ovarian cancer by using SELDI-TOF MS to profiling of the serum peptidome. Villanueva et. al. [27] used MALDI-TOF MS-based peptides profiling to successfully distinguish prostate, bladder, and breast cancer patients from healthy persons. However, there were only several reports on the study of peptidome in urine [28–31]. The large number of small peptides in urine provided the potential for disease diagnosis of the kidney function [32,33], while the peptidome of urine was not explored in-depth.

Sample preparation is the first and most important step for study of the urinary peptidome. Solid phase extraction (SPE) is currently the most commonly used method to extract the peptides from urine [28–31], and is mainly based on their hydrophobicity but has no size selectivity. Nanoporous materials have shown great promise for sample pretreatment of body fluid in recent years [17,34,35]. The silica materials with highly ordered mesoporous structure such as M41s [36] and SBA-15 [37] have unique properties of high surface areas, extremely narrow pore size distribution and perfectly adjustable pore size. Another obvious advantage of these materials is their significantly higher in-pore surface area than the external surface area. All of the above mentioned properties make these type of material promising for selective adsorption of biomolecules based on the size-exclusion mechanism [38]. We have compared three highly ordered nanoparticles with pore size of 2 nm, 8 nm and 12 nm to extract the peptides in plasma and found that MCM-41 with a pore size of 2 nm was most efficient for extraction of the peptides with a molecular weight cutoff of 12 kDa [39].

Here, we used MCM-41 nanoparticles to extract the peptides from human urine. Some high-abundant proteins such as HSA were excluded out of the pore, and only the low molecular weight peptides were adsorbed onto the nanoparticles. For the first time, the pH effect of urine preparation is studied to keep the proteins/peptides from degradation by endogenous proteases. The extracted peptides were further fractionated according to their molecular weight by using a size exclusion column, and the hydrophobic metabolites extracted by the nanoparticles were further separated from the peptides. The collected fractions were then analyzed by μRPLC–MS/MS for the identification of endogenous peptides.

## 2. Materials and methods

### 2.1. Chemicals

Formic acid, acetic acid and trifluoroacetic acid were purchased from Sigma (St. Louis, MO, USA). Chromatographic acetonitrile was obtained from Merck company (Darmstadt, Germany). Water used in all procedures was prepared using a Milli-Q system (Milford, MA, USA). Others chemicals were all analytical grade. The MCM-41 was prepared according to the procedures reported previously [36,40]. Briefly, the MCM-41 was synthesized using CTAB (cetyl trimethyl ammonium bromide) as a structure-directing agent and TEOS as the silica source. 1.8 g of CTAB was dissolved in 50 mL of deionized water followed by the addition of 10 mL of 25% (v/v) NH3 solution. 8 mL of TEOS was added and then stirred for 2–3 h, followed by the hydrothermal treatment for 48 h at 100 °C. Finally, the products were collected and calcined at 550 °C to remove the templates.

### 2.2. Sample preparation

Human urine was collected from one healthy female and four male donors in the morning and was immediately stored at −80 °C for further analysis. The slurry of MCM-41 nanoparticles was prepared by mixing 10 mg nanoparticles with 1 mL deionized water and then sonicated for 5 min to avoid agglomeration. Afterwards, the slurry was immediately mixed with 5 mL human urine directly (or adjusting the pH of urine to 2.5 with hydrochloric acid) and shaken at 25 °C for 2 h. The suspension was centrifuged at 5000 × *g* for 2 min, and then the nanoparticles were separated from the supernatant and washed with deionized water three times (1 mL for each wash). The peptides retained on silica surface were eluted with 1 mL solution containing 80% acetonitrile with separation by centrifugation. The supernatant solution with eluted peptides was lyophilized for the following experiments. When extracting the blend of yeast and urine samples 10 μg of yeast proteins were added into 5 mL urine sample and then the peptides were extracted with the conditions described above.

### 2.3. Fractionation with size exclusion chromatography

Size exclusion column was TSK SuperSW2000 (4 μm, 125 Å, 300 mm × 4.6 mm i.d.) from TOSOH company (Tokyo, Japan). The mobile phase was delivered by an LC-10ADvp pump (Shimadu, Kyoto, Japan). The detector used was SPD-M10Avp UV–vis detector (Shimadzu, Kyoto, Japan) and the chromatographic data were collected with WDL-95 workstation (National Chromatographic R&A Center, Dalian, China). Chromatographic conditions were as follows: mobile phase, 45% ACN in 0.1% TFA buffer (isocratic elution); flow rate, 0.35 mL $min^{-1}$; UV detection wavelength, 214 nm. The lyophilized urine peptides were dissolved in 25 μL 0.1% formic acid. 20 μL of the extracted sample were injected onto the SEC column. The fractions were collected automatically every 30 s from 4.0 min. Each of the fractions was lyophilized and redissolved in 5 μL 0.1% FA.

### 2.4. MALDI-TOF MS

MALDI-TOF MS was performed on the Bruker AutoflexTM instrument (Bruker Co., Bremen, Germany). The instrument was

equipped with a nitrogen laser (λ = 337 nm) with available accelerating potential in the range of +20/−20 kV. The MALDI experiments used a ground-steel sample target. All mass spectra were obtained in the positive ion detection mode. 3 μL CHCA (alpha-cyano-4-hydroxycinnamic acid) containing 0.1% TFA (trifluoroacetic acid) were added to 1 μL peptide sample, and then 1 μL aliquot of the resulting solution was deposited onto the target for analysis.

### 2.5. Peptide identification by μRPLC–MS/MS analysis

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 $min^{-1}$. 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 packed in-house 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 positive ion detection mode. A voltage of 1.8 kV was applied to the cross and the temperature to heat capillary was set at 170 °C. Normalized collision energy was 35.0 and 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 of 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 (0–5 min), 90%A–65%A (5–85 min), 65%A–20%A (85–95 min), 20%A–20%A (95–105 min), 20%A–98%A (105–110 min), 98%A–98%A (110–120 min).

### 2.6. Data analysis

Protein/peptide identifications were searched against yeast database from website (ftp://genome-ftp.stanford.edu/yeast/data_download/sequence/genomic_sequence/orf_protein/orf_-trans.fasta.gz) or human database from IPI (human v3.04) using the Sequest algorithm from Thermo Electron (San Jose, CA). Search parameters used were as follows: no enzyme, no static modifications were set; variable modification was set for oxidation on Met, the mass type of peptide was set at average. Output results were combined together using software Armone written in-house to remove keratins and the redundant data. The false positive rate of the peptide identification was determined by performing Sequest search against a composite database that included both regular and reverse protein sequences. To achieve high confident identification of the peptides, the search results were filtered by a critical parameter to achieve a false positive rate (FPR) of less than 5% according to our developed method [41]. Briefly, the lowest $X_{corr}$ was set as 1.71, 2.89 and 3.66 corresponding to 1+, 2+ and 3+ charge states, and a minimum delta correlation ($\Delta C_n$) was set at 0.14, 0.16, 0.13 corresponding to 1+, 2+ and 3+ charge states.

## 3. Results and discussion

### 3.1. Extraction of the peptides from urine

It has been assumed that proteins are filtered and excreted intact, and the degraded proteins will be reabsorbed into the bloodstream. Until recently, studies have shown that large quantities of peptides also appeared in urine [30,42]. However, the composition of the peptides in urine is not clearly understood. The lack of analysis of peptides in urine is likely due to the technical difficulty of measuring peptides in the presence of proteins [17]. Singh et al. [43] has studied the variety of peptides in urine from patients with renal disease by combined Lowry method (which detects both proteins and peptides) and Bradford method (which detects only proteins) together. The results showed that the excretion of peptides decreased in the presence of proteinuric renal disease [43]. However, this method was controversial because other contaminants such as urinary phenols, free amino acids, other endogenous compounds, and common drugs could interfere with the assay [17]. It is well known that urine contains high concentrations of small metabolites such as nucleotides and organic compounds. The small molecules will coelute with the peptides and contaminate mass spectrometric analyses. The selective extraction and separation of urine samples by nanoparticles with ordered pores have the potential to greatly promote the research of peptides in urine. There are two aspects to be taken into account for the pretreatment of urine samples. Firstly, the peptides should be selectively extracted in the high background of inorganic salts and high MW proteins. Compared with SPE extraction previously reported, the nanoparticles can exclude the large proteins out of the pore and have a higher efficiency for peptide extraction. Secondly, the peptide mixture should be kept stable, which means that there should be no additional peptides generated during the extraction process. The urine is typically relatively stable in its native state as it contains protease inhibitors

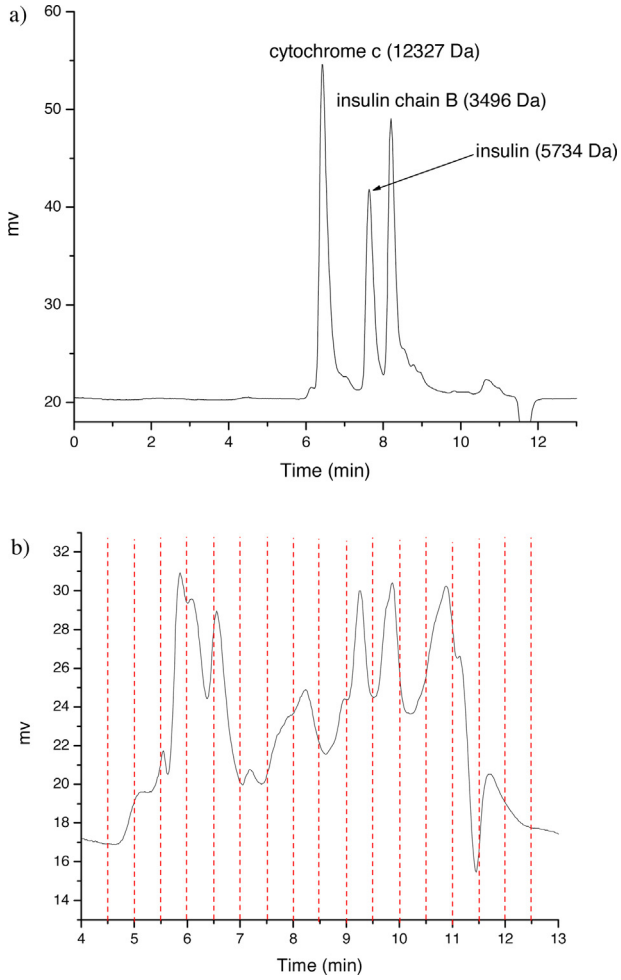


**Fig. 1.** Chromatogram of SEC fractionation of (a) the standard peptides and (b) extraction from human urine. Flow rate, 0.35 mL $min^{-1}$; UV detection, 214 nm.

(such as urinary trypsin inhibitor, inter-α-trypsin inhibitor) to protect the proteins from degradation. However, protease inhibitors may have reduced potency at low pH [44]. For example UTI (urinary trypsin inhibitor), which was a glycoprotein with the molecular weight of 67 kDa, was stable in alkali condition, but can be degraded into small peptides at pH < 3.5 [45]. On the other hand, some proteases in the urine still keep their activity at pH 2.7, thus still capable of degrading other proteins into fragments. It was reported that urine and other body fluid samples were acidified to pH 2.7 with hydrochloric acid immediately to prevent bacterial growth before analysis [6,30,46]. To check whether adjusting of the pH would affect the stability of the peptides in urine, the peptides in native state urine sample and acidified urine sample (pH 2.7) were extracted using the same procedure. The extracted peptides were then analyzed by μRPLC–MS/MS. Database search results identified only 30 peptides from the extract of urine at the native state. However, 217 peptides were identified in the sample with pH adjusted to 2.7. A similar phenomenon was also observed when working with the plasma samples [47].

To understand how the peptides were affected by the pH adjustment, we added yeast lysate into the urine samples and then separated the mixture into two parts. One was kept at the native pH (normally varied from 5.7 to 7.4 for different samples) and labeled as native urine. The other part was first adjusted to pH of 2.7, incubated for 30 min and then reverted back to the native pH. This sample was labeled as pH-processed urine. Both samples were subsequently extracted according to the same procedures. The extracts were then analyzed by μRPLC–MS/MS. The search results showed that 115 yeast peptides were identified from the native urine while 456 yeast peptides were identified from pH-processed urine. As the pH values were the same when the peptides were extracted, and with the high percentage of organic solvent (80% ACN) for elution, there will be very little interaction between the peptides and proteins. Therefore, the increasing number of the identified peptides from the yeast lysate can be attributed to the possibility that the peptides were generated in vitro at low pH. We concluded that the significantly increased number of the identified peptides may be produced due to the degradation of proteins by proteases which were activated at low pH. To avoid the generation of artificial peptides, we carried out all of the subsequent experiments with the urine peptides extracted in the native state.

### 3.2. Prefractionation by SEC for mass spectrometry analysis

The urine is a complex biofluid that contains metabolites excreted during the circulating process. Although the peptides can be selectively extracted by the nanoparticles according to the size exclusion mechanism, there were lots of metabolites and high MW peptides extracted simultaneously with the standard peptides. It is well-known that only peptides with a molecular weight below 3000 Da can be routinely analyzed by MS/MS [21]. The peptides with the molecular weight of over 3000 Da are less likely to be identified and they might interfere in the MS/MS quality of other peptides. At the same time, the high content of metabolites can overwhelm the MS signals from the peptides; therefore the peptides should be further separated from the small molecules before analysis. Size exclusion chromatography (SEC) is known to be able to separate samples according to their molecular weight based on the size sieving mechanism, which makes it easier to identify the peptides by MS/MS. We have reported previously that low molecular weight peptides can be efficiently separated by SEC by using mobile phase without salt [48]. The SEC have very good reproducibility compared to other chromatographic separations due to its pure separation according the molecular weight of the analytes [48]. Here, we used a size exclusion column to separate the extracted peptides into fractions. The chromatograms of SEC

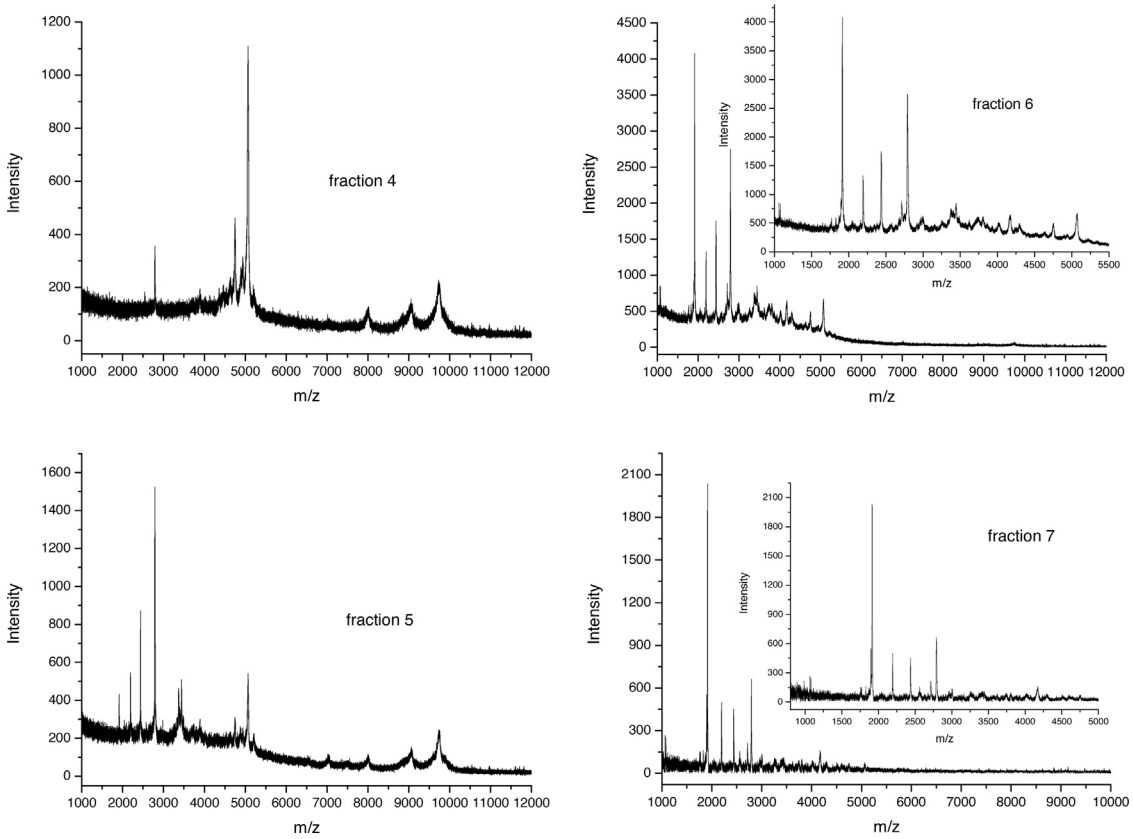


**Fig. 2.** MALDI-TOF MS spectra for the high molecular weight peptides in fractions 4–7.

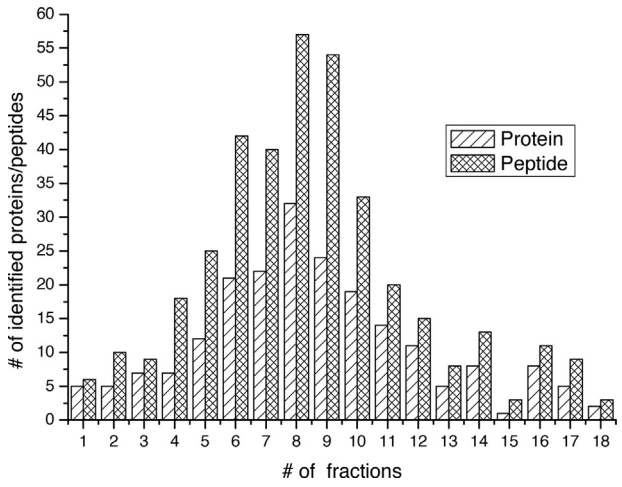


**Fig. 3.** Peptides and proteins identified for each fraction collected from the elution off SEC column.

separation of standard peptides and extraction from human urine are shown in Fig. 1. The eluted fractions were collected every 30 s from 4 min start time. We can see from UV chromatogram that most peptides began to elute off the column in the 4th fraction, and the MS peak signals from MALDI-TOF MS were also much weaker in the first 3 fractions. The selected MALDI-TOF MS spectra of the fractionated peptides from fractions 4–7 are shown in Fig 2. It can be clearly seen that the molecular weights of the extracted peptides were below 12 kDa, which also demonstrated that the large proteins were not extracted with the peptides. The mass profiling of the fractions demonstrates that the molecular weight varied from high to low as the fractions eluted, which indicated that the peptides were separated according to their molecular weights.

Each of the collected fractions was then analyzed by μRPLC–MS/MS. In total, 193 peptides were identified by combining all fractions. This was a fivefold increase from the number identified by direct analysis, which indicates that the SEC prefractionation was necessary for improving the identification of peptides. The numbers of identified peptides and proteins in each fraction are shown in Fig. 3. There were 57 peptides identified in fraction 8, which was almost double the number identified by direct analysis. We have found that most peptides were identified in fraction 6–10. Only a small part of peptides were identified from the early eluted fractions because HMW peptides eluted in these were not easily identified by MS/MS. For the late eluted fractions, the content of peptides was low and most of molecules were organic metabolites. Majority of our identified peptides had a molecular weight below 3500 Da, though there were also a significant number of peptides with HMW. So far, we can only get the molecular weight of the HMW peptides by MALDI-TOF MS or identify their progenitor protein by trypsin digestion [48]. With further development of MS technology such as TOF-MS [49] and FT-MS [50], the identification of the HMW peptides will be become more accessible. The molecular weight and normalized retention time distribution is shown Fig. 4. We found that the distribution patterns were obviously different. The peptides extracted at native state had a wide distribution of molecular weights and retention times compared to the peptides extracted at pH 2.7. This further suggested the possibility that these peptides were proteolytic fragments generated in vitro from the proteins in urine.

The identified number of the peptides was not as high as other reports, such as from the serum/plasma [21,39] or tissue peptidome [48]. The reasons for phenomenon probably are: (a) the peptide amounts in urine are actually low because the kidney will reabsorb some peptides/proteins released into the urine. (b) The urine peptides were relatively endogenous compared to the peptides in serum and in tissues [21]. We believe that some potential peptide biomarkers can be uncovered when quantitative methods such as the isotope-labeling were adopted for urine peptidomics.

### 3.3. Cleavage pattern and function analysis of the urine peptides

Since peptides are protease-induced cleavage product from proteins, we characterized the cleavage pattern to see what kinds of proteases played the dominant role during the generation of these peptides. The cleavage patterns of the peptides identified from human urine are shown in Fig. 5. It illustrates that the cleavage sites were relatively average for the peptides extracted at native state, while for the peptides extracted at pH 2.7 leucine (L) dominated the cleavage site at the C-termini 1 and 2; leucine (L) and valine (V) were the most cleaved sites at the N-termini 1 and 2. We also characterized the peptides identified from yeast. As shown in Fig. 6, the cleavage pattern of yeast peptides in acidified urine sample was consistent with that of urine peptides, which means that they are generated by the same type of proteases. By searching the database of peptidases [51] (http://merops.sanger.ac.uk/index.htm) for cleavage of these bonds, we found that cathepsin D, an aspartic protease, specifically cleaves at leucine/leucine and leucine/valine sites. Cathepsin D is an endopeptidase most active at acidic pH, and it has also been previously detected in human urine [52], likely involved in protein degradation [13,44,53]. Therefore, we presumed that the peptides in the acidified urine sample were likely generated by cathepsin D and other acid peptidases present in the urine.

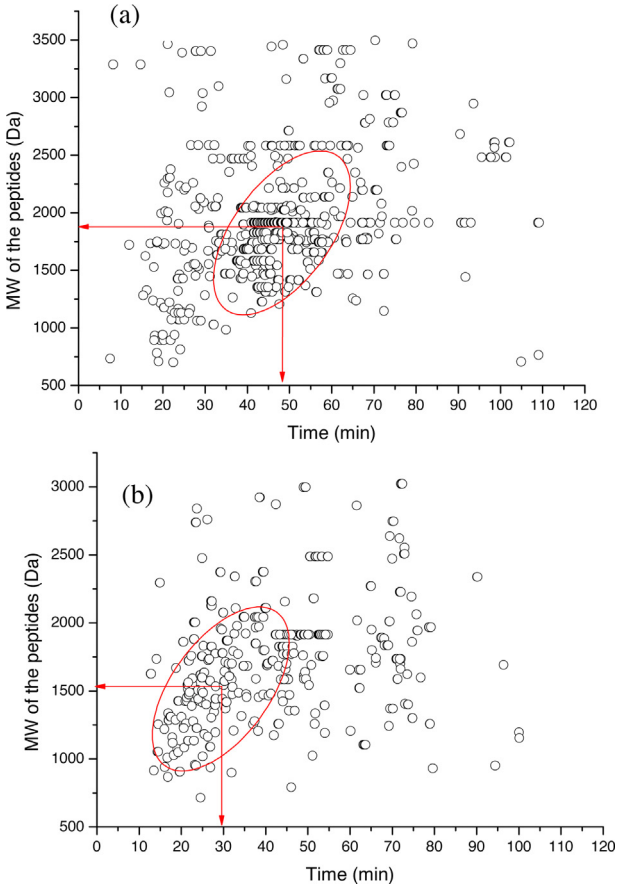


**Fig. 4.** Profiling of the molecular weight and retention time for the peptides identified at (a) native state and (b) pH 2.7.

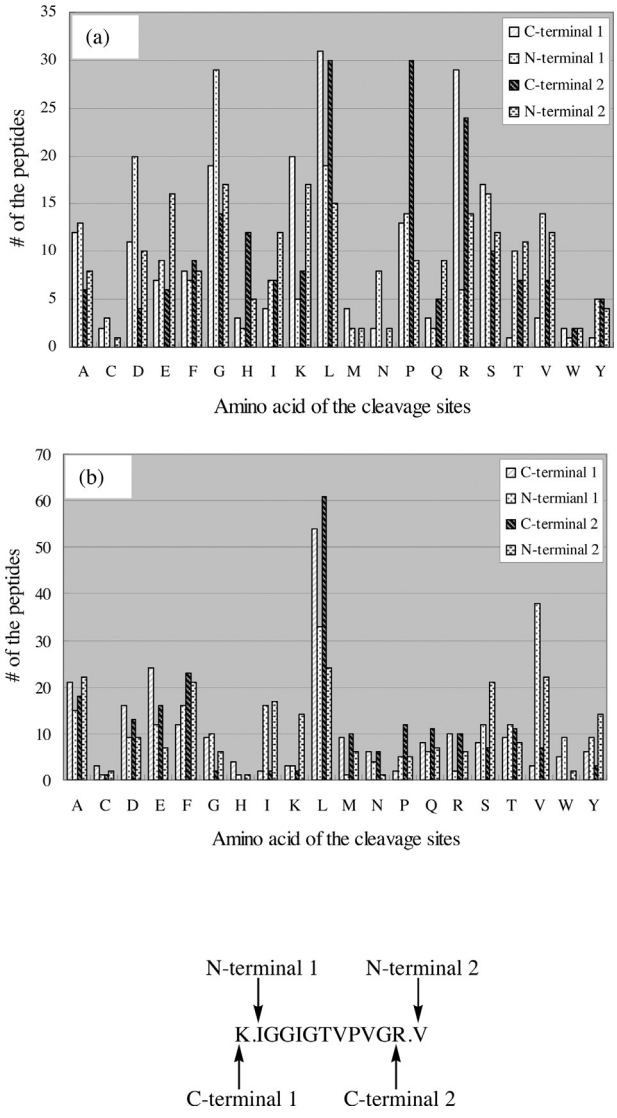





**Fig. 5.** Peptides cleavage patterns for the peptides identified at (a) native state and (b) pH 2.7.

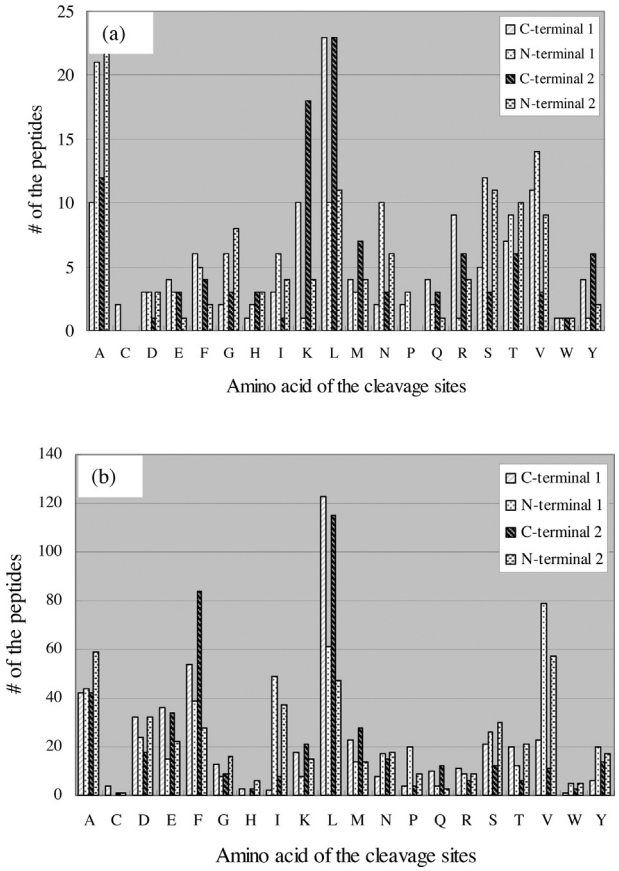


**Fig. 6.** Peptides cleavage patterns for the yeast peptides identified at (a) native urine sample and (b) pH-processed sample.

Mann et al. [13] established the most comprehensive proteome database for human urine. By comparison with the proteins identified in their report, only 41 proteins out of the 121 proteins can be found in the database. Of the top ten abundant proteins in Mann's report only two proteins (ALB protein and kininogen) were identified in our experiments. The high abundant proteins which were identified by means of proteome analysis were rarely identified here. The low overlap between the proteins identified from the endogenous peptides and from the digestion of the proteome indicated that the peptidome was complementary to the proteome in urine and not just a subfraction of proteome. For the proteins identified in the extract sample at pH 2.7, 67 proteins were overlapped with Mann's report, which was much higher than in the native state. This also suggests that some of the peptides were protein degradation products and not the endogenous peptides in vivo. Though an increasing number of peptides were identified at pH 2.7, only 21 peptides were also found in the native state data.

The most abundant proteins identified from the endogenous peptides were uromodulin and dermcidin. Uromodulin is a major glycoprotein produced by kidney cells [54] and it was recently found to be responsible for the clinical changes in interstitial renal disease, polyuria, and hyperuricaemia [55]. Dermcidin is an antibiotic peptide secreted by sweat glands first reported in 2001 [56]. The dermcidin peptide plays a key role in the innate immune response of the skin and it was reported recently that the levels of dermcidin-derived peptide in sweat were decreased in patients with atopic eczema [57]. Up to now, there have been no reports on the presence of dermcidin in urine. Besides the high abundant peptides, there were also a number of low abundant regulatory peptides and protein fragments identified, such as growth factors and protease inhibitors.

## 4. Conclusions

The peptidome in urine contains tremendous informations that needs to be further explored. The peptides can be efficiently extracted with silica nanoparticles without any pretreatments and sufficiently characterized by two-dimensional SEC/μRPLC–MS/MS analysis. Our study also indicates that the sample preparation should be performed to avoid the potential protein proteolysis, which can generate artificial peptides. The method we developed will provide a new approach to study the urine peptidome, and the profiling of the endogenous peptides in human urine could become an emerging field for biomarker discovery.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.04.040.

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