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# Development of an Online Microbore Hollow Fiber Enzyme Reactor Coupled with Nanoflow Liquid Chromatography-Tandem Mass Spectrometry for Global Proteomics

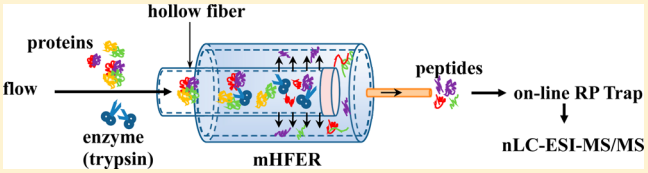
Jin Yong Kim,[†] Sun Young Lee,[‡] Sook-Kyung Kim,[‡] Sang Ryoul Park,[‡] Dukjin Kang,*[,‡] and Myeong Hee Moon*[,†]

[†]Department of Chemistry, Yonsei University, Seoul, 120-749, Korea

[‡]Center for Bioanalysis, Division of Metrology for Quality of Life, Korea Research Institute of Standards and Science, Daejeon, 305-340, Korea

Supporting Information

**ABSTRACT:** In this study, we report the development of a microbore hollow fiber enzyme reactor (mHFER) coupled to nanoflow liquid chromatography-tandem mass spectrometry (nLC-ESI-MS/MS) for the online digestion or selective enrichment of glycopeptides and analysis of proteins. With mHFER, enzymatic digestion of protein could be achieved by continuous flow within a very small volume (~10 μL) of mHF inserted in a PEEK tube. Digested peptides exited through the pores of the hollow fiber membrane wall to external single or multiplexed trap columns for nLC-ESI-MS/MS analysis. Evaluation of online mHFER-nLC-ESI-MS/MS system was made with bovine serum albumin (BSA) by varying the temperature of digestion and the amount of protein injected. We evaluated the ability of the mHFER system to enrich glycopeptides by injecting a mixture of lectin (concanavalin A) and digested peptides from α-1-acid glycoprotein (AGP) into the mHFER, followed by delivery of PNGase F for endoglycosidic digestion. Nonglycosylated peptides unbound to lectins eluted at the first breakthrough run while N-linked glycopeptides eluted after the endoglycosidic digestion. The developed method was applied to urine samples from patients with prostate cancer and controls; 67 N-linked glycopeptides were identified and relative differences in glycopeptide content between patient and control samples were determined.




In proteomic analysis, the bottom-up approach of liquid chromatography coupled with mass spectrometry (MS) has become a well-established protocol for identifying proteolytic peptides and their post-translational modifications (PTMs). The growth of top-down proteomics, in which biological samples are directly analyzed at the protein level, has also increased in recent years,[1−5] and advanced MS/MS platforms with multidimensional liquid chromatography are powerful tools for identifying proteins and their functions in cells, such as protein−protein interactions, intercellular recognition, and signal transduction.[6−8]

Prior to proteomic analysis, proteolytic preparation, which is an essential step for protein identification, was commonly carried out by in-gel or in-solution digestion using various proteases (e.g., trypsin, chymotrypsin, Lys-C, AspN). Conventionally, proteolysis is carried out manually and requires at least 12 h for sufficient proteolytic cleavage. Moreover, for low concentration samples, a preconcentration step before enzymatic treatment is required to enhance protein−enzyme reactions. However, the preconcentration step may result in sample loss leading to the irreproducible recovery of peptides, which may affect confidence in the quantitative/qualitative determination results.[9] Recently, high pressure and ultrasound have been used to speed up proteolytic digestion. An external force-assisted enzymatic reactor (EFER) promotes digestion in less than 10 min and increases sample throughput in comparison to conventional overnight preparation procedures.[10−12] However, it is difficult to couple EFER with LC. To create an online proteolytic digestion platform integrated with multidimensional LC-MS/MS, on-column immobilized enzyme reactors (IMERs) packed with diverse enzyme-immobilized supports, such as silica, polymeric particles, and monolithic materials have been introduced.[9,13−15] IMERs can minimize unwanted enzyme autolysis caused by prolonged digestion and increase the reproducibility of proteolysis after repeated runs. However, automization of IMERs is not achievable with 1-dimensional HPLC but with 2D-HPLC devices[16,17] because the typical low pH mobile phase used for peptide analysis may deactivate the immobilized enzyme, and there may be activity loss of the immobilized enzyme because of repeated usage. Recently, a variety of modified enzyme reactors such as membrane-interfaced IMERs,[18] on-chip enzyme reactors,[19] and a fast online high pressure digestion system (FOLDS)[20] have been developed to overcome the

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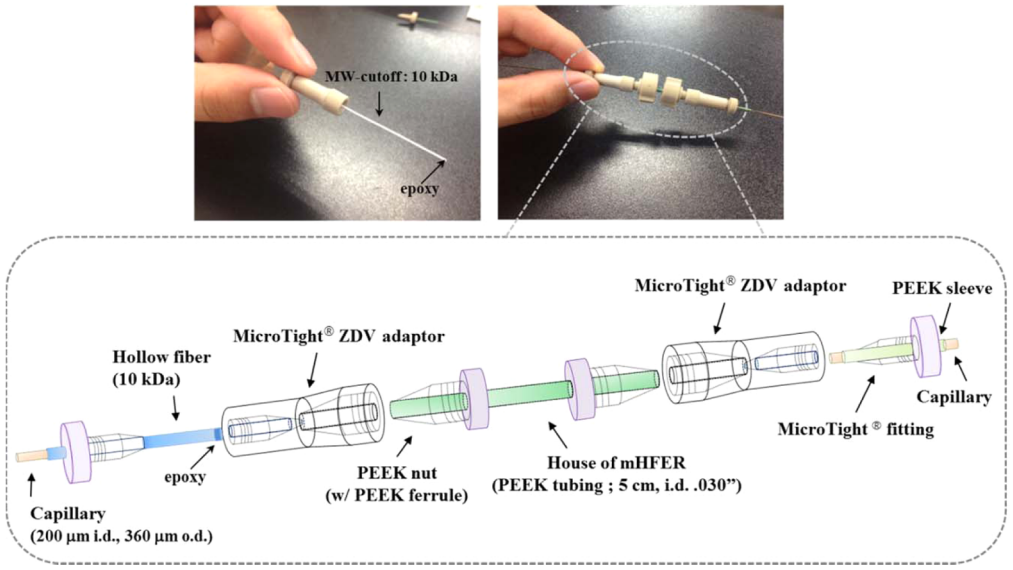


**Figure 1.** Assembly of a mHFER: An end-blocked microbore HF was inserted into a PEEK tube that had both ends connected to MicroTight adapters. The silica capillary on the left side was inserted inside the mHF and tightened without a sleeve.

limitations listed above. However, the reproducibility of formation of proteolytic peptides within a reactor and difficulty in regulating pressure buildup still need to be addressed.

In this study, we developed a microbore hollow fiber enzyme reactor (mHFER) for online proteolysis prior to nLC-ESI-MS/MS. A mHFER is a simple, highly reproducible, inexpensive to assemble, user-friendly device for global shotgun proteomic analysis. A mHFER is composed of a short (~5 cm long and ~10 $\mu$L in volume) porous microbore hollow fiber (mHF, molecular weight cutoff of 10 kDa) inserted into PEEK tube of which the inner diameter is slightly larger than that of the mHF. Both ends of the container are connected to capillary tubing. Because the mHF is porous and one end of the mHF is closed by a seal, a proteome sample can be simultaneously concentrated and digested inside the mHF when protease is loaded in sequence. The resulting peptides can then exit the mHF through the pores of the mHF wall by pump flow and leave the container toward a sample-trapping column prior to nLC-ESI-MS/MS. The entire process can be fully automated by integrating the system with an autosampler and a switching valve. We evaluated the performance of a mHFER for proteolytic preparation of bovine serum albumin (BSA) by examining the reproducibility and minimum handling amount of proteins. A mHFER can also be utilized to selectively enrich targeted N-linked glycopeptides; purification/enrichment of glycopeptide is an essential step in glycoproteomics. A mixture of tryptic peptides of $\alpha$-1-acid glycoprotein (AGP) and concanavalin A (ConA) were loaded in the mHF and nonglycosylated peptides were screened out first. The remaining glycopeptides bound to ConA underwent endoglycosidic digestion by injecting PNGase F. The resulting deglycosylated peptides from the mHFER were then analyzed by nLC-ESI-MS/MS.

## MATERIALS AND METHODS

**Materials and Chemicals.** Ammonium bicarbonate ($NH_4HCO_3$), dithiothreitol (DTT), formic acid, urea, calcium chloride, manganese chloride, magnesium chloride, and the three protein standards (concanavalin A (ConA) from *Canavalia ensiformis*, bovine serum albumin (BSA), and alpha-1-acid glycoprotein (AGP) from human plasma) were purchased from Sigma (St. Louis, MO, U.S.A.). Sequencing grade trypsin and Lys-C were obtained from Promega Corp. (Madison, WI, U.S.A.). For deglycosylation of glycopeptides and glycoproteins, peptide-*N*4-(*N*-acetyl-beta-glucosaminyl) asparagine amidase (PNGase F) was purchased from New England BioLabs, Inc. (Ipswich, MA, U.S.A.). HPLC-grade acetonitrile and water for a binary gradient elution were obtained from J. T. Baker (Deventer, Netherlands). Fused-silica capillaries (25, 50, 75, and 100 $\mu$m-i.d.; 365 $\mu$m-o.d.) used for the capillary LC column and tubing connections were obtained from Polymicro Technology LLC (Phoenix, AZ, U.S.A.). Fittings, adapters, and PEEK tubing were purchased from Upchurch Scientific (Oak Harbor, WA, U.S.A.) of IDEX Health & Science, LCC.

**mHFER.** The mHFER was assembled as shown in Figure 1. The mHF used in this study was made of polysulfone (surface-modified hydrophilic polysulfone, molar mass cutoff of 10 kDa) obtained from Kolon Central Research Institute (Yongin, Korea). The dimensions of the mHF were as follows: 4.8-cm long, 500-$\mu$m i.d., 650-$\mu$m o.d., and a geometrical fiber volume of approximately 10 $\mu$L. Before inserting the mHF into the mHFER module, one end of the mHF was blocked with an epoxy (epoxy resin/polythiol) from Tushin Trading Co. Ltd. (Tokyo, Japan) so that proteolytic peptides could exit only through the pores of the mHF by pump flow.

To assemble the mHFER module, both ends of a PEEK tube (5-cm long, 0.030 in.-i.d. 1/16 in.-o.d.) were connected to MicroTight ZDV adapters, the opposite ends of which can be connected with a MicroTight Fitting for 1/32 in. o.d. tubing. The end-blocked mHF was inserted through the MicroTight adapter from one end of the PEEK tube (left side of Figure 1), while the capillary tube from the injector was inserted into the mHF up to the midpoint of the Microtight adapter to enable the mHF and silica capiliary (200 $\mu$m i.d. and 360 $\mu$m o.d.) to be tightened using the MicroTight Fitting without a sleeve. The opposite end (right side) of the PEEK tube was connected via an adapter to another silica capillary using a 1/32 in. o.d. PEEK tubing sleeve so that the filtrate from the mHF wall would exit through the end of the PEEK tubing.


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Online operation of the mHFER and nLC-ESI-MS/MS was accomplished using a 10-port switching valve and a column switching valve (for a multiplexed trap system) obtained from IDEX Health & Science, LCC (Oak Harbor, WA, U.S.A.), as shown in Figure 2. For online proteolysis, the protein sample

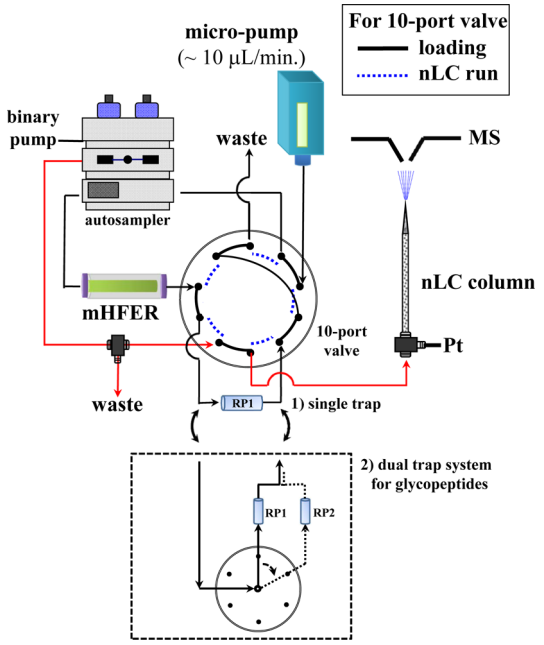


**Figure 2.** System configuration of the online mHFER-nLC-ESI-MS/MS system: (1) single trap for ordinary proteolysis and (2) dual trap system for glycopeptide enrichment; the single trap was replaced with a the column switching valve for multiplexed trap selection. The line notation (loading and nLC run) at the top of the figure indicates the 10-port valve configuration.

was loaded directly into the mHFER by the autosampler (Agilent Technologies, Waldbronn, Germany) at a flow rate of 5 μL/min using an external microHPLC pump from FLOM Corp. (Tokyo, Japan), followed by the injection of trypsin. The carrier solution for the delivery of the protein sample and trypsin was a buffer solution (referred to as B1) consisting of 50 mM ammonium bicarbonate ($NH_4HCO_3$) solution with or without 10 mM dithiothreitol (DTT). During proteolysis, the mHFER module was heated in a column heater from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.) to speed up proteolysis. Digestion was performed by varying the incubation temperature (25−80 °C). After digestion, solution B1 was pumped by the micro-HPLC pump to the mHFER so that the digested peptides would exit the mHF membrane through the pores of the membrane wall toward a sample trapping column, which was connected to the 10-port switching valve with the single trap (RP1), as shown in Figure 2. For online purification of *N*-glycopeptides with lectins, a mixture of glycopeptides and lectins was loaded into the mHFER, which in this example served as a selective isolator of *N*-glycopeptides. In this case, the single RP1 shown in the box at the bottom of Figure 2 was replaced with a column-switching valve equipped with two trapping columns, so that nonglycosylated peptides (RP1) and *N*-glycopeptides (RP2) could be trapped in each trapping column, respectively.

**Proteolytic Digestion of Glycoproteins and Endoglycosic Digestion of N-Linked Glycopeptides.** To evaluate the online isolation of N-glycopeptides using the mHFER, α-1-acid glycoprotein (AGP) was utilized as a glycoprotein standard and injected into the mHFER after digestion. AGP was first digested using dual enzymes (Lys-C followed by trypsin) in solution. One hundred milligrams of AGP was dissolved in 0.1 M phosphate-buffered saline (PBS) solution containing 8 M urea and 10 mM DTT and incubated for 2 h at 37 °C. The remaining thiol groups were alkylated with 20 mM iodoacetamide (IAM) in an ice bath for 2 h in the dark. To remove the remaining IAM, cysteine (40-fold excess of IAM) was added. The mixture was then diluted to a final concentration of 1 M urea with 0.1 M phosphate buffer, digested with 5 μg of sequencing grade Lys-C for 18 h at 37 °C, followed by secondary digestion with 5 μg of sequencing grade trypsin for 18 h at 37 °C. The resulting mixture was desalted using an Oasis HLB cartridge from Waters (Milford, MA, U.S.A.). Washed peptides were lyophilized and stored in the freezer.

Lectin−peptide complexation was achieved by mixing 100 μg (4 nmol) AGP peptide powder with concanavalin A (ConA, 64 μmol/L) in a total volume of 1 mL binding buffer solution with vortexing for 1 h. The binding buffer (B2) was 50 mM Tris-HCl solution (pH 7.40) containing 1 mM calcium chloride, 1 mM manganese chloride, and 1 mM magnesium chloride. While loading the lectin−peptide complex mixture into the mHFER with B2, free peptides unbound to lectins exited the mHFER by breakthrough and were loaded into the RP1 trapping column of the dual trap system shown in Figure 2. Endoglycosidic digestion of N-linked glycopeptides bound to ConA within the mHFER was performed by introducing 5 μL of PNGase F (≥5000 units/mL) into the mHF. Free deglycosylated peptides exited through the pores of the mHF by pump flow and were trapped in the RP2 trapping column for nLC-ESI-MS/MS analysis.

The same procedure used for AGP was applied to urine samples from three healthy controls and three prostate cancer patients obtained from Severance Hospital (Seoul, Korea) under informed consent. To extract urinary proteins, two protease inhibitor cocktail tablets from Hoffmann-La Roche Ltd. (Basel, Switzerland) were added to 10 mL raw urine and then the mixture was centrifuged at 3000*g*. The supernatant solution was decanted and concentrated using Amicon Ultracel-3K membrane kit from Millipore (Danvers, MA, U.S.A.). The filtrate was dispersed in B1 buffer to a concentration of 2 mg/mL and the protein concentration was analyzed by Bradford assay. Urinary proteins (10 μg) were digested in-solution and the resulting peptides were mixed with 200 μg of ConA. Thereafter, the samples were processed as described above for AGP.

**nLC-ESI-MS/MS.** During nLC-ESI-MS/MS analysis, separation was carried out in a pulled tip capillary column using a binary gradient elution and the model 1260 capillary LC system from Agilent Technologies (Waldbronn, Germany) interfaced with an LTQ Velos ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, U.S.A.). The trapping columns and analytical column were prepared in our laboratory as described in previous studies.[21−23] All trapping columns (3 cm in length) were packed in 200 μm-i.d. capillary with 5 μm-200 Å Magic C18AQ from Michrom Bioresources, Inc. (Auburn, CA, U.S.A.), while the analytical column (pulled tip capillary column, 17 cm × 75 μm-i.d.) was packed with 5 μm-100 Å Magic C18AQ. The mobile phase composition of the binary gradient elution for nLC was 98/2 (v/v) water/acetonitrile for mobile phase A, and 95/5 acetonitrile/water for mobile phase


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B, and 0.1% formic acid was added to both mobile phases. The binary gradient elution conditions are listed in Table S1 of Supporting Information. During one nLC-ESI-MS/MS run, the used protease in the mHF membrane was automatically washed out by inverting the washing solution from the mHFER outlet to the inlet (dotted line configuration in Figure 2) for the next proteolytic digestion.

For electrospray ionization of the eluted peptides, the outflow rate of the analytical column was kept at 200 nL/min by controlling the length of the capillary tube (20 $\mu$m-i.d.) that was attached to the microtee for splitting the flow from the HPLC pump, as shown in Figure 2. The voltage for ESI was set at 2.5 kV for the MS precursor scan ($m/z$ 300−1800). For each precursor scan, three intense precursor ions were selected for data-dependent MS/MS scans that were analyzed using Proteome Discoverer software (version 1.2.0.208) with the false positive option based on the nrNCBI proteome database. Dynamic exclusion conditions for MS/MS were as follows: repeat duration of 10 s, an exclusion duration of 180 s, and f mass exclusion of ±2.50 Da. The mass tolerance values were 1.0 Da for precursor peptide and 0.8 Da for fragment ions. The results were screened based on the following requirements: $\Delta Cn$ score of 0.1 and a cross-correlation (Xcorr) value larger than 2.4, 2.8, and 3.7 for singly-, doubly-, and triply charged ions, respectively. The variable modification for the identification of glycopeptides was set as oxidation of methionine. Deglycosylated modification of asparagine was included to identify N-glycopeptides of alpha-1-acid glycoprotein (AGP).

## RESULTS AND DISCUSSION

**Evaluation of Online Digestion in the mHFER.** Automated operation of an online mHFER for nLC-ESI-MS/MS experiments involves three steps: (i) introduction of protein or a lectin-peptide mixture followed by protease or endoglycosidase (e.g., trypsin or PNGase F) into the mHFER module from the autosampler, (ii) elution of digested peptides through the mHF wall, and (iii) online analysis of the eluted peptides from the mHFER using nLC-ESI-MS/MS. Because the mHF comprised a porous membrane and one end of the mHF was plugged with epoxy, we could concentrate and desalt (or buffer-exchange) the loaded protein sample in the mHF by pump flow. In this step, free peptides included in the sample mixture penetrated the pores of the mHF wall and exited the mHFER to be trapped in the RP1 trap, while salts and other impurities passed through RP1. Immediately after sample loading, a buffered protease solution placed in a different vial of the autosampler was delivered in sequence to the mHF at a weight ratio of 1:1 (protease/protein). The ratio was selected from an optimization study, which will be discussed later. During digestion (30 min), the carrier buffer B1 without DTT was pumped continuously into the mHFER and digested peptides were loaded into the RP1 trap. The continuous pumping of delivery buffer increases the probability of protein−enzyme reaction because proteins and enzymes are confined near the inner wall of the mHF. Furthermore, continuous pumping facilitates the removal of peptide products from the mHF, which thermodynamically favors further digestion. After digestion, the 10-port switching valve was arranged in the dotted line configuration shown in Figure 2, so that the binary pump flow was delivered to the RP1 trap to transfer trapped peptides to the analytical column for nLC-ESI-MS/MS analysis.

We evaluated the digestion efficiency of the developed mHFER module first by varying the ratio of the amount of enzyme to that of substrate. When the ratio (enzyme:protein) was varied from the typical 1:50 to 1:10 and further to 1:1, the sequence coverage of BSA as listed in Table 1 increased from 74.6% to 81.7% with in-solution digestion. When it was tested with mHFER, the sequence coverage increased from 83.1 to 91.3% even without the presence of urea in the buffer solution. These results indicate that digestion efficiency can be maximized by using an amount of trypsin equivalent to that of substrate protein.

**Table 1. Efficiency of Tryptic Digestion at Different Ratios of Trypsin to Substrate (Fixed as 1 $\mu$g BSA) between In-Solution and mHFER Digestion Methods[a]**

| digestion method | enzyme/BSA | sequence coverage (%) | number of peptides |
|---|---|---|---|
| in-solution digestion (trypsin w/10 mM DTT, 1 M urea) | 1:50 | 74.6 ± 3.6 | 56 ± 3 |
| | 1:10 | 80.6 ± 5.0 | 62 ± 5 |
| | 1:1 | 81.7 ± 1.3 | 62 ± 2 |
| in-solution digestion (trypsin w/10 mM DTT, no urea) | 1:50 | 72.9 ± 1.4 | 53 ± 2 |
| | 1:10 | 74.1 ± 1.5 | 54 ± 2 |
| | 1:1 | 75.6 ± 1.8 | 56 ± 3 |
| mHFER digestion (trypsin w/10 mM DTT, no urea) | 1:50 | 83.1 ± 2.6 | 72 ± 3 |
| | 1:10 | 85.3 ± 0.9 | 75 ± 1 |
| | 1:1 | 91.3 ± 1.3 | 83 ± 2 |

[a]Each data point is based on triplicate measurements.

The digestion efficiency of the mHFER module was further evaluated by varying the incubation temperature of the column heater and the amount of albumin standard loaded. The efficiency of tryptic digestion at different temperatures was monitored by comparing sequence coverage at different temperatures. A comparison of the base peak chromatograms (BPCs) of digested albumin peptides from the mHFER obtained by nLC-ESI-MS/MS for different temperatures (25, 40, 60, and 80 °C) is provided in Figure 3. In each run, 10 $\mu$g of albumin (150 pmol) and 10 $\mu$g of trypsin (410 pmol) were loaded into the mHFER. The peak intensities of peptides increased dramatically until 60 °C, but then dropped rapidly. Moreover, the sequence coverage values of the identified

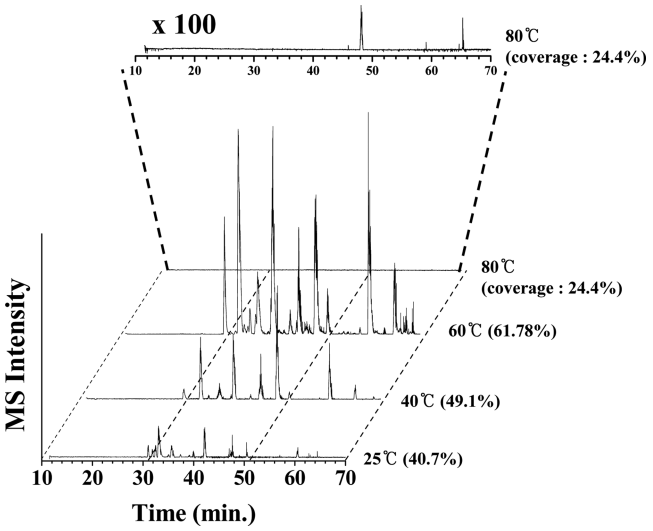


**Figure 3.** Base peak chromatograms (BPCs) of BSA peptides digested by online mHFER at different temperatures (25, 40, 60, and 80 °C) obtained by nLC-ESI-MS/MS.


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peptides over the entire amino acid sequence of albumin were 40.7% at room temperature, 49.1% at 40 °C, and 61.78% at 60 °C. However, sequence coverage decreased to 24.4% at 80 °C, indicating that trypsin was deactivated at this temperature as reported previously.[24,25] The sequence coverage values shown in Figure 3 are worse than the typical coverage values obtained for ordinary in-solution digestion, because we did not include DTT to reduce disulfide bonds in the 50 mM ammonium bicarbonate solution that we used to deliver the protein sample to the mHFER (sequence coverage was greatly improved later by adding DTT to delivery buffer B1; this will be discussed in more detail later). Amino acid (AA) sequence (covering multiple peptide chains) of albumin identified at different temperatures was marked with different colors in Supporting Information Table S2. The amino acid (AA) sequences identified at 25 °C are marked in gray while additional AA sequences obtained after increasing the digestion temperature are marked in cyan (40 °C) and yellow (60 °C). These results indicate that tryptic digestion can be performed at elevated temperatures without preliminary reduction of the protein because at elevated temperatures the protein is in a more accessible form that i.e. steric clashed between the enzyme and protein are minimized.[26]

When 10 mM DTT was added to the carrier solution used to deliver protein to the mHFER, sequence coverage was greatly enhanced. Figure 4 shows the superimposed BPCs obtained from three consecutive mHFER-nLC-ESI-MS/MS runs of BSA (10 $\mu$g) along with the MS scan spectrum at $t_r$ = 58.10 min. A typical MS/MS spectrum of $m/z$ 926.40 ($[M + 2H^+]^{2+}$) at $t_r$ = 51.2 min (see Figure S1 of Supporting Information) was identified as LFTFHADICTLPDTEK from all three runs. The peptide sequence coverage was 99.1 ± 1.0% ($n$ = 3), indicating that online mHFER with nLC-ESI-MS/MS provides excellent digestion efficiency. The high reproducibility of proteolytic cleavage obtained using this system indicates that the quantitative analysis results obtained for targeted proteins or biomarkers, when analyzing proteome samples from sera, cells, and tissues, will be highly accurate. We examined the autolysed trypsin peptides and it was found that an average number of 3.7 trypsin peptides in each run was detected (sequence coverage of 25.1 ± 9.9%). However the intensities of star marked peaks shown in Figure 4 were relatively lower than those of BSA peptide peaks. The full scan MS spectrum obtained at $t_r$ = 58.10 min supported that the intensity of a trypsin peptide ($m/z$ 1106.7, $[M + 2H^+]^{2+}$) marked with star was relatively lower than those of BSA peptides and autolysis of trypsin was not serious in mHFER.

The current operation was based on the digestion of 10 $\mu$g (150 pmol) BSA. When the amount of BSA injected was decreased to 5 $\mu$g, 1 $\mu$g, 500 ng, 100 ng, and 1 ng (15 fmol), while maintaining the same ratio (1:1) of protein to trypsin, the sequence coverage values decreased from 99.1% to 30.4%; however, sequence coverage was maintained at around 90% until the quantity of BSA was reduced to 100 ng, as shown in Table 2. The number of peptides identified decreased gradually from 101 with 10 $\mu$g BSA to 66 with 100 ng BSA and further to 11 with 1 ng BSA. It is noteworthy that the sequence coverage was 87.0% at 100 ng (1.5 pmol of BSA) of protein. This result indicates that mHFER-nLC-ESI-MS/MS can be utilized to analyze trace amounts of target protein from a proteome sample. Low abundant biomarkers (subnanogram amounts in 1 mL plasma samples) are often encountered in practice, for example, carcinoembryonic antigen (CEA, 0.5 ng/mL) in

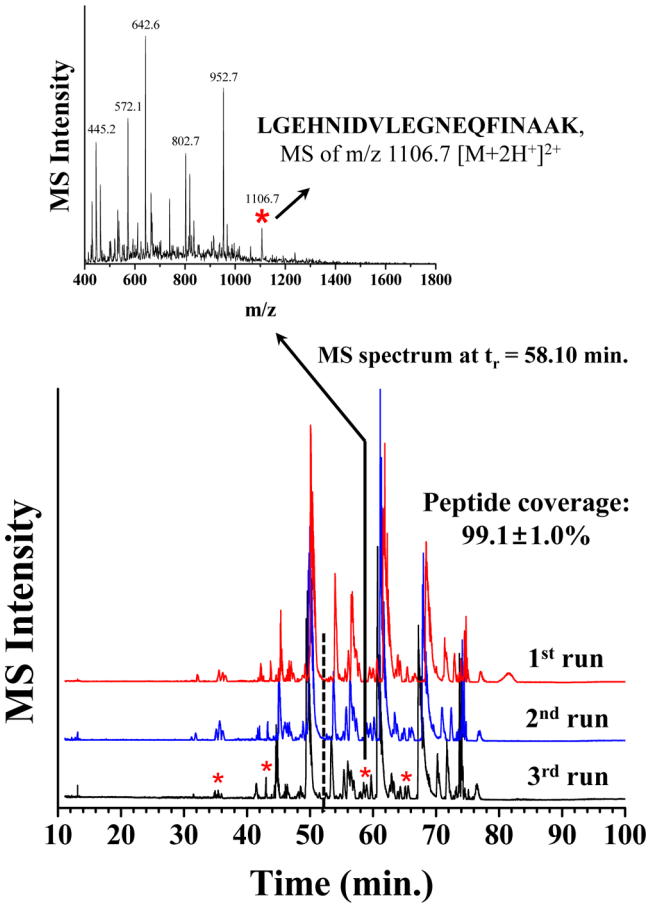


**Figure 4.** Reproducibility of mHFER digestion of BSA (10 $\mu$g) illustrated with BPCs obtained from three consecutive mHFER-nLC-ESI-MS/MS runs. Elution of autolysed trypsin peptides was marked with star in the chromatogram along with the MS spectrum at $t_r$ = 58.10 min showing a number of BSA peptide ions along with an autolysed trypsin peptide ion ($m/z$ 1106.7, $[M + 2H^+]^{2+}$) marked with star. mHFER digestion was performed at 60 °C.

peritoneal cancer, vascular endothelial growth factor (VEFG, 0.2 ng/mL) in breast cancer, and gastrin (0.1 ng/mL) in prostatic cancer.[27−29] Table 2 also shows that the number of miscleaved peptides in the mHFER appears to be larger than that obtained using the in-solution method for each test. This number increased as the number of identified peptides increased.

After each round of online proteolysis, the mHFER was cleaned by back-flushing with buffer B1 for 30 min at 5 $\mu$L/min. To evaluate if there was carry-over between runs, the effluent was collected during back-flushing and subjected to in-solution digestion for nLC-ESI-MS/MS analysis of unreacted BSA. It was not possible to calculate the amount of undigested BSA in the back-flushed effluent. After the mHFER module was washed, a blank mHFER-nLC-ESI-MS/MS analysis was carried out. On the basis of 1 $\mu$g BSA treatment, the average loss calculated from the peak area of a specific peptide from back-flushing compared to the first mHFER proteolysis was 10.6 ± 1.5% ($n$ = 3) for the peptide KVPQVSTPTLVEVSR and 9.7 ± 1.0% ($n$ = 3) for LVVSTQTALA. However, blank online proteolytic digestion after back-flushing and a subsequent nLC-ESI-MS/MS run yielded an average peak area of 0.0 ± 0.0% ($n$ = 3). From this experiment, it is clear that it is essential to wash the mHFER between runs by back-flushing for at least 30 min.


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Table 2. Comparison of Sequence Coverage (%) and the Number of Identified and Miscleaved Peptides of BSA Obtained between Conventional In-Solution and mHFER Digestion Methods for Different Amounts of BSA[a]

| | sequence coverage (%) | | number of identified peptides | | number of miscleaved peptides | |
|---|---|---|---|---|---|---|
| amounts of BSA | in-solution digestion | mHFER | in-solution digestion | mHFER | in-solution digestion | mHFER |
| 10 μg | 77.9 ± 1.0 | 99.1 ± 1.0 | 62 ± 1 | 101 ± 2 | 37 ± 2 | 61 ± 3 |
| 1 μg | 75.6 ± 1.8 | 91.3 ± 1.3 | 56 ± 3 | 83 ± 2 | 25 ± 2 | 51 ± 2 |
| 100 ng | 51.7 ± 3.8 | 87.0 ± 5.1 | 23 ± 3 | 66 ± 2 | 18 ± 1 | 42 ± 2 |
| 1 ng | 0.0 ± 0.0 | 30.4 ± 2.4 | 0 ± 0 | 11 ± 1 | 0 ± 0 | 9 ± 1 |

[a]Enzyme/protein ratio was fixed at 1:1 and $n$ = 3.

To test the durability of the mHFER, 50 consecutive online digestions (1 μg BSA) were performed. The sequence coverage after 50 runs decreased to 83.6% (65 of 85 peptides) indicating that the mHFER is highly durable (Table 3).

Table 3. Durability of mHFER

| number of mHFER runs | sequence coverage (%) | number of peptides |
|---|---|---|
| 1st run | 92.6 | 85 |
| 10th run | 89.5 | 76 |
| 20th run | 85.7 | 67 |
| 50th run | 83.6 | 65 |

**Online Enrichment of *N*-Glycopeptides by mHFER.** To examine the efficiency of mHFER as a tool for selective enrichment of *N*-glycopeptides, the single trap column was replaced with a dual trap system with the aid of a column switching valve, as shown in Figure 2. For the selective isolation of glycopeptides, tryptically digested AGP was mixed with ConA, a lectin with specific binding affinity for high mannose-type glycans, to induce the formation of lectin-glycopeptide complexes. The resulting mixture was injected into the mHFER. Endoglycosidic digestion of *N*-glycopeptides in the lectin−glycopeptide complexes was then achieved by adding an enzyme to the mHFER. In this experiment, 10 μL (21 μg) of ConA-AGP peptide mixture (20:1 in mass) was fed into the mHF from the autosampler with the column switching valve positioned to RP1. The carrier solution was the binding buffer solution B2 containing metals for ConA-glycopeptide complexation. At this stage, we expected that peptides smaller than the MW-cutoff (10 kDa) of mHF and glycosylated peptides with poor affinity for ConA or nonglycosylated peptides containing an N-glycosylation site would pass through the pores of the mHF during injection and automatically be trapped in the first trapping column (RP1). Simultaneously, *N*-glycopeptides bound to ConA would have been retained inside the mHF because of the size of ConA (~104 kDa at pH 6 in multimeric form). After the breakthrough run (30 min), the column switching valve was directed to RP2 (inside the square box shown in Figure 2) and then 5 μL of PNGase F (≥5000 units/mL) was delivered from the autosampler for endoglycosidic digestion. The carrier solution was pumped for 30 min to ensure dissociation of glycopeptides from ConA. The resulting deglycosylated peptides exited through the pores of the mHF by pump flow and were then trapped in the RP2 trap column for nLC-ESI-MS/MS analysis. The sequences of peptides from the RP1 (nonglycosylated peptides) and RP2 (*N*-glycopeptides) peptide fractions were identified separately using nLC-

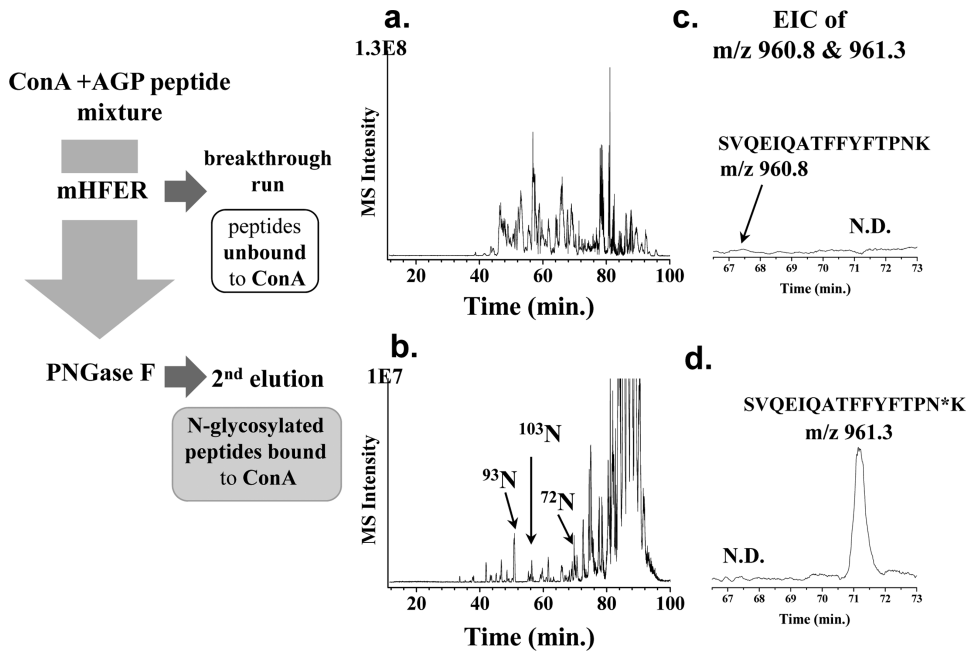


Figure 5. Scheme for online enrichment of N-glycopeptides with ConA in the mHFER and BPC of each peptide fraction from (a) the breakthrough elution and (b) the second elution showing peptides of three N-glycopeptides with glycosylation sites $^{72}$N, $^{93}$N, $^{103}$N, and (c−d) EICs of $m/z$ 960.4 and 961.3 from BPCs in a and b, respectively.


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ESI-MS/MS, as shown in Figure 5. The scheme used to isolate glycopeptides is shown in Figure 5 along with the two BPCs of both nLC-ESI-MS/MS runs in Figures 5a and 5b, respectively. The two chromatograms shown in Figures 5c and 5d are the extracted ion chromatograms (EICs) of the two ions, $m/z$ 960.8 and 961.3, representing the same peptides with and without glycosylation at asparagine, respectively. The CID spectra of these two ions are shown in Supporting Information Figure S2 along with fragment $y$-ions marked with circle to show the increase of $m/z$ value by 1 Da for each corresponding $y$-ion. The peptides identified from each run are listed in Supporting Information Table S3. It was found from the analysis of the breakthrough fraction that a nonglycosylated peptide ($^{58}$SVQEIQATFFYFTPNK$^{73}$) containing an N-glycosylation site with very low peak intensity was detected, as shown in Figure 5c. It is known that AGP contains five N-linked glycosylation sites.[30] However, peptides eluted from the mHFER after endoglycosidic digestion included three N-glycopeptides with glycosylation sites $^{72}$N, $^{93}$N, and $^{103}$N as indicated in Figure 5b, as well as N*(asparagine) in Supporting Information Table S3. Moreover, peptides with N-glycosylation (SVQEIQATFFYFTPN*K, $m/z$ 961.3 showing a typical increase of 1 Da because of the deglycosylation of the peptide resulting in an exchange of D to N) were clearly resolved as shown in Figure 5d, while the same unglycosylated ion was not detected. These results demonstrate that mHFER can be used to selectively isolate glycopeptides online.

We applied this method to human urinary proteome samples (samples from three prostate cancer patients and three controls). The resulting N-glycopeptides identified are listed in Table S4 of Supporting Information, along with the ratio of the relative peak area of cancer patients and controls. Peak area was calculated from EIC of each ion. In total, 55 N-linked glycoproteins from 67 N-glycopeptides and 24 glycopeptides were commonly found in both types of samples. Zinc alpha-2-glycoprotein was 2.17 ± 0.44 fold more abundant in patient samples than control samples; this protein has previously been identified as a potential serum[31] and urine[32] biomarker of prostate cancer.

## CONCLUSIONS

Our initial evaluation of mHFER for online proteolysis revealed that this system can be used to analyze samples containing very low amounts of protein when combined with nLC-ESI-MS/MS. Complete online operation from digestion to analysis prevents the possible loss of digested peptides that tends to occur during off-line digestion and purification processes. Because no enzyme-immobilized substrate is utilized in mHFER and the used enzyme can be back-flushed after digestion, possible carry-over in consecutive runs can be minimized. In addition, pressure build-up by clogging or pH limitations in reactor operation are not limitations of mHFER in contrast to IMER. When we used a mHFER to selectively isolate glycopeptides, online endoglycosidic digestion was performed by injecting a premixed mixture of lectin and digested peptides into the mHFER module so that non-glycosylated peptides and deglycosylated glycopeptides could be isolated in sequence. However, it should be possible to create a complete online purification and analysis system for glycopeptides by introducing a microscale mixer for the complexation of lectin and glycopeptides between two mHFER modules so that proteolytic digestion could be performed in one mHFER module and endoglycosidic digestion in the other.

## ASSOCIATED CONTENT

### Supporting Information

Additional material as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

### Corresponding Author

*Address: Center for Bioanalysis Korea Research Institute of Standards and Science 1 Doryong-Dong, Yuseong-Gu, Daejeon, 305-340, Korea (D.K.); Department of Chemistry Yonsei University Seoul, 120-749, Korea (M.H.M.). Phone: (82) 42 868 5160 (D.K.); (82) 2123 5634 (M.H.M.). Fax: (82) 42 868 5801 (D.K.); (82) 2 364 7050 (M.H.M.). E-mail: djkang@kriss.re.kr (D.K.); mhmoon@yonsei.ac.kr (M.H.M.).

### Notes

The authors declare no competing financial interest.

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