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Hydrazide-functionalized magnetic microspheres for the selective enrichment of digested tryptophan-containing peptides in serum

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ABSTRACT

The extreme complexity of protein samples is becoming a great challenge for proteomic analysis, especially for those having large dynamic range of protein abundance. To solve this problem, and to overcome the limitation of the current proteomic technologies, a new method using hydrazide-functionalized magnetic microspheres was established in this study. With this method, tryptophan (Trp)-containing peptides can be selectively and sensitively enriched from complex and low-volume samples. Furthermore, combined with 1D-LC–MS/MS analysis, the strategy was successfully applied to the proteomic study of mouse serum. The proportion of Trp-containing peptides was increased from 19.4% to 80.2% through enrichment, and the complexity of the sample was reduced more than two times. An additional 113 Trp-containing peptides and 48 novel proteins were detected compared to the conventional method. This enrichment method provides a means for identifying more proteins as potential biomarkers in serum and other complex samples.

1. Introduction

The proteomic study of extremely complex biological samples presents a technical challenge for comprehensive analysis. The latest research predicted that about 20,000 and 16,000 protein-coding genes are expressed in human beings and mouse, respectively. Moreover, a large dynamic range of protein abundance further complicates the proteome analysis, especially 12 orders of magnitude of abundance in serum proteins [1]. To solve this problem, a shotgun proteomic strategy based on chromatographic techniques has been developed and has become a widely used approach. However, the complexity of the proteins and digested peptides is still far beyond the capacity of available analytical platforms, not only because of a large dynamic range in protein abundance but also because of a digestion-related problem. As one protein can generate dozens of peptides, there are a large number of peptides present in protein digests. If one or a few representative peptides could be isolated from each protein, the complexity of the samples for proteome profiling would be reduced by at least one to two orders of magnitude [2]. Therefore, various chemoselective affinity enrichment methods directed towards certain amino acid residues, terminal and post-translational modification peptides have been developed. For example, some methods are focused on selective enrichment of methionine [3], tryptophan [4–6], terminal peptides [7–9] and on peptides carrying post-translational modifications such as phosphorylation [10] and glycosylation [11].

Tryptophan is an interesting target for chemical tagging; it is an amino acid of low abundance with a 1.1% frequency [12], but approximately 90% of proteins contain at least one tryptophan residue in their sequence [13]. Several well known methods have been developed for the derivatization of tryptophan residues in peptides and proteins. The principal reaction of the indole ring of the tryptophan side chain with dicarbonyl compounds like malondialdehyde (MDA) was already briefly described in the 1960s by Teuber et al. [14,15]. Lindner and his co-workers improved the reaction conditions to enhance the specificity of the derivation and capture of Trp-containing peptides [6,16]. Based on their method, we optimized the enrichment technology with magnetic spherical particles to get a higher sensitivity and detect more proteins in complex samples.

Magnetic spherical particles of micro- and nanometer size are gaining increasing attention. The fast and effective separation of magnetic carriers from the reaction mixture without filtration or centrifugation has promoted the widespread application of magnetic materials in chemistry, biochemistry, biology and medicine. Furthermore, many types of functional groups can be bound to the chemically modified surface of magnetic particles. For example,

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magnetic chelating resins have broad applications in IMAC [17]. Deng and his co-workers synthesized superparamagnetic microspheres modified with the TiO_2 or Ce^{4+} chelate to selectively enrich phosphopeptides from liver or serum samples [18] and functionalized with aminophenylboronic acid group to capture glycopeptides and glycoproteins from mixture solution [19]. In this work, we developed a technology with hydrazide-functionalized magnetic microspheres to selectively enrich tryptophan-containing peptides from standard mixture samples. In addition, the method was applied successfully to a digest of mouse serum to reduce the complexity of samples and to identify more proteins in a single analysis process.

2. Materials and methods

2.1. Material and chemical

The magnetic microspheres coated with hydrazide were purchased from Chemicell GmbH (Berlin, Germany). The hydrodynamic diameter of the microspheres is 1.0 μm. The number of particles per gram is 1.8×10^{12}. And the type of magnetization is superparamagnetic. All standard synthetic peptides were bought from Int Biotech BioScience & Technology Ltd. (Shanghai, China). The standard protein myoglobin, hydrazine dihydrochloride, 1,1,3,3-tetramethoxypropane (TMP) and urea were purchased from Sigma Chemical (St. Louis, MO, USA); Trifluoroacetic acid (TFA) was purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Molsheim, France). All other chemicals were of analytical grade and were used as received.

2.2. Sample preparation and protein digestion

The nude mouse sera were collected from 25 SKOV-3 (an ovarian cancer cell line)-xenografted nude mice. Three weeks after the abdominal injection of the SKOV-3 cells, 0.5–1 mL of serum was collected from the mice. The sera were separated by centrifugation of the clotted blood samples at 5000 × *g* for 5 min. All serum samples were processed no later than 4 h after collection and were stored at −80 °C until further analysis. The protein concentration was measured using the PlusOne 2-D Quant Kit (GE, Amersham).

1 mg of serum proteins was dissolved in 55 μL of water and boiled for 5 min. Then 20 μL of 100 mM aqueous ammonium bicarbonate solution and 20 μg trypsin (proteomics grade, Sigma, dissolved in 5 μL water) were added; the digestion was performed at 37 °C for 16 h. The digested samples were concentrated by lyophilization for further reaction and analysis.

2.3. Labeling with malondialdehyde (MDA)

A total of 10 μg synthetic standard peptides (mixture of four Trp-containing peptides and four non-Trp-containing peptides) were dissolved in 10 μL of 80% TFA in water, and 0.18 μL of TMP (MDA dimethylacetal) was added. For serum proteins, 1 mg of digested peptides was dissolved in 100 μL of 80% TFA and 1.8 μL of TMP were added for MDA labeling. In all cases, the mixture was diluted approximately 1:20 with water after a reaction time of 1 h at room temperature; the excess reagent was removed by solid-phase extraction using C-18 cartridges (Phenomenex, Torrance, CA). The C-18 column was activated with 1 mL methanol and balanced with 1 mL 0.1% TFA in water. Then the sample was loaded on the column and washed with 2 mL 0.1% TFA in water. Finally, the sample was eluted with 1 mL 50% methanol and concentrated by lyophilization.

2.4. Enrichment procedure for Trp-containing peptides

1 mg of MDA-treated peptide mixtures (concentrated with lyophilization) was dissolved in 400 μL of 100 mM ammonium acetate (pH 5) and added to 20 mg hydrazide-coated magnetic particles in a ratio of 1:20 (w/w). The mixture was vortexed at 37 °C for 8 h. After centralizing the material beads by high magnetic force, the supernatant was collected and the beads were washed twice with 800 μL of methanol, twice with 800 μL of 8 M urea and once with 800 μL of 100 mM aqueous ammonium acetate (pH 5) at room temperature for 5 min each time. The peptides were cleaved from the surface of the beads with pyrrolidine as follows: the beads were shaken twice with 750 μL of 500 mM pyrrolidine in water for 30 min, then twice with 400 μL of 500 mM pyrrolidine in water/methanol (50:50, v/v) for 15 min at room temperature. For hydrazine-induced cleavage method, the material beads were shaken at 50 °C, and the pyrrolidine reagent was changed to hydrazine dihydrochloride in 100 mM aqueous ammonium acetate (pH 3, adjusted with acetic acid). The corresponding fractions were pooled, and the solvent was concentrated by lyophilization for further separation and detection.

2.5. Reverse phase liquid chromatography (RPLC) separation, mass spectrometry detection and database search

The enriched standard peptides were dissolved in 50 μL of 1% TFA solution and desalted with ZnO-poly (methyl methacrylate) nanobeads [20]. One microliter of the peptide solution was mixed with 0.5 μL of the matrix (5 mg/mL of α-cyano-4-hydroxycinnamic acid diluted in 50% acetonitrile/0.1% TFA) and spotted onto a plate. The MS measurements were carried out on the 4700 MALDI-TOFTOF mass spectrometry analyzer (Applied Biosystems, USA). The instrument setting was reflector mode with 200 Hz (355 nm) frequency, 8 kV accelerating voltage and 7 kV decelerating voltage. Laser shots at 2500–3000 per spectrum were used to acquire the spectra in the mass range of 500–2000 Da. The digested fragment peaks of horse myoglobin served as internal standards for mass calibration.

The enriched peptides of serum samples were resuspended with solvent A (A: water with 0.1% formic acid). One sixth of the samples were analyzed by a nano-scale RPLC system (Shimadzu Company, Japan) coupled online with a LTQ-Orbitrap XL mass spectrometer (Thermo Electron Corp., Bremen, Germany). Fifteen micrograms of untreated samples were detected as the control. The samples were separated on a nano-scale C18 reverse-phase column (3.5 μm, 100 Å, 0.1 mm × 150 mm, MICHROM Bioresources) with a linear gradient starting from 5% B to 45% B (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid) at 500 nL/min for 90 min. The LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra with two microscans (*m/z* 400–2000) were acquired in the Orbitrap with a mass resolution of 100,000 at *m/z* 400 and were followed by eight sequential LTQ-MS/MS scans. The dynamic exclusion option was selected with a repeat count of 2, a repeat duration of 10 s and an exclusion duration of 60 s. For MS/MS, the precursor ions were activated using 35% of the normalized collision energy at the default activation *q* of 0.25.

The mass spectra were searched against the mouse Swiss-Prot database (Release 14.9, March 3, 2009; 16,098 entries) using the Bioworks software (Version 3.3.1; Thermo Electron Corp.) based on the SEQUEST algorithm. The parameters for SEQUEST searching were as follows: enzyme, Partially Trypsin; missed cleavages, two; variable modifications, oxidation (*M*, *W*) peptide tolerance, 10 ppm; MS/MS tolerance, 1.0 Da. The results of the database searches were statistically analyzed using PeptideProphet & Proteinprophet soft-

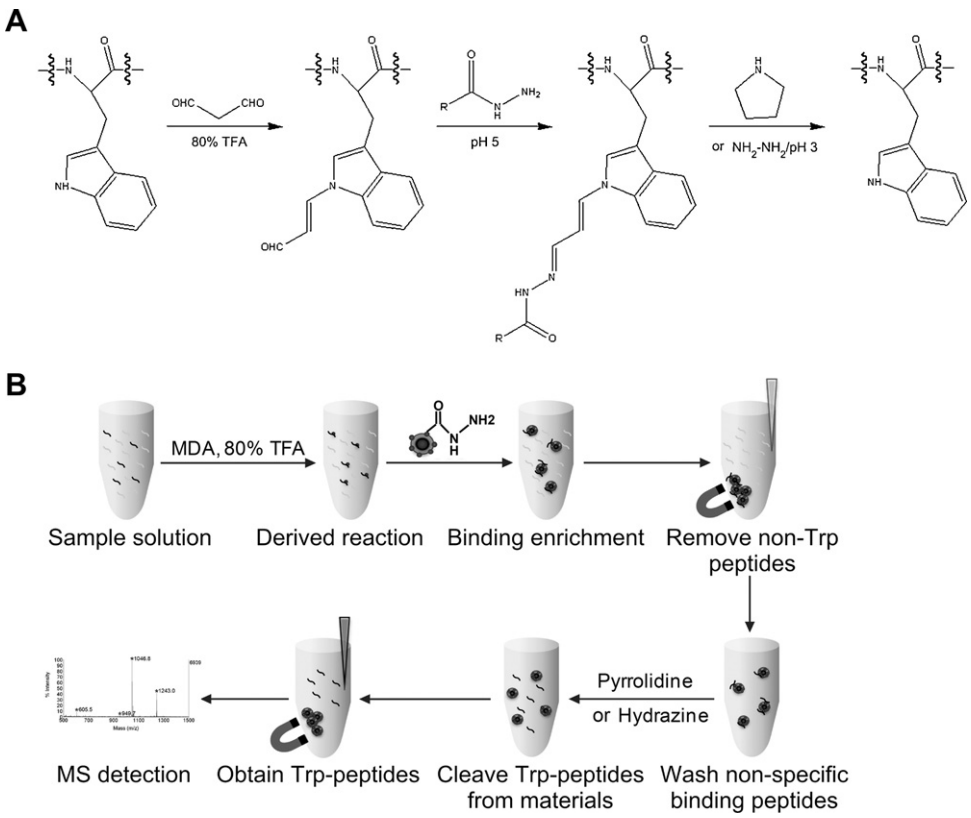

Fig. 1. Reaction equation and scheme for the enrichment of tryptophan-containing peptides. The magnetic microspheres coated with hydrazide were used to enrich derived Trp-containing peptides. The peptides of dark color represent Trp-containing peptides, and the peptides of light color represent non-Trp-containing peptides.

ware [21,22]. A minimum PeptideProphet probability score (*P*) filter of 0.9 was used to filter out low-probability peptides. Apex is used for protein quantification [23]. It considers the number of peptides that can be expected for each protein. To calculate the APEX values, the result files of Proteinprophet were used. The identified peptides of the top 50 proteins were used during the machine learning step to predict the "detectability" of the peptides in general (as recommended in the APEX manual). The proteins concluded in the Apex result have less than 1% FDR.

3. Results and discussion

3.1. Optimization of enrichment strategy with hydrazide-functionalized magnetic microspheres

Hydrazide-functionalized magnetic microspheres were used for enriching Trp-containing peptides in this study for the first time. The enrichment principle and scheme were shown in Fig. 1. Based on the reversible reaction concept about tryptophan N-heterocycles, the peptides were mixed with MDA in 80% TFA. One of the aldehyde groups of MDA reacts with the indole nitrogen, and a reactive acrolein-like α,β-unsaturated aldehyde is formed from the second aldehyde group [6]. This group can further react with hydrazide on the surface of materials. Therefore, the Trp-containing peptides were captured selectively by materials. Following three steps of washing to remove non-specific binding peptides (methanol, urea and ammonium acetate pH 5 were used for washing hydrophilic, hydrophobic and other non-specific binding peptides, respectively), the Trp-containing peptides were cleaved to native forms for further detection with MALDI-MS or RPLC-MSMS.

To reach high specificity and sensitivity, the experimental conditions were optimized using a mixture of eight synthetic peptides composed of Trp-containing peptides (L**W**MR, KNPYIE**W**, HPKRP**W**IL, KYRLKHLV**W**) and non-Trp-containing peptides (Pyr-LYENK, RPPGFSPFR, DRVYIHPFHL, PHPFHFFVYK) (Fig. 2A). After the reaction with MDA, the derived Trp-containing peptides drift +54 Da (for –CH=CH–CHO) per tryptophan residue in mass spectra (Fig. 2B). The enrichment conditions such as binding time, the ratio of materials to peptides, and the material density were optimized in the experiment (Figs. S1 and S2). The relative concentration of peptides in the supernatant was measured by the relative peak intensity of each peptide (peak intensity versus total peak intensities) in the mass spectra. Based on this relative concentration, the optimized conditions were as follows: the peptides were enriched for 8 h in 50 μg/μL materials, and the ratio of materials to Trp-containing peptides was higher than 50:1 (M/M). Meanwhile, two different cleavage methods using either pyrrolidine or hydrazine were compared in this study. They were both previously found to cleave the N–C bond at the nitrogen of the indole system [16]. Although the system with hydrazine (pH 3) was more compatible with mass spectrometry detection, its reversibility of the reaction was not complete and the peaks of derived peptides appeared in the mass spectra (Fig. 2C). With the other method, all the Trp-containing peptides were completely recovered to their native forms (Fig. 2D). For enhancing the signals of mass spectra and increasing sensitivity of this method, the final enriched solution was neutralized with 1% TFA and desalted. Therefore, pyrrolidine preferred to cleave the Trp-containing peptides from hydrazide-modified microspheres.

The specificity of this enrichment strategy was also examined with more complex standard samples. The samples were composed of digested peptides from myoglobin protein and a derived Trp-containing peptide HPKRP**W**IL (1100.7 Da) in different ratios (20:1, 100:1 and 500:1 M/M). The mass spectra of peptides before and after enrichment are shown in Fig. 3. The peak

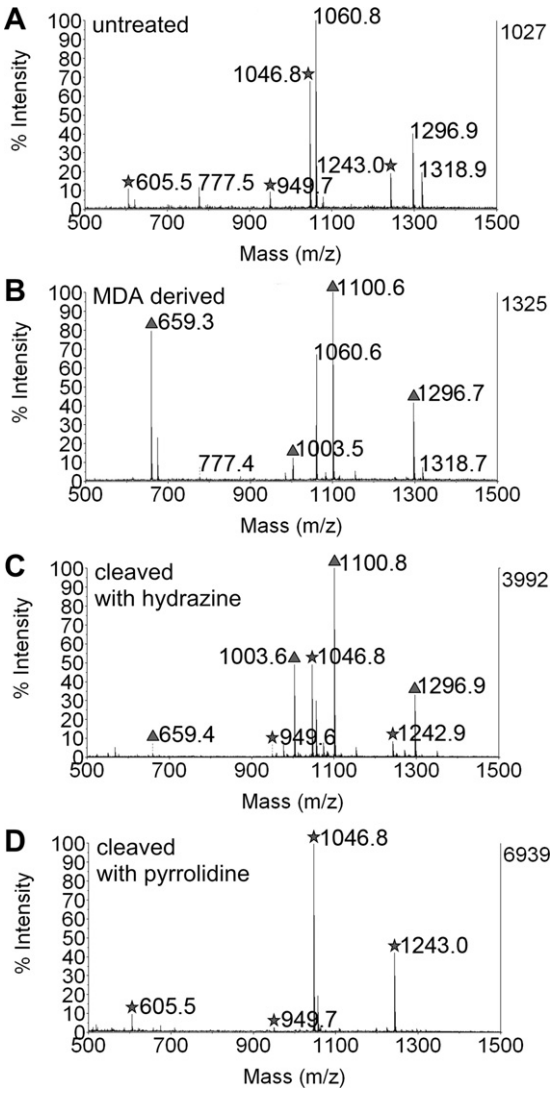

Fig. 2. Mass spectra of standard synthetic peptides before and after Trp-enrichment. A. Mass spectrum of mixed standard Trp and non-Trp-containing peptides. B. Mass spectrum of the peptides derived with MDA. C. Mass spectrum of enriched peptides cleaved with hydrazine. D. Mass spectrum of enriched peptides cleaved with pyrrolidine. The peaks labeled with stars were Trp-containing peptides and the peaks labeled with triangles were derived Trp-containing peptides.

of highest relative intensity (1606.9 Da) among the digested peptides was selected to compare with the Trp-peptide peak (the mass of its derived form is 1100.7 Da, and the mass of its native form is 1046.7 Da). When the myoglobin peptides and the derived Trp-peptide were mixed in 100:1, the ratios of the Trp-peptide peak intensity to the 1606.9 Da peak intensity were increased from 0.15 to 279 after enrichment. When the myoglobin peptides and the derived Trp-peptide were mixed in 500:1, the peak of the derived Trp-peptide (1100.7 Da) was hardly detectable in the mass spectrum, but its intensity could reach 82 times higher than the intensity of myoglobin peptide peak (1607.0 Da) after enrichment.

Application of hydrazide-functionalized magnetic microspheres made the enrichment strategy more effective, sensitive and easier to manipulate. Compared to conventional filtration and centrifugation technologies, the strong magnetism of the materials made the separation of solid materials from the supernatant much easier, while minimizing the loss of Trp-containing peptides during the washing steps. The large specific surface area of the microspheres enhanced the reaction effect, and the washing and cleavage times were reduced to half of those specified in the Lindner's method [6]. The comparison of protocol and results between their and our methods were shown in Table 1. In the results of standard peptide samples, the specificity of Trp-containing peptides enrichment was increased from 90% to 99% with current method. For the complex samples, the specificity of these two methods was similar (86% and 80%), but much more Trp-containing peptides (313 vs. 169) and proteins (105 vs. 16) were identified with current method for its high sensitivity. Moreover, the magnetic materials are commercially available, and the hydrazide group is precisely attached to the surface of the materials. It saved a reaction step and enhanced reproducibility of the results.

Table 1
Comparison between previous and current methods.

	Previous method[a]	Current method
Enrichment time		
Reaction time	1 h	1 h
Binding time	4 h or overnight	8 h
Washing time	10–15 min × 5	5 min × 5
Cleavage time	60 min × 2 and 30 min × 2	30 min × 2 and 15 min × 2
Specificity		
Standard peptide samples	More than 90%	More than 99%
Complex samples[b]	86%	80%
Sensitivity		
Complex samples[b]	169 Trp-peptides and 16 proteins identified	313 Trp-peptides and 105 proteins identified

[a] The previous method was set up and done by Lindner's group [6].
[b] The complex samples analyzed in these experiments were different. The previous method was applied to analyze yeast cell lysate while the current method was used for mouse serum samples.

3.2. Application of the enrichment strategy in mouse serum

To thoroughly evaluate our optimized Trp-enrichment strategy, we applied the developed method to analyze a crude fraction of mouse serum with ovarian cancer. A serum sample, composed of hundreds of proteins with large orders of magnitude, is a complex system. The presence of high abundant proteins, such as albumin and IgG, makes it more difficult to detect biomarkers in serum [1]. Enrichment of Trp-containing peptides can effectively reduce the complexity of protein samples. When comparing the base peak chromatograms of the untreated and Trp-enriched samples (Fig. 4A and B), the constitution of peptides and their concentration in samples were changed after enrichment. Combined with identification results in mass spectrometry, most of the non-Trp-containing peptides were proved to be removed from serum samples after enrichment. Meanwhile, the Trp-containing peptides were enriched to make it possible to detect more peptides of low abundance. Through Trp-enrichment, the complexity of sample system was reduced and more proteins were detected.

With the RPLC-MSMS analysis, 1116 and 416 peptides, including 239 and 313 Trp-containing peptides, were detected successfully in untreated and enriched samples respectively (Fig. 4C). Among the 137 newly detected peptides in the enriched samples, 113 peptides contain one or more tryptophan residues. The Trp-peptide enrichment method made a great contribution to their detection. As we all know, the proportion of spectra was correlated to the concentration of peptides in a sample [24]. We compared the proportion of spectra corresponding to Trp-peptides before and after enrichment, and evaluated the specificity of this enrichment method. The proportion of Trp-containing spectra increased from 19.4%

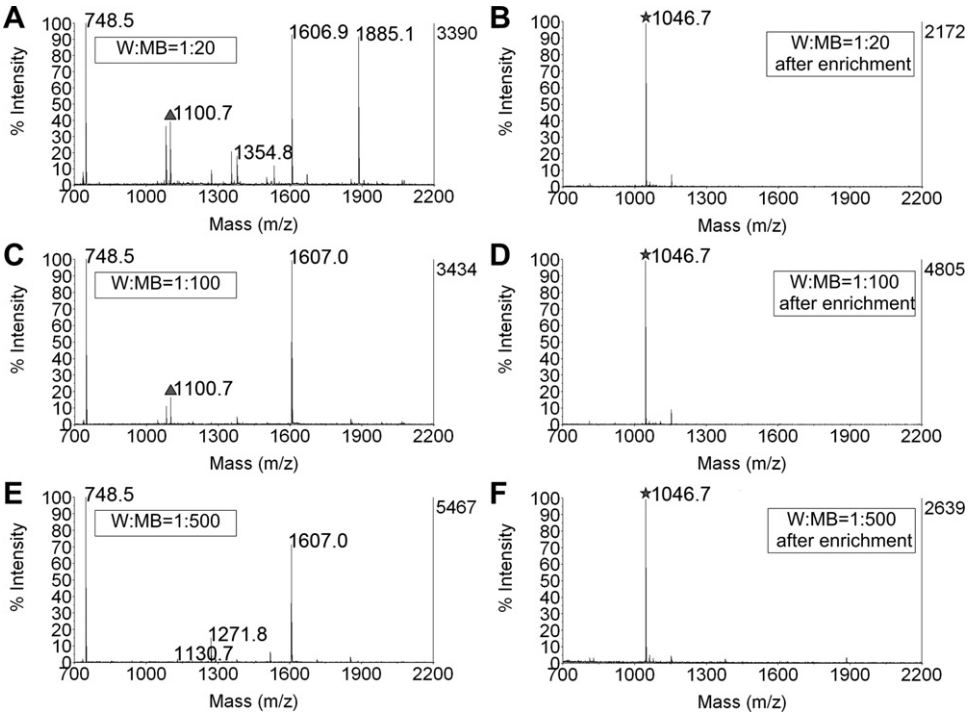

Fig. 3. Mass spectra of the standard Trp-containing peptide and digested myoglobin peptides mixed with different ratios before enrichment (A, C, E) and after enrichment (B, D, F). The peaks labeled with stars were Trp-containing peptides, and the peaks labeled with triangles were derived Trp-containing peptides. W and MB are short for Trp-containing peptide and the myoglobin peptides, respectively.

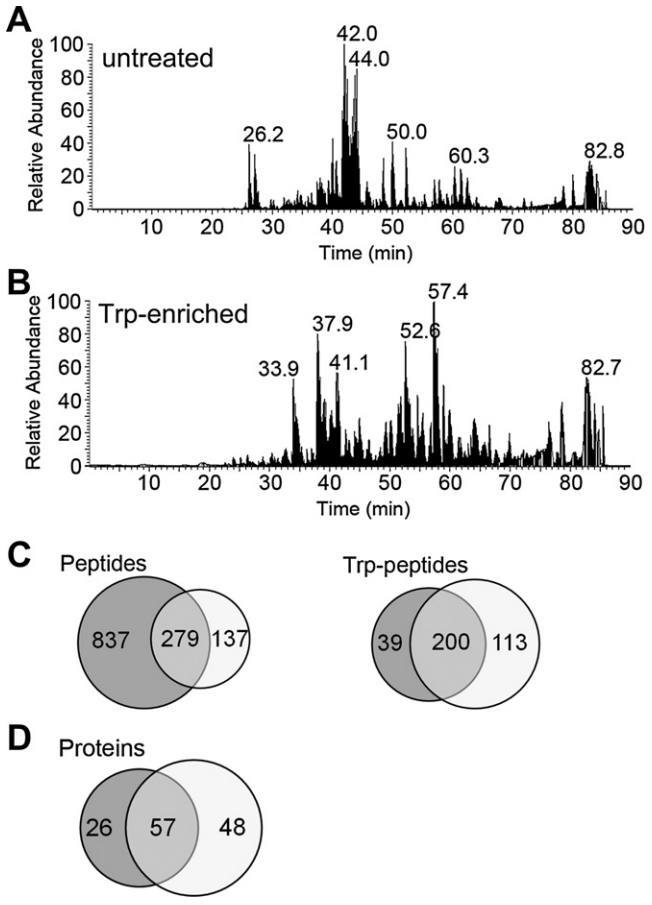

Fig. 4. Identification results of mouse serum before and after Trp-enrichment. Base peak chromatograms for the untreated sample (A) and the Trp-enriched sample (B). Identification results of peptides and Trp-containing peptides (C) and proteins (D) in untreated samples (dark circles) and Trp-enriched samples (light circles).

(909/4680) in untreated samples to 80.2% (1486/1852) after enrichment, implying high specificity of the Trp-enrichment strategy. Though in the enriched samples, 39 Trp-peptides were not detected compared with untreated samples. It might be due to misdetection in mass spectrometry, a normal phenomenon for the mass spectrometry technology that not all spectra can be detected between any two runs [25]. Even the simplest protein digestions yielded more peptide ions than LC–MS/MS could identify during a single experiment. The peptide lists from pairs of technical replicates overlapped by 35–60% [26]. However, the proportion of 113 novel Trp-peptides in the enriched samples was much higher than that of the 39 Trp-peptides in the untreated samples, especially when the total numbers of spectra and peptides were reduced greatly in the enriched samples. The 113 novel Trp-peptides can be detected mainly due to high specificity of the enrichment strategy. Meanwhile, we analyzed the characters of non-Trp containing peptides in the enriched samples and found that most of the non-Trp peptides were the high abundant ones in serum. The main reason may be non-specific binding of these peptides on the surface of materials.

In terms of proteins, 83 and 105 proteins were identified in the untreated and enriched serum samples, respectively (Fig. 4D). The ratios of identified peptides to proteins were 13.4 (1116 peptides: 83 proteins) before enrichment and 4.0 (416 peptides: 105 proteins) after enrichment. This result showed that the Trp-enrichment strategy reduced the complexity of the sample more than two times. More importantly, the utilization of peptides was increased with the enrichment strategy and more proteins can be identified especially in the limited speed of MSMS analysis. Taking the most abundant protein Serum albumin as an example, the numbers of identified peptides were 45 before enrichment and 15 after enrichment. The numbers of albumin related spectra were changed from 585 to 129, which decreased almost four times (Table 2). Compared to the results of untreated samples, 48 proteins were novelly detected. The enrichment strategy gives a great supplementary for protein identification.

Table 2
Identified peptides of serum albumin in samples before and after Trp-enrichment. The numbers in brackets were spectra numbers of the peptides.

Peptides before enrichment (585)	Peptides after enrichment (129)
LSQTFPNADFAEITK (113), LGEYGFQNAILVR (81), YNDLGEQHFK (47), TPVSEHVTK (43), APQVSTPTLVEAAR (28), DVFLGTFLYEYSR (27), GLVLIAFSQYLQK (25), LVQEVTDFAK (24), ENPTTFMGHYLHEVAR (19), HPDYSVSLLLR (17), GTVLAEFQPLVEEPK (15), TVDETYVPK (14), DDNPSLPPFERPEAEAM (13), MGHYLHEVAR (11), QTALAELVK (9), RHPDYSVSLLLR (8), DVFLGTFLYEY (6), DVFLGTFLY (5), ALTVDETYVPK (4), DYSVSLLLR (4), ENPTTFMGHY (4), ENPTTFMGHYLH (4), FSALTVDETYVPK (4), GFQNAILVR (4), KLVQEVTDFAK (4), LAEFQPLVEEPK (4), LGEYGFQNAIL (4), LGTFLYEYSR (4), SALTVDETYVPK (4), SLHTLFGDKL (4), VLAEFQPLVEEPK (4), YGTVLAEFQPLVEEPK (4), AEFQPLVEEPK (3), DDNPSLPPFERPEAE (3), ENPTTFMGH (3), SLHTLFGDK (3), EFQPLVEEPK (2), HPYFYAPELLY (2), RHPDYSVSLL (2), HTLFGDK (1), IAFSQYLQK (1), KQTALAELVK (1), LGEYGFQNA (1), PDYSVSLLLR (1), QVSTPTLVEAAR (1)	GLVLIAFSQYLQK (58), HPDYSVSLLLR (13), DVFLGTFLYEYSR (11), LGEYGFQNAILVR (8), RHPDYSVSLLLR (8), LSQTFPNADFAEITK (6), APQVSTPTLVEAAR (4), ENPTTFMGHYLHEVAR (4), GTVLAEFQPLVEEPK (4), LVQEVTDFAK (4), VFLGTFLYEY (3), DDNPSLPPFERPEAEAM (2), FGERAFKAWAVAR (2), LPCVEDYLSAILNR (1), VLAEFQPLVEEPK (1)

4. Conclusion

The method proposed here used hydrazide-functionalized magnetic microspheres for selectively enriching Trp-containing peptides for the first time. The chemoselective enrichment strategy was highly efficient and easy to manipulate. With the hydrazide-functionalized microspheres, less time was needed especially in the washing and cleavage steps. Meanwhile, it was much easier to achieve the separation of peptide solution and solid materials owing to the strong magnetism of the materials. In addition, the magnetic materials are commercially available and are convenient for other related studies.

Furthermore, the enrichment procedure was optimized in this study and successfully applied to the analysis of mouse serum. The specificity of this method was higher than 80% when enriching Trp-containing peptides from serum without removing high abundant proteins. Compared to the conventional method, an additional 113 tryptophan-containing peptides and 48 proteins were detected. The complexity of the system was clearly reduced more than two times compared to untreated samples. With the optimization of method being more compatible with mass spectrometry, the Trp-containing peptide enrichment can identify more proteins, including potential biomarkers of less abundance, in serum and other complex samples.

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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.2011.05.008.

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