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Short communication

Integrated protein analysis platform based on column switch recycling size exclusion chromatography, microenzymatic reactor and μRPLC–ESI-MS/MS

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ABSTRACT

An integrated platform with the combination of proteins and peptides separation was established via the unit of on-line proteins digestion, by which proteins were in sequence separated by column switch recycling size exclusion chromatography (csrSEC), on-line digested by an immobilized trypsin microreactor, trapped and desalted by two parallel C8 precolumns, separated by μRPLC with the linear gradient of organic modifier concentration, and identified by ESI-MS/MS. A 6-protein mixture, with Mr ranging from 10 kDa to 80 kDa, was used to evaluate the performance of the integrated platform, and all proteins were identified with sequence coverage over 5.67%. Our experimental results demonstrate that such an integrated platform is of advantages such as good time compatibility, high peak capacity, and facile automation, which might be a promising approach for proteome study.

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1. Introduction

For proteome research, “bottom-up” approach has been regarded as one of the most popular analytical strategies. However, the simultaneous separation of thousands of peptides brings great challenges, not only to 2D-HPLC for peptides separation, but also to MS/MS for accurate proteins identification [1–4]. Therefore, proteins separation before digestion is of significance to decrease the complexity of samples prior to “bottom-up” based analysis. Recently, some approaches have been developed through off-line protein separation, digestion, peptide separation and protein identification [5,6]. However, by such methods, sample loss or contamination is unavoidable. Furthermore, it is difficult to achieve the automatic operation of whole systems. Therefore, the development of an integrated platform with combination of on-line protein separation, digestion, peptide separation and identification is imperative.

The key unit to integrate proteins and peptides separation is the rapid on-line protein digestion by immobilized enzymatic reactors (IMERs). In recent years, various IMERs have been developed [7–9], and the digestion time could be shortened even within 30 s [9]. Furthermore, IMER was also on-line coupled with HPLC for protein digestion and peptides separation. Liu et al. combined a gold nanoparticle assembly microfluidic reactor with 2D-HPLC/MS/MS for analyzing protein extracted from mouse morphages, and 497 proteins were identified within 20 h [10]. Schriemer and co-workers proposed an integrated platform to achieve protein separation by RPLC, on-line digestion by IMER, and protein identification by MS/MS [11,12]. However, to improve the biocompatibility of IMER with RPLC, the decreasing of organic modifier concentration in mobile phase by introducing another kind of buffer might also result in the dilution of samples. Furthermore, in the above-mentioned platforms, single-dimension separation could not offer sufficient resolving power and peak capacities for proteome analysis.

An ideal platform for complex protein sample analysis should be composed of on-line protein separation, digestion, peptide separation and protein identification. Furthermore, it was critical to keep good compatibility of each unit, no matter with the consideration of solvent polarity, flow rate, or operation time. Therefore, to meet the above-mentioned requirements, in our recent study, column switch recycling size exclusion chromatography (csrSEC), IMER and μRPLC–MS/MS was integrated, and successfully applied into the separation and identification of proteins mixture.

2. Experimental

2.1. Apparatus

MAGIC MS4 HPLC system was purchased from Michrom Bioresources (Auburn, CA, USA). Syringe pump was brought from Longer Precision Pump (Baoding, China). An LCQ^{Duo} electrospray ion trap

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mass spectrometer was ordered from Thermo Fisher Scientific (San Jose, CA, USA).

2.2. Chemicals and materials

Myoglobin (horse heart), transferrin (horse), ribonuclease (bovine pancreas), β-lactoglobulin (bovine milk), chicken egg albumin(ALB), carbonic anhydrase (bovine erythrocytes) and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Shanghai Milk Company (Shanghai, China). Acetonitrile (ACN) of HPLC-grade was ordered from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, MD, USA). All other chemicals and solvents were of analytical grade.

SEC *SRT*-150 Å-5 μm and SEC *nanofilm*-250 Å-5 μm particles were purchased from Sepax Technologies (Newark, Delaware, USA). XBP C18 particles (5 μm, 200 Å) were ordered from Bona Inc. (Tianjin, China). Acrylic polymer particles with epoxy groups (5 μm, 1000 Å) were donated by Shenzhen Nano-micro Technology Inc. (Shenzhen, China). C8 captrap precolumns (2 mm × 0.5 mm I.D.) were purchased from Michrom Bioresources.

2.3. Sample preparation

A 6-protein mixture (BSA, myoglobin, transferrin, carbonic anhydrase, ribonuclease and β-lactoglobulin) was denatured by 8 M urea, dissolving in 50 mM NH_4Ac buffer (pH 8.0), at 37 °C for 1 h, and then reduced with dithiothreitol at 56 °C. After being alkylated with iodoacetamide at room temperature, it was diluted with 50 mM NH_4Ac buffer until the total concentration of proteins reached 2 mg/mL.

2.4. Column packing

SEC *SRT*-150 Å-5 μm and SEC *nanofilm*-250 Å-5 μm particles were slurried in a mixture of water and alcohol (v/v = 2:1), and then packed into steel tubes (150 mm × 4.6 mm I.D. or 250 mm × 4.6 mm I.D.) under a constant pressure of 4000 psi.

C18 packing materials were slurried in bromoform, and packed into a Peeksil tube (50 mm × 0.3 mm I.D.) under a constant pressure of 6000 psi.

2.5. Preparation of trypsin immobilized microenzymatic reactor

Acrylic polymer particles with epoxy groups (about 40 mg) were added into 25% (v/v) NaOH solution, and then reacted at 40 °C for 3 h. After centrifuged at 1000 × *g* for 5 min, the supernatant was discarded, and the residual particles were packed into a steel tube (10 mm × 4.6 mm I.D.). After 5% (w/v) glutaraldehyde solution was continuously flushed into the column for 6 h using a syringe pump, 5 mg/mL trypsin dissolved in 50 mM borate buffer (pH 8.2) was pumped into the column overnight at room temperature, and trypsin was subsequently immobilized onto particles. Finally, trypsin immobilized microreactor was treated with 25 mM sodium cyanoborohydride overnight, and stored at 4 °C. Before usage, IMER was flushed with 10 mM ammonium acetate containing 50% (v/v) ACN to remove residual unbound trypsin.

2.6. SEC operation

SEC experiments were performed on a Magic MS4 HPLC system. Proteins were separated by a home-packed serially coupled SEC column (SEC *SRT*-150 Å and SEC *nanofilm*-250 Å). The mobile phase used for SEC was composed of 10 mM Tris–HCl, containing 150 mM NaCl and 5% (v/v) acetonitrile (pH 8.0). Proteins were eluted isocratically at a flow rate of 150 μL/min. Effluents were detected at 214 nm.

Recycling SEC (rSEC) experiments were performed on a home-set-up system, composed of an HPLC pump, two high-speed 10-port valves, two serially coupled SEC columns and a UV detector, based on which column switch recycling SEC (csrSEC), was developed. Fractions eluted from csrSEC were in sequence transferred into IMER for on-line digestion.

2.7. On-line protein digestion and peptides analysis by μRPLC–ESI-MS/MS

0.05 mg/mL BSA was pumped through IMER at 37 °C with the flow rate of 50 or 200 μL/min, and then the yielded peptides were collected, followed by the separation by a C18 column and identification by ESI/MS/MS.

The mobile phases for peptides separation was 2% (v/v) ACN containing 0.1% (v/v) formic acid (A), and 98% ACN containing 0.1% formic acid (B). The gradient was set as follows, 5% (0 min) → 5% (10 min) → 40% (70 min) → 80% (75 min) → 80% (80 min) B, at the flow rate of 5 μL/min. After each μRPLC separation, the column was equilibrated with the initial mobile phase.

2.8. Analysis of a 6-protein mixture by integrated platform

A 6-protein mixture (40 μg) was analyzed by our developed integrated platform, as shown in Fig. 1. To achieve good compromise between the resolution for proteins separation and time matching for peptides separation by μRPLC–ESI-MS/MS, proteins were

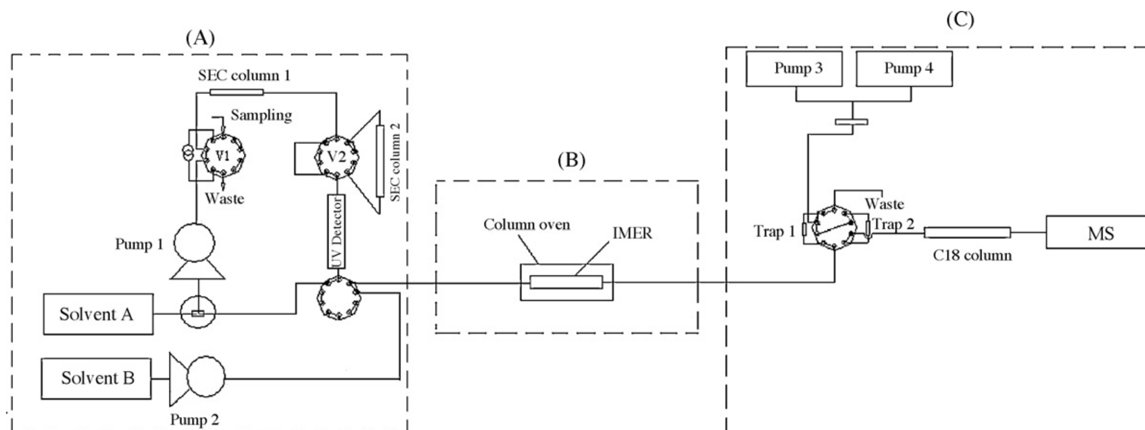

Fig. 1. Schematic diagram of integrated platform for protein analysis. (A) csrSEC; (B) IMER; (C) μRPLC–ESI/MS/MS.

separated by csrSEC (Fig. 1A). In brief, they were first separated by serially coupled SEC, and then divided into four fractions. In turn each fraction was switched back to the first SEC column, cycled for separation and transferred to IMER when satisfactory resolution was achieved. Simultaneously, the other fractions eluted by serially coupled SEC were tapped in the second SEC column. The buffer for csrSEC was 150 mM ammonium acetate containing 5% (v/v) acetonitrile (pH 8.0), and the flow rate was 50 μL/min. A total of 16 fractions generated by csrSEC were in turn transferred into IMER by another pump with 50 mM ammonium acetate containing 5% (v/v) acetonitrile (pH 8.0) as the buffer, under the flow rate of 50 μL/min, and on-line digested by IMER (Fig. 1B). The resulted peptides were then captured in turn by two parallel C8 precolumns, and further separated by μRPLC (Fig. 1C). The mobile phases for peptides separation were 2% (v/v) ACN containing 0.1% (v/v) formic acid (A) and 98% ACN containing 0.1% formic acid (B). For desalting, microcolumn of RPLC was flushed with 5% ACN for 10 min, and the following separation gradient was set as follows: 5% (0 min) → 5% (10 min) → 40% (60 min) → 80% (65 min) B → 80% (70 min), under the flow rate of 5 μL/min. After each μRPLC separation, the μRPLC column was equilibrated with the initial mobile phase.

2.9. ESI-MS/MS identification

ESI-MS/MS was hyphenated with μRPLC for protein identification. If not specially stated, spray voltage was 2.0 kV, and ion transfer capillary temperature was 150 °C. Collision energy was set as 35%. During μRPLC–ESI-MS/MS analysis, the effluents were sprayed directly into ESI source using a laboratory-made interface without sheath or auxiliary gas. All MS and MS/MS spectra were acquired in data-dependent mode, by which MS acquisition with *m/z* range of 400–2000 was automatically switched to MS/MS acquisition with the automated control of Xcalibur software. The two most intense ions of full MS scan were selected as parent ions, and subjected to MS/MS scan with isolation width of *m/z* 2.0. The dynamic exclusion function was set as follows: repeat count: 2, repeat duration: 30 s, and exclusion duration: 180 s.

2.10. Database searching

Acquired MS/MS spectra were searched against protein database using Bioworks software (v3.1) with SEQUEST program. Trypsin was set as enzyme for database searching. Peptides were searched using fully tryptic cleavage constraints, and up to two missed internal sites were allowed for tryptic digestion. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. Cysteine residues were searched as a static modification of 57.0215 Da. Database searching of six standard proteins was performed in *horse*.fasta database and *bovine*.fasta database. The SEQUEST results were filtered by cross-correlation score (X_{corr}). Peptides were considered positively if X_{corr} was larger than 1.9, 2.2 and 3.75, respectively, for singly, doubly and triply charge peptides, and ΔCn cut-off values were $\geq$0.1.

3. Results and discussion

3.1. Fast protein digestion by IMER

In our experiments, trypsin immobilized polymer particles were packed into a column (4.6 mm I.D. × 10 mm), working as IMER, as shown in Fig. 1B. When 50 μg/mL BSA was pumped through IMER at the flow rate of 50 μL/min, and the resulting peptides were analyzed by μHPLC/MS/MS (as shown in supplemental Fig. s-1). The sequence coverage of 51.89% was obtained, with 160 s residence time. To further shorten the digestion time, BSA was introduced at the flow rate of 200 μL/min, corresponding to the residence time of 40 s, and the sequence coverage of 45.30% was obtained (as shown in supplemental Table S1). All these results indicated that by such an IMER, fast speed protein digestion could be achieved with good sequence coverage.

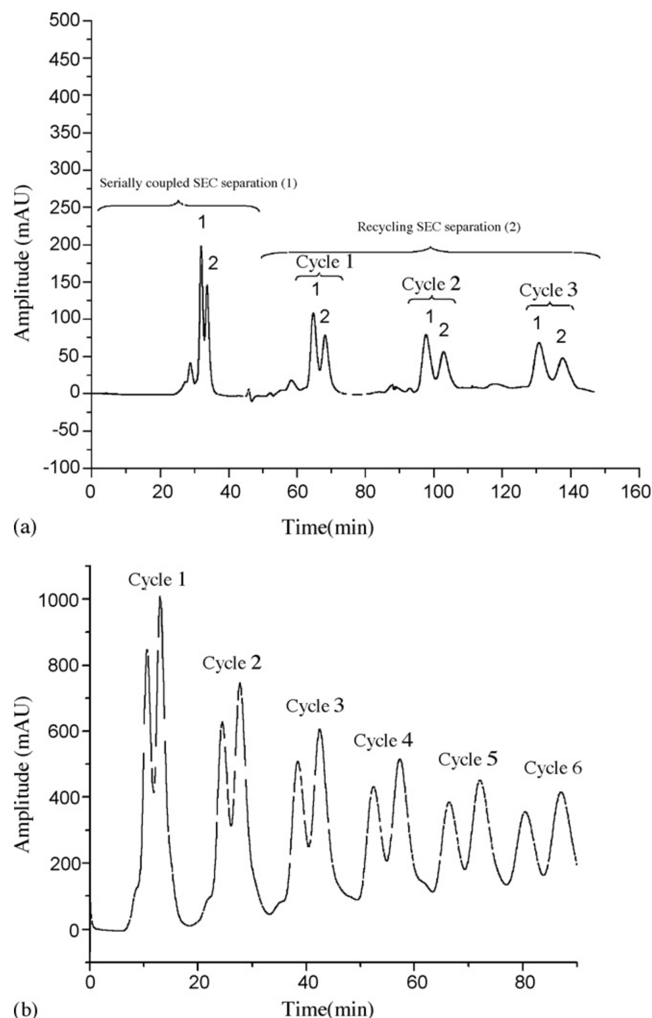

Fig. 2. ALB transferrin separation by serially coupled SEC (a1), rSEC (a2) and csrSEC (b). Experimental conditions were described in Section 2.6.

3.2. Protein separation by rSEC

To overcome the disadvantages of SEC such as low separation resolution, poor peak capacity and narrow separation window, rSEC was firstly applied for proteins separation with two serially coupled columns. From Fig. 2a it could be seen that, ALB and transferrin could be not separated completely by serially coupled SEC (R = 0.95). However, the resolution was obviously improved, and reached 1.2 by rSEC after 3 times cycling. Furthermore, compared to SEC, the separation window of rSEC, defined as the period between dead time and retention time of the latest eluted peak, could be increased by over 3 times, which was favorable to ensure time synchronization with further peptide separation.

3.3. The peak capacity of csrSEC

To further improve the peak capacity of SEC, csrSEC was proposed with the combination of serially coupled SEC, sample parking, fraction switching and rSEC, as shown in Fig. 1A. In such a system, proteins were firstly separated by two serially coupled SEC columns, and then parked in SEC column 2. Subsequently, the first

fraction with similar molecular weight (Mr) was eluted from column 2 back to column 1, and separated in terms of closed-loop till the satisfactory resolution was obtained. Then the separated eluants were transferred out of column 1 for the following operation. The other fractions parked in column 2 were analyzed by the same procedure. As shown in Fig. 2, the peak capacity of serially coupled SEC is about 10 (about 30 min of elution time divided by an average peak width of 3 min), and that of rSEC is about 13 (about 80 min

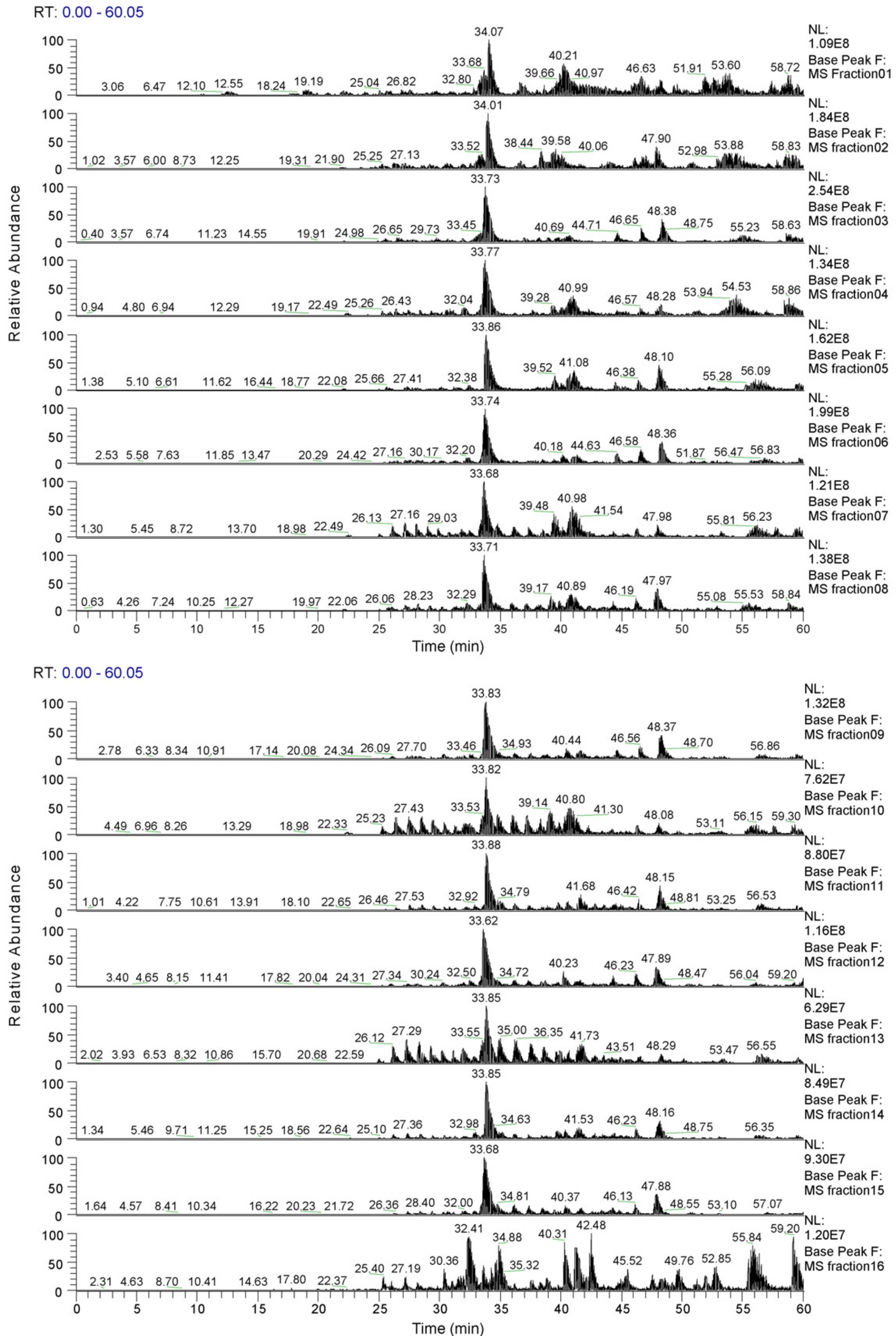

Fig. 3. Base peak chromatogram of 6-protein mixture digests analyzed by integrated platform. Experimental conditions were shown in Section 2.7.

of elution time divided by an average peak width of 6 min). Thus, the peak capacity of csrSEC should be 130, the multiplicative peak capacity of serially coupled SEC and rSEC.

3.4. Construction of integrated platform

Due to good biocompatibility with IMER, improved resolution and broadened separation window for proteins separation, and good time synchronization with peptides separation by μRPLC, csrSEC is used as the first dimensional mode, as shown in Fig. 1A. To ensure all fractions eluted from SEC column 1 could be maintained in column 2, the column lengths for 1 and 2 are, respectively, 15 cm and 25 cm. When fractions with satisfactory resolution is obtained, they are in sequence transferred to IMER for on-line digestion by 50 mM ammonium acetate containing 5% (v/v) acetonitrile under the flow rate of 50 μL/min, as shown in Fig. 1B. Subsequently, the yielding peptides were trapped on trap column 1, and after desalting were further separated by μRPLC and identified by ESI-MS/MS. During separation, another digested fraction was trapped on the parallel trap column 2 for desalting, so that the total analysis time for peptide separation could be shortened, as shown in Fig. 1C.

Due to the good enzymatic activity and time compatibility of csrSEC, IMER and μRPLC–ESI-MS/MS, these units were connected without any interface, thus greatly simplified the construction of an integrated platform for protein separation, on-line digestion, peptide separation and protein identification.

3.5. Proteins analysis

To evaluate the performance of such an integrated platform, a 6-protein mixture, including BSA, β-lactoglobulin, carbonic anhydrase, myoglobin, transferrin and ribonuclease, with Mr ranging from 10 kDa to 80 kDa was analyzed, and the results were shown in Fig. 3. For csrSEC, proteins were divided into four fractions, and for each fraction, it was cut into other four parts, and in sequence sent to IMER for digestion and μRPLC–ESI-MS/MS for separation and identification. Thus, in total 16 figures were obtained.

By further data searching, as shown in supplemental Table S2, all proteins were successfully identified with the sequence coverages of 37.40%, 74.69%, 40.15%, 58.82%, 5.67% and 17.74%, respectively. Except for ribonuclease (glycoprotein), more than three matched peptides were identified, which demonstrated high reliability of such an integrated platform for complex proteins analysis.

4. Conclusion

A novel integrated platform for proteome analysis with the combination of protein separation by csrSEC, on-line digestion by an IMER, as well as peptides separation and proteins identification by μRPLC/MS/MS was established, and successfully applied for the analysis of proteins mixture with high resolution, fast and high efficient on-line digestion, and automatic operation capacity. All these results demonstrate that such an integrated platform might be promising in proteome study.

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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.chroma.2009.06.019.

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