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Short communication

Urea free and more efficient sample preparation method for mass spectrometry based protein identification via combining the formic acid-assisted chemical cleavage and trypsin digestion

Shuaibin Wu[a,b], Kaiguang Yang[a,*], Zhen Liang[a], Lihua Zhang[a], Yukui Zhang[a]

[a] Key Laboratory of Separation Sciences for Analytical Chemistry, National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] Graduate School of Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

A formic acid (FA)-assisted sample preparation method was presented for protein identification via mass spectrometry (MS). Detailedly, an aqueous solution containing 2% FA and dithiothreitol was selected to perform protein denaturation, aspartic acid (D) sites cleavage and disulfide linkages reduction simultaneously at 108 °C for 2 h. Subsequently, FA wiped off via vacuum concentration. Finally, iodoacetamide (IAA) alkylation and trypsin digestion could be performed ordinally. A series of model proteins (BSA, β-lactoglobulin and apo-Transferrin) were treated respectively using such method, followed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The identified peptide number was increased by ~80% in comparison with the conventional urea-assisted sample preparation method. Moreover, BSA identification was achieved efficiently down to femtomole (25 ± 0 sequence coverage and 16 ± 1 peptides) via such method. In contrast, there were not peptides identified confidently via the urea-assisted method before desalination via the C18 zip tip. The absence of urea in this sample preparation method was an advantage for the more favorable digestion and MALDI-TOF MS analysis. The performances of two methods for the real sample (rat liver proteome) were also compared, followed by a nanoflow reversed-phase liquid chromatography with electrospray ionization tandem mass spectrometry system analysis. As a result, 1335 ± 43 peptides were identified confidently (false discovery rate <1%) via FA-assisted method, corresponding to 295 ± 12 proteins (of top match = 1 and requiring 2 unique peptides at least). In contrast, there were only 1107 ± 16 peptides (corresponding to 231 ± 10 proteins) obtained from the conventional urea-assisted method. It was serving as a more efficient protein sample preparation method for researching specific proteomes better, and providing assistance to develop other proteomics analysis methods, such as, peptide quantitative analysis.

1. Introduction

Mass spectrometry (MS) [1] is one of the most important techniques applied to identify proteins in proteomics, especially for highly complex protein mixtures such as whole proteomes in cells or tissues. For the favorable MS based protein identification, protein sample preparation [2,3] must be performed firstly using enzymes [4] or chemicals that cleave proteins at some amino acid (AA) sites for obtaining peptides. As the pivotal process, in the conventional urea-assisted protein sample preparation method, detergents [5–7] routinely used to denature proteins, such as urea and guanidine hydrochloride will disturb MS analysis to some extent. For instance, they will interfere with the whole process of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) working by suppressing the response signal of peptides and degrading the quality of mass spectrum.

Along with the unceasing progress in proteomics research, various sample preparation methods [8,9] have been always developed for more efficient protein identification based on MS. Pangburn and co-workers [10] presented a protein sample preparation method utilizing the temperature gradient and validated by several model proteins. In this method, native proteins could be digested efficiently by trypsin in an aqueous solution without urea, and this method in conjunction with MS identification obtained more peptides than that of the conventional urea-assisted sample preparation method. However, it might be not suitable for large-scale proteome identification via electrospray ionization tandem mass spectrometry (ESI–MS/MS) due to the possible protein precipitation along with heating. An acid-labile surfactant [11,12], which could decompose into insoluble degradation products was

* Corresponding author. Tel.: +86 411 84379720; fax: +86 411 84379560.
E-mail address: yangkaiguang@dicp.ac.cn (K. Yang).

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introduced recently to replace urea. However, the decomposition process may lead to the loss of hydrophobic peptides. Likewise, organic solvents [13–15] were useful for an efficient and clean protein digestion due to organic solvents can be removed by lyophilization, which is a more efficient "cleanup" step to reduce sample loss, compared to urea removal via prevalent columns packed with hydrophobic media such as C18 particles, simultaneously leading to more efficient protein identification [13].

The aspartic acid (D) site cleavage by 2% formic acid (FA) introduced by Fisher and co-workers [16] demonstrated that an aqueous solution containing FA only could be utilized as the solvent system for the cleavage of protein occurred at either side or at both sides (C- and N-terminal) of D site. The reaction provides advantages in terms of speed of cleavage and urea free operation. Those properties are advantageous for a clean protein identification via MS. Regretfully, its utility as a sample preparation method for protein identification via MS is limited, in that the peptides derived from D site cleavage [17–20] were not well suited for MS identification considering the sequence length of peptide (average ~16 AA), in comparison with peptides (~8 AA) obtained from the conventional urea-assisted protein sample preparation method, which commonly uses trypsin to cleave proteins specifically at the basic residues, arginine and lysine. Given to the drawback above mentioned, commonly a variety of acid cleavage [21,22] was combined with protease digestion for membrane protein analysis. However, such combination method was not investigated thoroughly, aiming at developing a urea free and more efficient protein sample preparation method for MS (MALDI-TOF MS and ESI–MS/MS) identification against the conventional urea-assisted protein sample preparation method for individual protein and the whole proteome analysis.

Here, a protein sample preparation method (FA-assisted sample preparation method) was reported based on the combination of the chemical D site cleavage by FA as a pretreatment procedure ahead of trypsin digestion. It was verified by model proteins and a complex proteome sample (rat liver proteome). In comparison with the result of MALDI-TOF MS and nano ESI–MS/MS identification obtained from the conventional urea-assisted protein sample preparation method, an obvious improvement in the performance of protein identification was achieved.

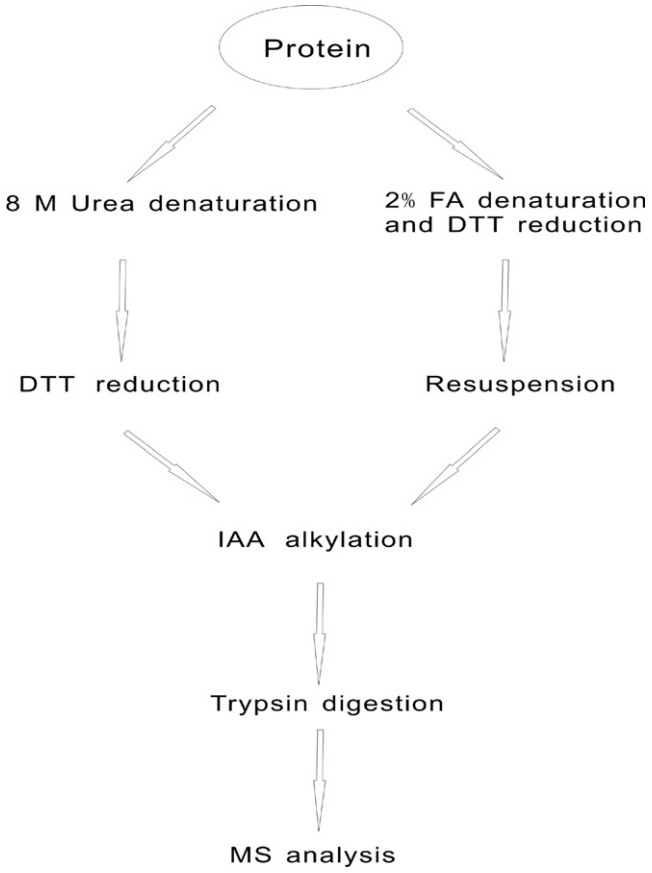

Fig. 1. Flowcharts of the urea (left) and FA (right)-assisted protein sample preparation method.

2. Experimental

2.1. Chemicals and reagents

Bovine serum albumin (BSA), β-lactoglobulin, apo-Transferrin, TPCK-treated trypsin (from bovine pancreas, activity ≥10,000 BAEE units/mg protein) and protease inhibitor mixture for protein extraction were all purchased from Sigma–Aldrich (St. Louis, MO). Dithiothreitol (DTT) and iodoacetamide (IAA) were from Acros (Geel, Belgium). Sinochrom ODS-AP C18 particles (5 μm, 300 Å) were obtained from Dalian Elite Analytical Instrument Co., Ltd. (Dalian, China). Urea (≥99.5%) was purchased from Shenyang Chemical Reagents (Shenyang, China). C18 zip tip was purchased from Millipore (Milford, France). Acetonitrile (ACN, HPLC grade) was ordered from Merck (Darmstadt, Germany). Ammonium bicarbonate (AR grade) and FA (AR grade) were supplied by Kermel (Tianjin, China). Deionized water purified by a Milli-Q system (Millipore, Milford, France) was used in all experiments.

2.2. Instrumentation

A SpeedVac (Thermo Fisher, San Jose, CA) was used to lyophilize samples. Acid cleavage at 108 °C by FA was subjected to an electric constant temperature drying oven (Zhonghuan experiment electric stove Co., Ltd., Tianjin, China). A Tissue Tearor (Bartlesville, OK) was used for homogenate. An Allegra 64R centrifuge (Beckman Coulter. Inc., Miami) was used for sample purification. MALDI-TOF MS experiments were performed on an Ultraflex III MALDI-TOF/TOF MS instrument (Bruker Daltonics, Bremen, Germany) equipped with a high rate (100 Hz) diode pumped all-solid-state SmartBeam laser (third harmonic at 355 nm) to analyze peptides in the positive reflection mode. A nanoRPLC (nanoflow reversed-phase liquid chromatography)-ESI–MS/MS system was constructed by combining the nanoRPLC with a Finnigan LTQ XL IT mass spectrometer (Thermo, San Jose, CA).

2.3. Rat liver proteome extraction

The rat liver ordered from Dalian Medical University (Dalian, China) was cut out about 0.3 mg. Immediately, the liver was put in a vessel freezed by ice cubes, and cut to pieces with continuous rinse via 1 mL ice-cold PBS buffer (0.68 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl). After that, 2 mL 8 M urea or 2% FA together with 1 mM protease inhibitors cocktail were added into the fragments at a volume of 100:1 (v/v), followed by homogenate at 30,000 rpm for 3 min. Then the sample was purified at 4 °C with a speed of 15,000 × *g* for 1 h. The supernatant was taken out and diluted with 50 mM ammonium bicarbonate buffer (pH 8.2) for the further protein concentration measurement using a Bradford protein assay kit.

2.4. Urea-assisted protein sample preparation

Proteins (1 mg) were dissolved and denatured by 100 μL urea (8 M), and further 20 μL DTT (100 mM) was added for the reduction

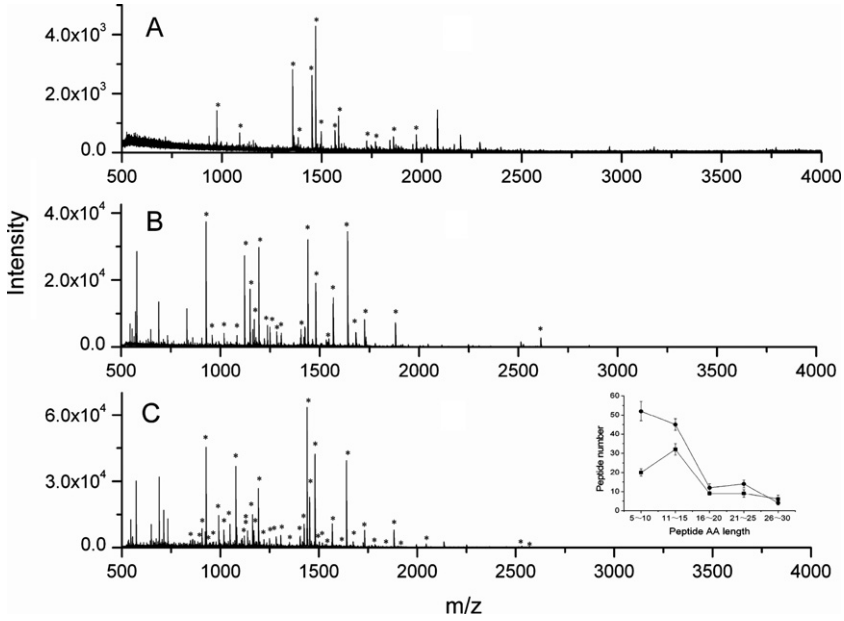

Fig. 2. MALDI-TOF MS spectra of BSA (100 ng/μL) via chemical cleavage at D site by FA (a), the urea (b) and (c) FA-assisted protein sample preparation method. All matched peptides were marked with "*". Inset figure: the length distribution of peptide obtained from three model proteins (BSA, β-lactoglobulin and apo-Transferrin) using Urea (■) and FA (●)-assisted protein sample preparation method.

of disulfide linkages at 60 °C for 1 h. After cooled down to room temperature, 20 μL IAA (200 mM) was added for alkylation in the dark for 30 min, followed by dilution with 50 mM ammonium bicarbonate buffer (pH, 8.2) to decrease the urea concentration below 1 mol/L. In solution trypsin digestion was carried out by adding trypsin into the protein solution with an enzyme-to-protein ratio of 1:50 (w/w) at 37 °C for 12 h for model proteins. Finally, FA was added to quench the digestion process. For proteome samples, the trypsin-to-protein ratio was increased to 1:25, and keeping trypsin digestion for 24 h.

2.5. FA-assisted protein sample preparation

Proteins (1 mg) dissolved in different aqueous FA solutions (0.5%, 2%, 5%, and 10% (v/v)) including 20 mM DTT at 108 °C reacted for 2 h for optimization. After cooled down, the peptide solution was vacuum-concentrated. 10 μg of the peptides was resolubilized in 100 μL of 50 mM ammonium bicarbonate buffer, followed by adding 1 μL IAA (40 mM) for alkylation in the dark 30 min. Trypsin digestion of model proteins was carried out as described in the conventional urea-assisted protein sample preparation digestion. For proteome samples, the trypsin-to-protein ratio ranged from 1:1, 1:5, 1:25, 1:50 and 1:100 for optimization, and keeping digestion for 24 h.

2.6. MALDI-TOF MS analysis

External calibration of MALDI-TOF MS was performed with ten commercial standard peptides. Spectra were acquired from the accumulation of 1000 laser shots. The voltage was set as following: acceleration, 21.85 kV; lens, 9.2 kV; reflector 1, 26.39 kV; reflector 2, 14.0 kV. A 7 mg/mL CHCA in 70% (v/v) aqueous ACN with 0.1% (v/v) TFA solution was prepared by adding CHCA (solid) to the organic solvent, followed by the addition of water and acid. The matrix solution was thoroughly vortexed and centrifuged. The sample and matrix solution were mixed with 1:1 (v/v) in a 300 μL eppendorf tube, and 0.5 μL of the mixed solution was deposited onto a MALDI target plate. MALDI-TOF MS analysis was performed in the same condition for comparing methods, and two spots were applied for each sample.

2.7. NanoRPLC-ESI–MS/MS analysis

A 16 cm long capillary (75 μm i.d.) with pulled spray tip was packed with C18 particles at 5000–7000 psi by a high-pressure pump overnight. Meanwhile, a 2 cm long capillary (200 μm i.d.) packed with the same particles was prepared as the pre-column. Two kinds of buffer solutions for NanoRPLC were (A) H_2O with 2% (v/v) ACN containing 0.1% (v/v) FA and (B) ACN with 2% (v/v) H_2O and 0.1% (v/v) FA, respectively. The gradient was set as follows for optimizing conditions: 0–5 min, 0% B (v/v); 5–10 min, 0–10% B (v/v); 10–40 min, 10–40% B (v/v); 40–45 min, 40–80% B; 45–50 min, 80% B; 50–60 min, 100% B (v/v), and 200 ng sample was injected to the analysis system. The gradient was set as follows for comparing two methods: 0–10 min, 0% B (v/v); 10–100 min, 10–40% B (v/v); 100–110 min, 40–80% B (v/v); 110–120 min, 20% B (v/v), and 2 μg sample was injected to the analysis system. The ESI voltage was all set at 1.8 kV for LTQ, and the spray capillary was heated to 180 °C. Total ion current chromatograms and mass spectra ranging from m/z 400 to 1800 were recorded with the Xcalibur software (v 3.1). The MS was set as one full MS scan followed by seven MS/MS scans. Each sample was analyzed by the nanoRPLC-ESI -MS/MS system twice.

2.8. Database searching and interpretation

MALDI-TOF MS spectra were determined based on the MASCOT (http://www.matrixscience.com) PMF search program by querying the SwissProt 57.1 (462 764 sequences; 163 773 385 residues) database assisted by BioTools 3.0 program (Bruker Daltonik). For urea-assisted protein sample preparation, mass tolerance was set to 100 ppm, carbamidomethyl (C) as fixed modification and oxidation as variable modification; allowed up to two missed cleavage sites. For FA-assisted protein sample preparation, a new 'enzyme' Formic acid + Trypsin was set up (cleavage at K/R/D sites) firstly in silico following instructions in MASCOT user's manual. Other two variable modifications were added to suit acid cleavage:

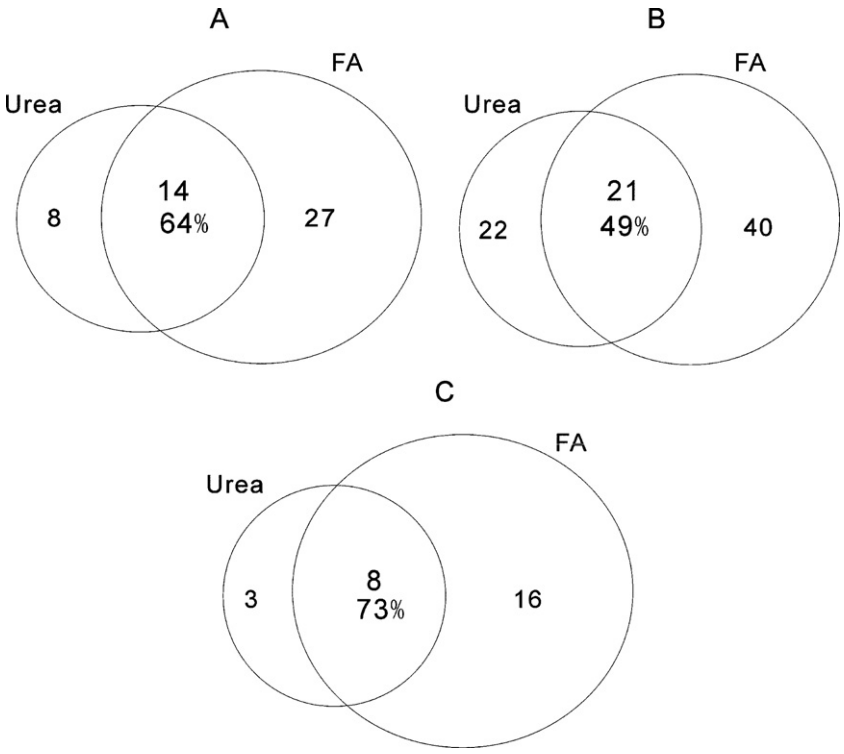

Fig. 3. Venn diagram showing comparison of detected peptides from three model proteins. (A) BSA. (B) Apo-Transferrin. (C) β-Lactoglobulin.

formylation and pyroglutamic acid [23], and allowed up to four missed cleavage sites. For Chemical D site cleavage by FA [16], formic acid was used as cleavage 'enzyme'. Without carbamidomethyl, other conditions were set as FA-assisted protein sample preparation method.

Tandem mass spectra detection and database searching were operated by the Sequest database search engine. Peptides searched using fully tryptic cleavage constraints and at most two and four internal cleavages sites were allowed for the conventional urea and FA-assisted protein sample preparation, respectively. Trypsin (cleavage at K/R sites) was used as the enzyme for the urea-assisted method. TrypsinFormic (cleavage at K/R/D sites) was set up in the Bioworks Browser software (v 3.1), and used as the enzyme for the urea-assisted method. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. Cysteine residues were searched as static modification of 57.0215 Da, and variable modifications were abandoned for the optimization of MS/MS spectra. Parameters for peptide filtering were set as follows: the cross-correlation score (X_{corr}) values were higher than 1.9, 2.2, and 3.75, respectively for singly, doubly, and triply charged peptides, and $\Delta C_n > 0.3$ and false discovery rate (FDR) was also controlled less than 1% based on the number of accepted decoy database peptides. FPR was calculated using the following equation, FPR = $2 \times n$ (rev)/(n (rev) + n (real)), where n (real) is the number of peptides matched

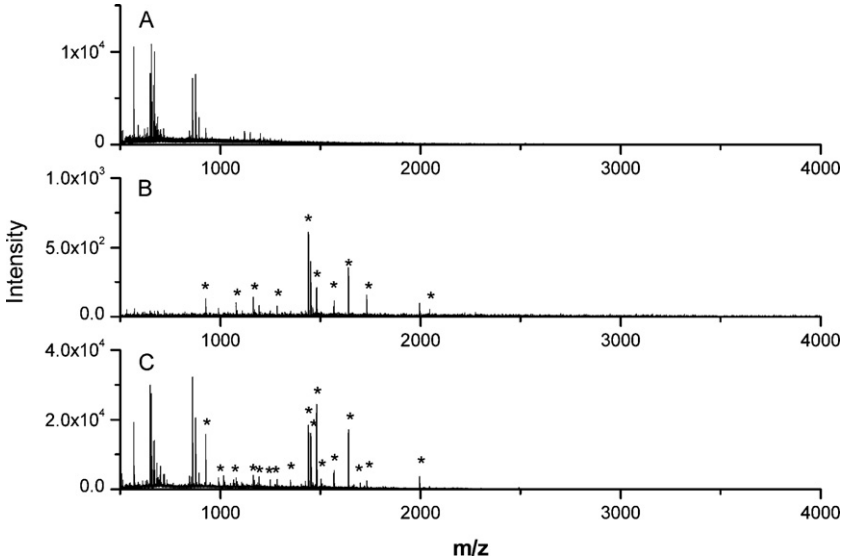

Fig. 4. MALDI-TOF MS spectra of BSA (1 ng/μL) via the urea (A and B) and (C) FA-assisted protein sample preparation method. All matched peptides were marked with "*". Urea was removed (B) via a C18 zip tip following the operation protocol in user's manual.

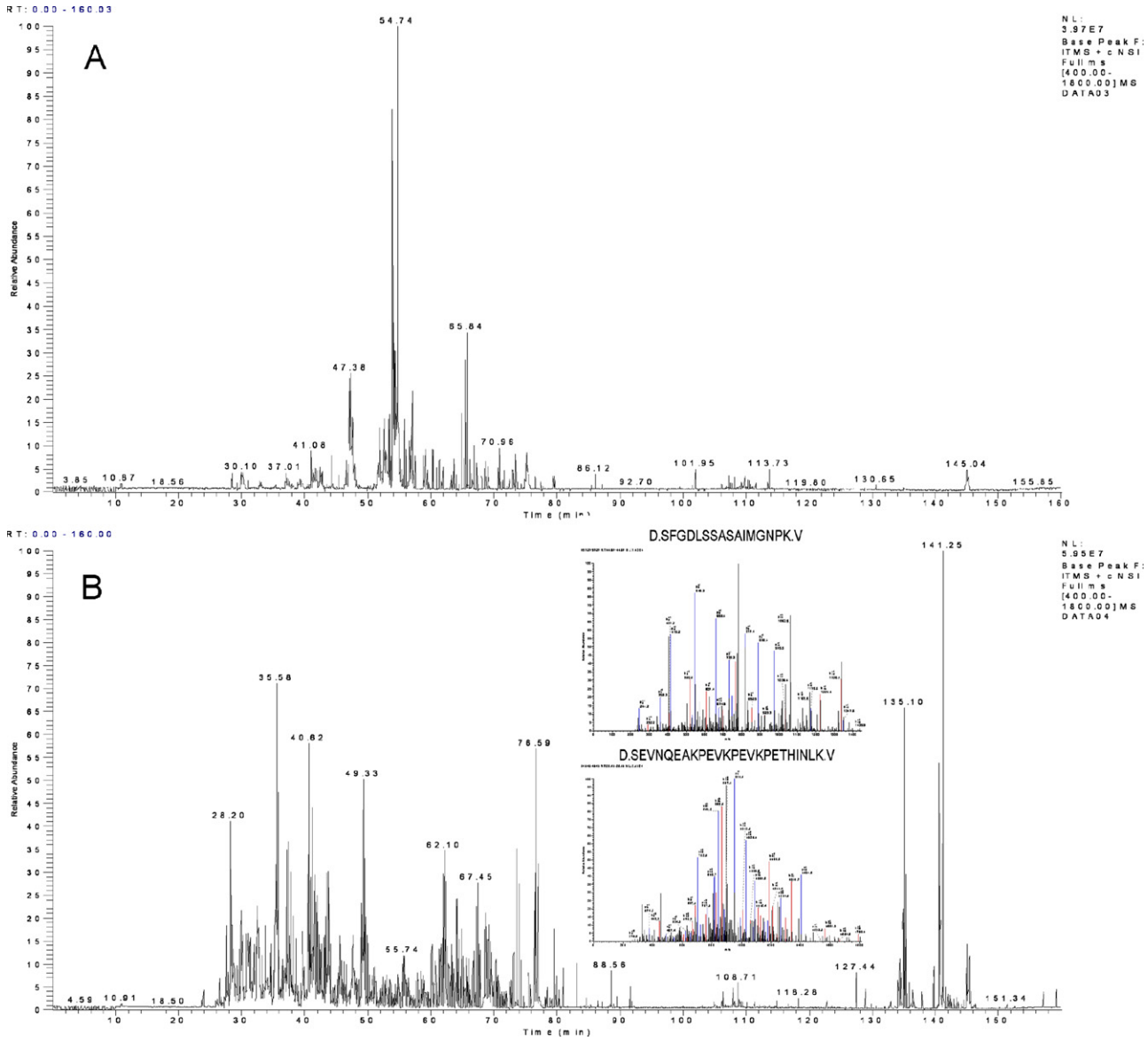

Fig. 5. Base peak chromatograms of nanoRPLC-ESI–MS/MS analysis of tryptic peptides obtained from digestion of rat liver proteome via the urea (A) and FA (B) -assisted protein sample preparation method; inset figure: tandem mass spectra of peptides, D.SFGDLSSASAIMGNPK.V (A) and D.SEVNQEAKPEVKPEVKPETHINLK.V (B) from rat liver proteome via the FA-assisted protein sample preparation method.

to "real" proteins, and n (rev) is the number of peptides matched to "reverse" proteins, respectively. To further increase the confidence of proteins, of top match was equal to 1, and a minimum of two unique peptides matches to a protein were required.

3. Results and discussion

Flowcharts of the urea and FA-assisted protein sample preparation method were illustrated contrastively in Fig. 1. 2% FA investigated in the Ref. [16] was recommended as the most appropriate solvent system for MALDI-TOF MS identification. 2 h was also investigated as the optimal acid cleavage time in that report. For comparison, two BSA aliquots (1 mg), a protein which is tightly folded and resistant to digestion, was treated respectively in vials according to two methods mentioned in Fig. 1. As a control, amounts of DTT and IAA for FA-assisted method were also calculated to keep accordance with that used in the urea-assisted method. Finally, trypsin digestions of two methods were operated in same conditions and batch as described in the experiment section. Simultaneously, BSA peptides obtained from the 2% FA cleavage in the same batch of FA-assisted method was also collected for comparison. Finally, three samples were analyzed simultaneously via MALDI-TOF MS using the dried spot method. Generally, the S/N of peptide represents the amount of the final peptides of digestion with a strong correlation to the digestion efficiency. As shown in Fig. 2a, a poor S/N was observed even after accumulation up to 1000 laser shots for BSA peptides obtained from the 2% FA cleavage. Whereas BSA treated with the FA-assisted protein sample preparation method (Fig. 2c) generated a similar MALDI signal intensity with the urea-assisted (Fig. 2b), which all clearly showed about a 10-fold increase in the absolute intensity of peptide peaks compared to that of chemical D site cleavage. The corresponding MASCOT searching result was listed in Table S1. There were not any peptide identified confidently from the 2% FA cleavage method. In contrast, 38 ± 2 peptides (40 ± 1% sequence coverage) and 22 ± 1 peptides (37 ± 3% sequence coverage) were obtained from the FA and urea-assisted protein sample preparation method, respectively.

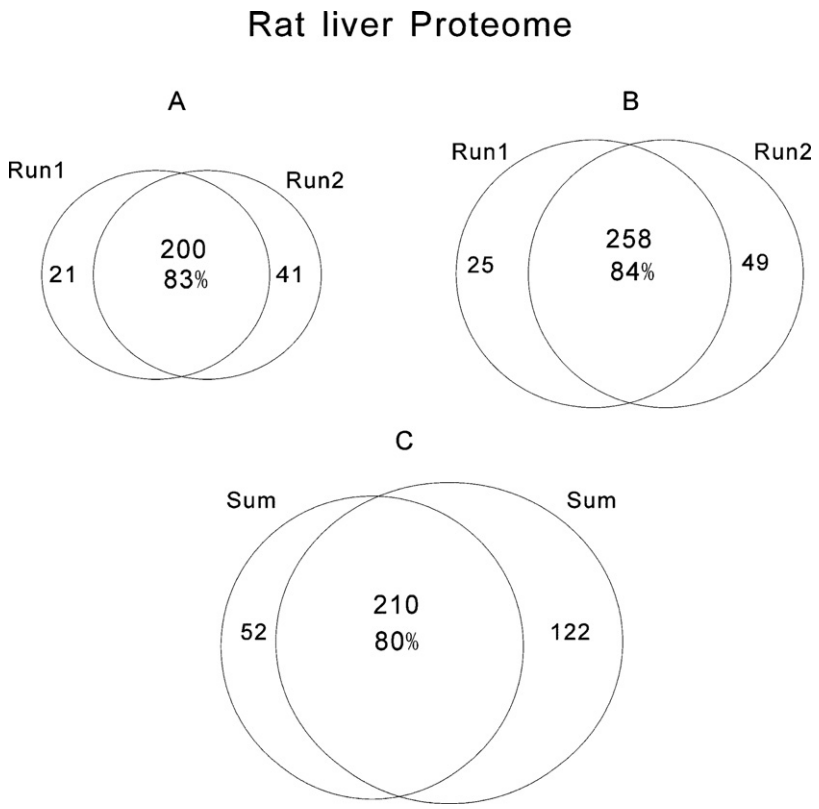

Fig. 6. Venn diagrams comparing the overlap in identified proteins. Run to run analysis via urea (A) and FA (B)-assisted method. (C) sum identification number via two runs for each method.

Apo-Transferrin and β-lactoglobulin were also treated respectively via the urea and FA-assisted protein sample preparation methods in the same conditions for comparison. Corresponding MALDI-TOF MS spectra and obtained peptides were displayed in Fig. S1, Table S2 and S3, respectively. Obtained peptides from the treatment of three proteins (BSA, apo-Transferrin and β-lactoglobulin) via two methods were classified according to the sequence length. As shown in Fig. 2 inset, although the total peptide number obtained from the FA-assisted method was far beyond that of the urea-assisted, there were only 4 ± 1 peptides with a sequence length of over 25 AA, in contrast to 6 ± 1 peptides procured from the urea-assisted method. It is well-known that such large peptides are comparatively hard to be analyzed, due to the difficulty to achieve clear and confident peaks from MALDI-TOF MS. To further evaluation, Overlap in these detected peptides for each model proteins via two methods was also analyzed and presented in Fig. 3. The identified peptide number via the FA-assisted method was increased by ~80% in comparison with the conventional urea-assisted method, corresponding to ~17% increase in sequence coverage concerning all of three proteins. Moreover, a majority of peptides obtained from the conventional urea-assisted method can be contained by the FA assisted method. For the FA-assisted method, most peptides were observed with m/z values around 1000–2500, during which MALDI-TOF MS identification could be performed well in terms of mass accuracy, resolution and sensitivity. Theoretically, BSA contains 40 D sites and 86 K/R sites, which reveals that there are 40, 86 and 126 cleavage sites provided for the chemical D site cleavage, the urea and FA-assisted protein sample preparation method, correspondingly. Thereupon, the increased peptide number could be mainly attributed to the difference of cleavage site between two sample preparation methods.

To investigate the digestion performance of this method for low concentration sample, 1 ng/μL BSA were treated by the FA-assisted method. As shown in Fig. 4, even a low amount down to 7 fmol (1 ng/μL BSA) was positively identified, yielding 25 ± 0 sequence coverage (16 ± 1 peptides). In contrast, there were not peptides identified confidently from 7 fmol BSA via urea-assisted method without desalination. After desalination via a C18 zip tip, BSA was identified confidently based on 15 ± 0 sequence coverage (10 ± 0 peptides), and identified peptides were listed in Table S4 for comparing with that from the FA-assisted method. In fact, the C18 zip tip can enrich peptides to some extent during the desalination procedure. The presence of urea in the FA-assisted method was beneficial for more favorable trypsin digestion and MALDI-TOF MS identification, giving rise to more peptides identification. For the FA-assisted method, not only protein could be denatured and solubilized well in 2% FA to facilitate trypsin digestion, but also more proper peptides were obtained for MALDI-TOF MS identification.

Samples of proteome represent a bigger challenge because of its extreme complexity and diversity. Initially, the amount of FA in the FA-assisted method was optimized to accommodate the complex sample and subsequent nanoRPLC-ESI–MS/MS analysis. When the FA content was 0.5%, there were so many undigested proteins into the sample, leading to a choke occurred on the packed C18 column. Therefore, proteins were not cleaved via 0.5% FA sufficiently, and many proteins were only denaturized in the high temperature, leading to the formation of aggregation, which would block the separation column gradually. When the amount FA was beyond 2%, there were not obvious increase concerning identified peptides (Table S5). Corresponding base peak chromatograms were illustrated in Fig. S2. 2% FA was enough to keep the efficiency and completeness of acid cleavage. It is well known that FA will affect protein solubilization and result in various

Table 1
Comparison of identification results of treated via the FA-assisted protein sample preparation method using different ratios of trypsin to protein.

Trypsin to protein ratio (w/w)	1:10	1:25	1:50
Peptide number	245 ± 7	376 ± 1	282 ± 6

chemical modifications on peptides due to the extremely acid reaction condition. Therefore, 2% FA is best choice which could not only maximize the acid cleavage efficiency, but also enhance the practicability and reproducibility of such method for various individual proteins with different physicochemical properties. The trypsin to protein ratio for such method was also optimized. Corresponding base peak chromatograms were illustrated in Fig. S3, and identification results were listed in Table 1 for comparison. It is the least number about identified peptide at 1:10 ratio (245 ± 7). Numbers of sample peptides with low intensity in the total ion chromatogram were ignored technically due to the high intensity trypsin autolysis peaks, which covered sample peptide peaks. On the other hand, it is well know that trypsin will deactivated gradually during digestion, and low ratios (1:50) leaded to a deficient digestion for such complex sample (282 ± 6). 1:25 was the optimal ratio for the FA-assisted method concerning the identified peptide number (376 ± 1).

For comparison, as a control, two aliquots of rat liver proteome were treated by the FA and urea-assisted methods respectively. Two representative base peak chromatograms for two sample preparation methods were illustrated in Fig. 5, and the inset illustrated representative MS/MS spectra of two peptides derived from the rat liver proteome via the FA-assisted protein sample preparation method. All of them showed a good signal to noise ratio. Statistical identification results were summarized in Fig. 6 detailedly. For each method, following database search and data filtering, overlap with respect to identified proteins between twice analyzes was beyond 80%. Meanwhile, overlap between two methods was 80% concerning identified proteins. More importantly, average 1335 ± 43 (770 ± 22 unique peptides) peptides corresponded to average 295 ± 12 proteins were positively identified via the FA-assisted method, showing a ∼21% increased average peptide, ∼29% increased unique peptides and ∼28% increased identified proteins, compared with the urea-assisted method, and peptides used for protein identification were put in four excel files. The FA-assisted protein sample preparation method was a more efficient method for analyzing the rat liver proteome, demonstrating the applicability of such method for real proteome.

4. Conclusion

A urea-free protein sample preparation method was confirmed that it is capable of being applied to model proteins as well as proteome samples successfully. Besides, more peptide and proteins could be obtained via such preparation method based on MS (MALDI-TOF MS and ESI–MS/MS) identification, compared to the standard urea-assisted protein sample preparation method. Its potential serving as a more efficient protein sample preparation method for researching specific proteomes better, and providing assistance to develop other proteomics analysis methods was exploring.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.08.052.

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