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ScienceDirect
Journal of Chromatography B, 849 (2007) 223–230
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

A bifunctional monolithic column for combined protein preconcentration and digestion for high throughput proteomics research☆

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Received 19 May 2006; accepted 9 November 2006
Available online 5 December 2006

Abstract

Enzymatic digestion of proteins is a key step in protein identification by mass spectrometry (MS). Traditional solution-based protein digestion methods require long incubation times and are limitations for high throughput proteomics research. Recently, solid phase digestion (e.g. trypsin immobilization on solid supports) has become a useful strategy to accelerate the speed of protein digestion and eliminate autodigestion by immobilizing and isolating the enzyme moieties on solid supports. Monolithic media is an attractive support for immobilization of enzymes due to its unique properties that include fast mass transfer, stability in most solvents, and versatility of functional groups on the surfaces of monoliths. We prepared immobilized trypsin monolithic capillaries for on-column protein digestion, analyzed the digested peptides through LC/FTICR tandem MS, and compared peptide mass fingerprinting by MALDI-TOF-MS. To further improve the digestion efficiency for low abundance proteins, we introduced C4 functional groups onto the monolith surfaces to combine on-column protein enrichment and digestion. Compared with immobilized trypsin monolithic capillaries without C4, the immobilized trypsin-C4 monolith showed improved digestion efficiency. A mechanism for increased efficiency from the combination of sample enrichment and on-column digestion is also proposed in this paper. Moreover, we investigated the effects of organic solvent on digestion and detection by comparing the observed digested peptide sequences. Our data demonstrated that all columns showed good tolerance to organic solvents and maintained reproducible enzymatic activity for at least 30 days.

Keywords: Protein digestion; Proteomics; Fourier transform ion cyclotron resonance mass spectrometry; LC/MS/MS; Monolithic column

1. Introduction

Separation and identification of expressed protein components of biological systems are key elements in proteomics research. Mass spectrometry (MS) based methods for protein identification have become a standard platform in this field [1–3]. However, the mass of a protein alone does not always provide sufficient information for its identification. The most popular strategies rely on digestion of proteins into peptides by a sequence-specific enzyme, followed by either peptide mass fingerprinting using MALDI-TOF-MS or tandem ESI-MS. Therefore, digestion and separation are critical steps in protein identification by MS prevalent in today's proteomics research.

Traditional solution-based protein digestion methods present a number of drawbacks. The standard protocol for in-solution tryptic digestion requires overnight incubation which limits the advancement of high throughput protein identification technology. Increased enzyme concentration can accelerate digestion and decrease analysis time; however, high concentration of enzyme will also tend to produce interfering and undesired autodigestion of the protease itself. To obtain high speed, high sensitivity, and high throughput protein digestion, development efforts have focused on the immobilization of enzymes [4–9]. In the past, immobilized enzyme digestion methods have been developed by several researchers to improve digestion efficiency [4–8,10–12]. In these studies, autodigestion is usually eliminated by immobilizing the enzyme on a solid support that isolates the enzyme moieties. Therefore, when the protein passes through a high concentration, immobilized enzyme reactor, the digestion process can be performed rapidly. In addition, the immobilized enzyme can also remain highly stable and active for extended periods of time. Because both the morphology and properties of monolithic supports have contributed to stability and activity of

☆ This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann.

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doi:10.1016/j.jchromb.2006.11.029

the enzyme, more recent efforts for enzyme immobilization have focused on the development of novel supports and preparation methods.

Recent developments of rigid, macroporous monoliths provide the possibility of improved supports to immobilize enzymes because they allow fast mass transfer and maintain high enzyme stability. Svec and coworkers first successfully immobilized trypsin on porous polymers and packed it into a LC column for digestion of proteins [13]. This group further improved the preparation method and introduced the immobilized trypsin monoliths into capillaries and microfluidic-chips [5,14,15]. The immobilized trypsin monolithic columns showed high enzyme activities and provided a high throughput approach to protein digestion for proteomics research. Palm and Novotny prepared a high flow through enzyme reactor, where the trypsin was immobilized by a simple and rapid method [6]. Their work demonstrated that catalytic activity of enzyme immobilized onto monoliths was stable for more than 20 weeks at room temperature. Ye et al. presented an enzymatic micro-reactor prepared in capillaries by immobilizing trypsin onto polymer monoliths and coupling digestion with online CE [4]. Their paper demonstrated the advantages of immobilized trypsin column in combination with micro-separation tools. Calleri et al. prepared a new trypsin-based bioreactor by immobilizing trypsin on an epoxy-modified silica monolithic support for protein digestion [11]. The silica monolithic bioreactor coupled through a switching valve to an LC-ESI-MS system showed better performance in the identification of proteins, as compared with conventional solution-phase digestion.

Currently, a significant challenge in proteomics research is related to the identification of low concentration proteins using on-column digestion. According to the process of enzyme kinetics, the speed of protein digestion is determined by the concentration and catalytic activity of the enzyme and substrate concentration [16]. Previous attempts to improve protein digestion have also been reported that focus on concentrating protein substrates [17]. Low concentration proteins were extracted from a mixture followed by the addition of trypsin for digestion.

A more recent approach for improved digestion in aqueous–organic solution has also been reported [18]. Russell et al. reported that protein digestion in organic solvent is more efficient than in 100% aqueous solutions based on the abundance of molecular ions from digested fragments observed in the mass spectra. Slysz et al. used a mixed aqueous–organic solution to couple with a packed column containing immobilized trypsin [7]. They obtained different digested peptide fragments by varying organic solvent concentration and their results suggested that organic solvents might help improve sequence coverage in on-column digestion of proteins.

LC/MS/MS has increasingly been employed in protein identification, which can provide important additional information through tandem mass spectrometry-based peptide fragments compared with peptide mass fingerprinting strategy [1,3]. Presently, Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) can provide unparalleled ultrasensitive, high mass accuracy measurement for characterization of proteolytic digests. FTICR-MS combined with LC has become a high throughput tool for proteomics analysis [19].

Described in this report are our efforts to develop novel immobilized trypsin monolithic capillaries and evaluate BSA digestion efficiency by LC/FTICR MS/MS and MALDI-TOF. To improve the digestion process of low concentration proteins, we developed a novel strategy for on-column digestion using a combination of immobilization enzymes and protein enrichment features. An immobilized trypsin-C4 monolithic column was developed to improve digestion and identification of lower abundance protein. Aqueous–organic solvent was also investigated with our columns as media to improve the digestion quality. Our results showed a notable increase in the protein digestion efficiency as compared with trypsin columns that lacked C4 functionality.

2. Experimental

2.1. Materials and chemicals

Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). Ethylene dimethacrylate (EDMA), glycidyl methacrylate (GMA), butyl methacrylate (BMA), azobisisobutyronitrile (AIBN), cyclohexanol, 1-dodecanol and sodiumcyanoboro hydride, γ-methacryloxypropyl trimethoxysilane (γ-MAPS), bovine serum albumin (BSA), casein, glutaraldehyde solution, trypsin, trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid, and dithiothreitol (DTT) were from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were from Fisher Scientific (Fair Lawn, NJ). All organic solvents were of HPLC grade.

2.2. Mass spectrometry

A Bruker Daltonics Apex-Q FTICR mass spectrometer with a 7.0 T superconducting magnet was used for the LC–MS experiments. All nano-ESI spray tips were made by etching from a 20-μm i.d., 360-μm o.d. fused-silica capillary with 49% HF. The voltage on the capillary inlet tube was set at -2 kV. All data sets acquired were 128 k points and the mass spectral data were acquired using Xmass 7.0.6 as the data acquisition software program.

LC separation was performed with an LC Packings Ultimate Nano-HPLC system equipped with a Famos micro autosampler and a Swichos micro column switching module (Dionex, Sunnyvale, CA). Reversed-phase solvents were (A) 0.1% TFA in 2% acetonitrile and (B) 0.1% TFA in 95% acetonitrile. Samples were first injected by the autosampler and loaded onto a micro trap column (C18 PepMap, 300 μm $\times$ 10 mm, 5 μm, LC Packings) at a flow rate of 50 μL/min with solvent A. A 20 μL aliquot of each on-column digestion solution was added into a small vial, dried by flowing nitrogen gas through the sample, and followed by addition of 0.1% TFA solution into the vial to result in a total volume of sample of 20 μL. A 10 μL aliquot of each solution was injected onto the LC column. The loaded sample was continuously washed with solvent A for 3 min to remove salts. Peptides were then eluted at a flow rate of 500 nL/min to an analytical column (C18 PepMap, 180 μm $\times$ 150 mm, 3 μm, 100 Å,

LC Packings) and separated using the following gradient: 0% B for 0–5 min, 0–15% B for 5–15 min, 15–25% B for 15–60 min, 25–40% B for 60–80 min, 95% B for 80–90 min, and 0% B for 91–120 min. The eluant from the analytical column was sprayed on-line with the mass spectrometer.

For the LC–FTICR-MS/MS experiment, the initial MS scan utilized an *m/z* range of 400–2000 followed by a tandem MS scan. Dynamic exclusion was activated to discriminate against previously selected ions. Collision cell energies were also selected according to the default energy file (peptides.cef) in XMASS 7.0.6. This file contains the slope and intercept values for calculating the optimal MS/MS collision energy for a specific charge state. For 1+ ions, the slope is 0.027 and the intercept is 17.044. For 2+ ions, slope is 0.0386 and the intercept is −3.5576. For 3+ charge states or higher, the slope is 0.0291 and the intercept is −1.0453.

All FTICR-MS/MS data were analyzed using the software package ICR-2LS [20]. Time-domain signals were apodized (Welch) and zero-filled (2) before Fourier transformation to the mass spectra. The MS/MS data were first transferred to .pek file by ICR-2LS software, and then converted to .mgf file using a macro function developed within Microsoft Excel. MASCOT was used for MS/MS searches using the current NCBInr database.

MALDI-TOF MS analysis was performed using an OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). C18 ZipTips (Millipore, Billerica, MA, USA) were used to concentrate and desalt protein digests. Spectra were acquired in the reflectron positive-ion mode.

2.3. Preparation of monolithic capillary column

The capillaries were internally treated with 3-(trimethoxysilyl)propyl methacrylate in the manner described by Svec and coworkers [5]. The capillary was first rinsed with a 1 M NaOH solution for 1 h, and then rinsed with water and methanol for 10 min. After drying the capillary using nitrogen gas, the capillary was filled with a methanol solution containing 50% (v/v) γ-MAPS and kept at room temperature for 1 day to react. Finally, the capillary was rinsed with methanol and dried with nitrogen. The polymerization reaction solution was prepared by mixing 0.35 mL GMA, 0.1 mL EDMA, 0.2 mL cyclohexanol, 0.4 mL dodecyl alcohol or 0.25 mL GMA, 0.1 mL EDMA, 0.1 mL BMA, 0.2 mL cyclohexanol, 0.4 mL dodecyl alcohol (for C4 column) and 4 mg AIBN. After mixing, the homogeneous mixtures were sparged with nitrogen for 10 min. Then, a piece of silanized capillary was attached to syringe and filled with polymerization solution to desired length. Both ends of the capillary were plugged with a piece of rubber tubing. Both the capillary and vial were submerged into a water bath at 55 °C for overnight.

2.4. Immobilization of trypsin

We immobilized trypsin according to the method described by Ye et al. [4]. Trypsin was covalently immobilized on a macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic capillary column. A 180 μm ID, 10 cm long column was used for digestion. The monolithic column was first flushed with water for 20 min, followed by 29% ammonium hydroxide solution. The reaction proceeded for 3 h at 40 °C. After amination, a 2.5% aqueous solution of glutaraldehyde in 0.1 M borate buffer (pH 8.2) was pumped through the column for 1 h at room temperature. After activation by glutaraldehyde, trypsin was coupled to the support by pumping 3 mg/mL trypsin in a 0.1 M, pH 8.2, borate buffer through the column for 24 h at 55 °C. Finally, the trypsin immobilized monolithic column was treated with 25 mM sodium cyanoborohydride overnight.

2.5. Protein digestion

13.2 μg BSA was first dissolved in 1 mL buffer of 100 mM ammonium bicarbonate, pH 8.0. The protein was then reduced and alkylated. One milliliter of 13.2 μg/mL protein were added to 0.01 mL of 45 mM dithiothreitol for incubation at 50 °C for 45 min, followed by 0.01 mL of 10 mM iodoacetamide and an additional incubation for 30 min at room temperature (in the dark). The protein solution was diluted to a variety of concentrations (1, 5, 10, 25, 50 and 100 μM) with buffer solutions. The buffers we used were: 100 mM ammonium bicarbonate, pH 8.0 (buffer A); 30 mM ammonium bicarbonate, pH 8.0 (buffer B); 30 mM ammonium bicarbonate, pH 8.0 and 12.5% (v/v) methanol (buffer C); 30 mM ammonium bicarbonate, pH 8.0 and 25% (v/v) methanol (buffer D); 30 mM ammonium bicarbonate, pH 8.0 and 10% (v/v) acetonitrile (buffer E); 30 mM ammonium bicarbonate, pH 8.0 and 20% (v/v) acetonitrile (buffer F). The column was rinsed with buffer B before use. The protein sample was pushed through the immobilized trypsin monolithic capillary at a constant flow rate using a syringe pump. When using immobilized trypsin-C4 monolithic capillary and aqueous buffer, we washed the column using 1–2 μL buffer D and added it to collected solution. Samples were kept at 4 °C prior to MS analysis.

3. Results and discussion

3.1. Fabrication of column and evaluation of on-column protein digestion

The monolithic columns were prepared adopting the method reported by Svec and coworkers [5,13] Since the folding state and stereo-space of the enzyme influence both its activity and digestion efficiency, it is necessary to adjust the concentration of monomers, cross-linkers and porogents to get suitable pore size and physical properties on the surface of monolithic supports. We selected the optimized experimental conditions to fabricate monolithic columns by evaluating the penetrability of monolithic supports for mobile phase and the activity of trypsin. We immobilized trypsin onto the surface of monoliths according to Ye and Dovichi's improved immobilizing trypsin method [4]. For monolithic immobilized trypsin-C4 column, we first introduced C4 groups on the monolith, and then immobilized trypsin as described above. All columns were stored in 30 mM borate buffer (pH 8.0) and rinsed with 30 mM ammonium bicarbonate buffer (pH 8.0) for 1 h before use. The digestion buffer pH

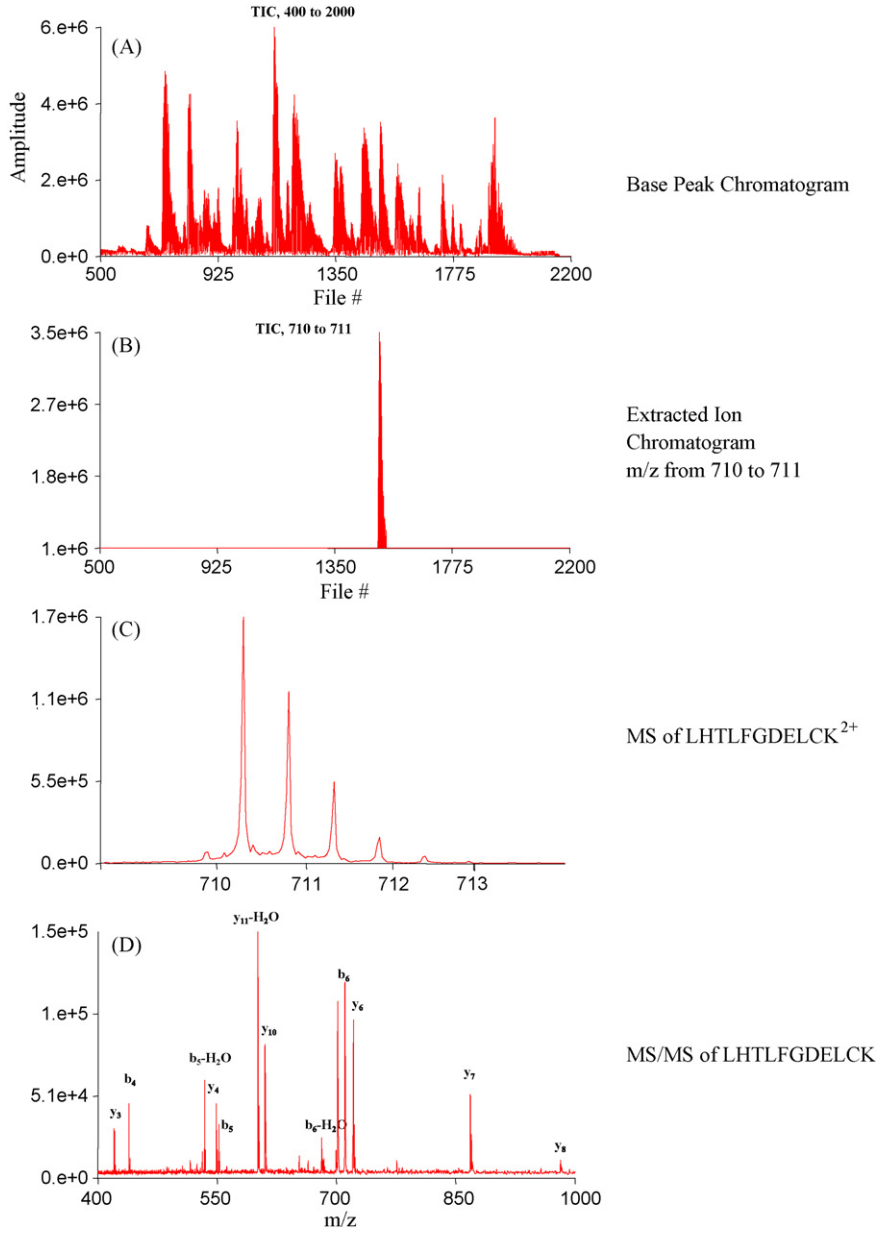

Fig. 1. LC/FTICR MS/MS spectra of peptides obtained from on-column digestion of BSA using a monolithic immobilized trypsin capillary. Condition: ODS capillary column (150 mm × ID 180 μm, OD 360 (m); injection volume 10 μL. Protein concentration of 50 μM in 100 mM ammonium bicarbonate buffer (pH 8). Flow rate through monoliths was 0.5 μL/min. (A) Base peak chromatogram from FTICR mass spectra obtained from capillary RPLC separation of BSA digest from monolithic immobilized trypsin capillary; (B) extracted ion chromatogram *m/z* from 710 to 711; (C) zoom-in MS showing the selected precursor ion in the extracted ion chromatogram; (D) CID MS/MS spectrum showing the fragment ions from selected *m/z* peptides.

was kept at 8.0 to maintain trypsin activity. After digestion, the pH of the sample solution was adjusted to 1.5 with addition of TFA. The evaluation of on-column digestion was first obtained with MALDI-TOF-MS. To obtain more precise and detailed digestion information, we evaluated the digestion efficiency by analyzing peptides with LC/FTICR-MS/MS. Fig. 1 shows a typical LC/FTICR MS/MS chromatogram of peptides obtained from on-column digestion of BSA using monolithic capillary immobilized trypsin. The separation of peptides was performed with gradient reversed-phase LC over 120 min. The base peak chromatogram in Fig. 1A shows that good separation was obtained under optimal conditions. Fig. 1B illustrates an extracted ion chromatogram of one of the peptides identified in this analysis, in this case a 2+ ion observed at $m/z = 711$. The mass of parent species and their fragments were obtained in successive measurement as shown in Fig. 1C and D. All LC/MS/MS spectra were transformed to Mascot generic file format and searched using Mascot [21]. The effects of digestion rate were evaluated by varying the flow rate of sample solution through the immobilized trypsin column. Both sequence coverage and Mascot score increase with decreased flow rate, as shown in Table 1. We focused on digest results obtained with a flow rate of 0.5 μL/min, because the flow rate is comparable with ESI and nano-LC.

Table 1
The effects of flow rate during digestion on BSA identification by LC/FTICR MS/MS.

Flow rate (μL/min)	MASCOT score	Peptide fragments	Sequence coverage (%)
0.2	872	27	49
0.5	529	17	31
1	417	12	22
2	309	11	21
5	303	11	21

BSA (50 μM) solutions were passed through the monolithic capillary at various flow rates as shown in the table.

3.2. Immobilized trypsin –C4 monolithic capillary

The effects of sample concentration on immobilized trypsin capillary digestion efficiency were further studied as shown in Fig. 2. The number of identified peptide sequences decreased with decreasing BSA concentration. The efficiency of the immobilized trypsin column was unsatisfactory for low concentration of samples such as 1 μM. To improve digest efficiency for low abundance proteins, the proteins could be concentrated using a hydrophobic column, followed by the addition of trypsin for digestion in solution [17]. Slysz and Schriemer presented a device which combined a C4 column and a trypsin immobilized column to realize real-time proteolytic digestion [22]. Peterson et al. [15] developed a dual-function microanalytical device to integrate solid-phase extraction and enzymatic digestion. The samples were extracted in the first segment of the column, and then eluted and digested in the second segment. We have developed a different method for improving the digestion process of minute amounts of protein using a combination of on-column immobilized enzyme and protein enrichment. We introduced butyl groups and enzyme onto a single monolithic column and

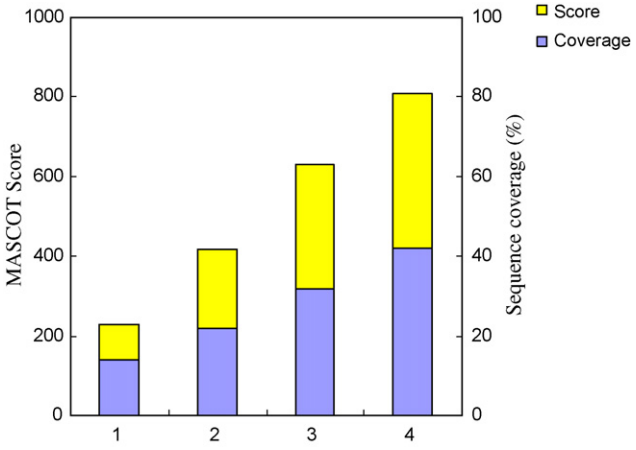

Fig. 2. The effects of sample concentration on BSA identification for peptide sequences from LC/FTICR-MS/MS. Various concentration BSA solutions were passed through the monolithic capillary at the flow rate of 1 μL/min, (1) 25 μM; (2) 50 μM; (3) 100 μM; (4) 200 μM.

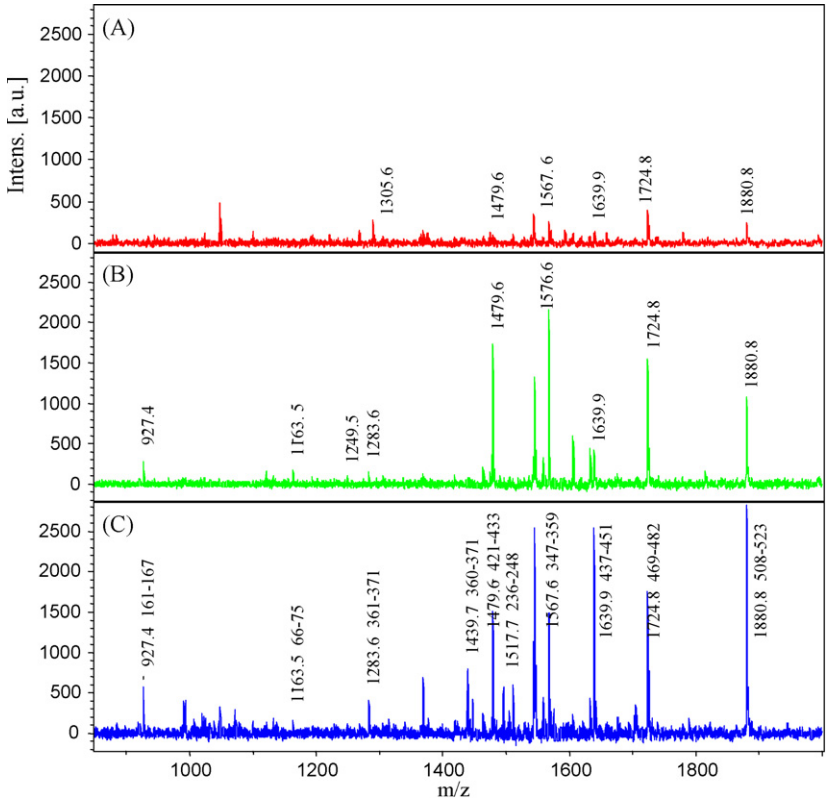

Fig. 3. MALDI-TOF spectra of peptides obtained from on-column digestion of BSA samples: BSA (1 μM × 10 μL); column: 180 μm (ID) ×10 cm; flow rate: 0.5 μL/min; room temperature; (A) immobilized trypsin monolithic capillary, 30 mM ammonium bicarbonate buffer (pH 8); (B) immobilized trypsin-C4 monolithic capillary, 30 mM ammonium bicarbonate buffer (pH 8); (C) immobilized trypsin-C4 monolithic capillary, 30 mM ammonium bicarbonate, pH 8.0 and 12.5% (v/v) methanol. (*) Labeled peaks are the identified peptide sequences of BSA tryptic digestion.

prepared an immobilized trypsin-C4 monolithic column. The hydrophobic functional groups, which were introduced onto the surface of supports in the vicinity of the trypsin, can enrich and retard the proteins in a mixture when the sample was injected and pushed through column. Therefore, the proteins were adsorbed onto the surface of supports which allowed improved substrate access to the active site of the enzyme. Further increase of the protein concentration and interaction between protein and enzyme provide the possibility of improved digestion and sensitivity for low abundance proteins.

Fig. 3 shows MALDI-TOF-MS data for 1 μM BSA digestion through an immobilized trypsin-C4 monolithic column. As compared to digestion results acquired from a trypsin column without C4 groups, we identified approximately 30% more peptide sequences with the addition of the C4 group. Furthermore, for peptides identified with both columns, we observed a significant increase in peak intensities with the use of the trypsin-C4 column (in some cases up to five times improvement). We also evaluated sensitivity with the two digested samples using LC–FTICR MS/MS. Ten picomole samples eluting from two monolithic columns were respectively loaded onto LC column and analyzed with the same experimental conditions. LC–FTICR-MS analysis of the digested sample from the immobilized trypsin-C4 monolithic column allowed positive identification of peptide sequences from BSA. However, the analysis of the sample from the trypsin column that lacked C4 functionality did not facilitate identification of BSA peptides. These results demonstrate two things. First, the use of the trypsin-C4 column increases the S/N and number of peptides we can identify from a given sample. Second, the trypsin-C4 column increases our ability to identify proteins from smaller amounts, as compared to results from immobilized trypsin columns that lack C4 groups.

3.3. Aqueous–organic solution as digest buffer

Recent studies showed that the protein digestion in aqueous–organic solvent may be more efficient than in aqueous solution as indicated by the abundance of molecular ions from digested fragments in mass spectra [7,9]. To further study the effects of organic solution on immobilized trypsin-C4 column performance, we introduced aqueous–organic solvent as media into the digestion system to improve the digestion quality. Aqueous–organic solvents present additional benefits when used with our columns since the C4 functional groups are hydrophobic and analyte retention can be tuned effectively by varying aqueous–organic solvent composition. Therefore, one can further optimize protein enrichment and digestion processes. Table 2 shows the peptide sequences observed in LC/FTICR-MS/MS using various aqueous–organic buffers with on-column digestion of BSA. Different peptide sequences were identified with different buffers, as shown in Table 2. The sequence cover-

Table 2
The effects of methanol on BSA digestion using immobilized trypsin-C4 monoliths

Matched sequence	Mr (expt)	Missed cleavage	Buffer B	Buffer C	Buffer D
YLYEIAR	926.49	0	y	y	y
ALKAWSVAR	1000.58	0	y	y	y
ADEKKFWGK	1017.56	0	y	y	y
CCTESLVNR	1137.5	0	y	y	y
KQTALVELLK	1141.71	1	y	y	y
LVNELTEFAK	1162.62	0	y	y	y
DTHKSEIAHR	1192.59	0	y	y	y
FKDLGEEHFK	1248.63	1	y	y	y
HPEYAVSVLLR	1283.7	0	y	y	y
HLVDEPQNLIK	1304.73	0	y	y	y
SLHTLFGDELCK	1418.7	1	y		
LGEYGFQNALIVR	1478.82	0	y	y	y
EYEATLEECCAK	1501.63	0			y
LKECCDKPLLEK	1531.78	1	y	y	
LKPDPNTLCDEFK	1575.8	1	y	y	y
KVPQVSTPTLVEVSR	1638.97	1	y	y	y
MPCTEDYLSLILNR	1723.87	0		y	y
DAFLGSFLYEYSR	1886.95	1	y		
HPYFYAPELLYYANK	1887.97	0	y	y	y
RPCFSALTPDETYVPK	1879.98	1	y	y	y
LKPDPNTLCDEFKADEK	2019.03	1		y	
DAIPENLPPLTADFAEDKDVCK	2400.03	1	y	y	y
LKPDPNTLCDEFKADEKK	2147.1	2		y	y
QEPERNECFLSHKDDSPDLPK	2540.2	2	y	y	y
VHKECCHGDLLECADDRADLAK	2611.17	2		y	y
SHCIAEVEKDAIPENLPPLTADFAEDKDVCK	3510.73	2		y	y
Matched peptides number			20	23	22
Coverage (%)			39	45	45

"y" means that the sequence were found. 5 μM BSA in solution, flow rate through monoliths was 0.5 μL/min and sample was injected in 20 μL. Buffer B: 30 mM ammonium bicarbonate, pH 8.0; buffer C: 30 mM ammonium bicarbonate, pH 8.0 and 12.5% (v/v) methanol; buffer D: 30 mM ammonium bicarbonate, pH 8.0 and 25% (v/v) methanol. Other conditions were the same as used for Fig. 1.

Table 3
The effect of acetonitrile on casein digestion using immobilized trypsin-C4 monoliths

Matched sequence	Mr (expt)	Missed cleavage	Solution B	Solution E	Solution F
YLGYLEQLLR	1266.8	0	y[a]	y	y
FFVAPFPEVFGK	1383.7	0	y	y	y
FFVAPFPEVFGKEK	1640.9	1	y	y	y
HQGLPQEVLNENLLR	1758.9	0		y	y
YKVPQLEIVPNSAEER	1871.0	1			y
HPIKHQGLPQEVLNENLLR	2234.2	1	y	y	y
EPMIGVNQELAYFYPELFR	2315.1	0	y	y	y
EGIHAQQKEPMIGVNQELAYFYPELFR	3207.7	1		y	y
Matched peptides number			5	7	8
Sequence coverage (%)			28	32	40

[a] "y" means that the sequence were found. 5 μM casein solution was passed through the column with a flow rate of 1 μL/min. Buffer B: 30 mM ammonium bicarbonate, pH 8.0; buffer E: 30 mM ammonium bicarbonate, pH 8.0 and 10% (v/v) acetonitrile; buffer F: 30 mM ammonium bicarbonate, pH 8.0 and 20% (v/v) acetonitrile. Other condition were the same as used for Fig. 1.

age obtained with aqueous buffer is 39%, and it increases to 45% when an aqueous–organic buffer is used. The use of a solution containing methanol resulted in improved digest efficiency. We also used acetonitrile as organic addition for digestion of casein as shown in Table 3. The sequence coverage obtained with the buffer containing 0, 10 and 20% acetonitrile is, respectively, 28, 32 and 40%. The results showed that increased sequence coverage was identified using aqueous-acetonitrile solution as opposed to aqueous solution alone with the use of the trypsin-C4 column.

We further studied the lifetime and stability of immobilized trypsin-C4 column. Between uses, the column was stored at 4 °C and 30 mM borate buffer (pH 8). Using the same column, we did two on-column digestions over a 30-day period. As shown in Fig. 4, a similar MS pattern can be observed in both digestion solutions: more than 70% peptide sequences are the same, including all high abundance peaks (such as *m/z* 927, 1439, 1479, 1567, 1640, 1881). This study demonstrated that the activity of the enzyme could be maintained for 30 days with proper column storage. In addition, the mixture of organic–aqueous solvent was

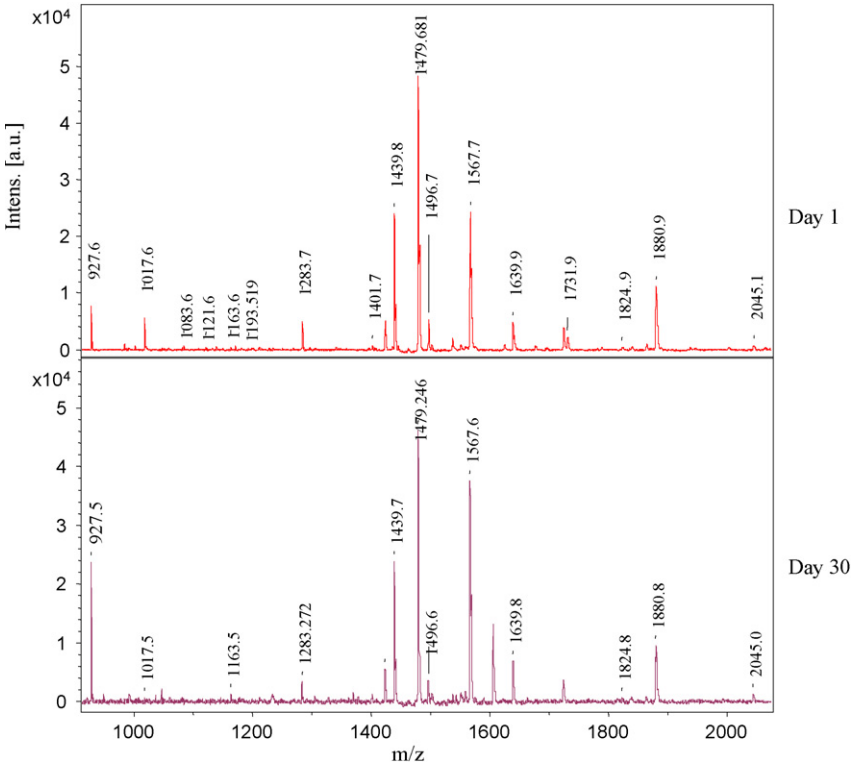

Fig. 4. MALDI-TOF mass spectra of peptides obtained from replicate on-column protein digestion experiments using immobilized trypsin-C4 column. (The digestion experiment first was done on day 1, and then the column was washed and kept with buffer B; the same experiment was performed after 30 days). Sample: 5 μM BSA in 30 mM ammonium bicarbonate, pH 8.0 and 12.5% (v/v) methanol. Other condition as the same with Fig. 1. (*) Labeled peaks are the identified peptide sequences of BSA tryptic digestion.

often used as digestion buffer, and no difference was observed in peptide mass fingerprinting after this operation, which further demonstrated that the immobilized trypsin-C4 columns have good tolerance to organic solvents.

4. Conclusions

The concentration, digestion and separation of protein samples are critical aspects that limit the current field of proteomics research. In this paper, we report development and initial evaluation of a novel monolithic column that increases the ability to extend immobilized enzyme digestion methods to lower concentration substrates. The on-column protein digestion from immobilized trypsin-C4 column was compared with that from immobilized trypsin column that lacked C4 functionality. Our work showed that the use of the trypsin-C4 column increases the S/N and number of peptides we can identify from a given sample. Moreover, the trypsin-C4 column increases the ability to identify proteins from small amounts, as compared to results from immobilized trypsin column that lacks C4 groups. It is very likely that further optimization of the ratio of C4 groups and trypsin coupled to the surface can further increase the ability to both enrich and digest low concentration substrates. Furthermore, the combination of immobilized trypsin-C4 column and aqueous–organic digestion solution is shown in our work to further increase the capability to digest low abundance protein. The introduction of aqueous–organic solution can further help improve digest efficiency for immobilized trypsin-C4 system. In addition, our data demonstrated that all columns showed good tolerance to organic solvents and maintained reproducible enzymatic activity. Immobilized trypsin-C4 column is a potential protein digestion tool, which is compatible with fast, high throughput methods for proteomic analysis. Finally, our results in this paper suggest that a single monolith with multiple functional groups can have a significant impact in the ability to digest protein samples with increased sensitivity and throughput in proteomics research.

Acknowledgments

This work was supported by grants from the Directorate of Biological Sciences, National Science Foundation, Grant No. DBI-0352451; and the Office of Science (BER), U.S. Department of Energy, Grant No. DE-FG02-04ER63924. The authors thank Gordon A. Anderson and Nikola Tolic at the Pacific Northwest National Lab for providing the software ICR-2LS and other software tools used for data analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2006.11.029.

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