RESEARCH ARTICLE

Rapid protein identification using monolithic enzymatic microreactor and LC-ESI-MS/MS

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A monolithic enzymatic microreactor was prepared in a fused-silica capillary by in situ polymerization of acrylamide, glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) in the presence of a binary porogenic mixture of dodecanol and cyclohexanol, followed by ammonia solution treatment, glutaraldehyde activation and trypsin modification. The choice of acrylamide as co-monomer was found useful to improve the efficiency of trypsin modification, thus, to increase the enzyme activity. The optimized microreactor offered very low back pressure, enabling the fast digestion of proteins flowing through the reactor. The performance of the monolithic microreactor was demonstrated with the digestion of cytochrome c at high flow rate. The digests were then characterized by CE and HPLC-MS/MS with the sequence coverage of 57.7%. The digestion efficiency was found over 230 times as high as that of the conventional method. In addition, for the first time, protein digestion carried out in a mixture of water and ACN was compared with the conventional aqueous reaction using MS/MS detection, and the former solution was found more compatible and more efficient for protein digestion.

Keywords:
Enzymatic reactor / LC-MS/MS / Monolithic column / Protein identification

1 Introduction

With the acceleration of proteome research, new technologies for a rapid and high throughput protein identification are highly required. Proteolytic digestion is an important step in PMF and MS/MS-based peptide sequencing. Trypsin, as one of the most popular digestion enzymes can selectively cleave proteins at lysine and arginine residues, and thus provides typically peptides in a mass range compatible with MS/MS for amino acid sequence determination [1, 2]. However, most of the current trypsin digestion protocols are performed in solution [3]. To avoid the autodigestion of trypsin, which might produce excessive amounts of undesired tryptic fragments and complicate the unambiguous assignment of the studied protein [4], digestion is generally performed with low concentration enzyme, leading to extended incubation times [5].

Enzyme immobilization is one of the methods to solve such problems since molecules immobilized through a proper spacer on the supports can reduce the probability of encountering each other and autodigestion [6]. Therefore, a higher density of trypsin can be employed for protein digestion. Additionally, the stability of trypsin toward chemical denaturants and organic solvents could be enhanced by immobilization on solid supports.

In recent years, several reports have demonstrated the feasibility of protein digestion using trypsin immobilized on various supports, such as a porous silicon matrix [7–9], glass [10] and gel [11] beads, polymer [12], sol-gel supports [13, 14],
membrane [15, 16] and porous monolithic materials [17–22]. In addition, porous polymer monolithic supports have recently been introduced as novel materials in which the diffusion resistance during mass transfer has been proved quite small. Therefore, they have also been regarded as ideal supports for the immobilization of enzymes and fast conversion of substrates [23–26]. Porous poly(glcidy) methacrylate-co-ethylene dimethacrylate) monolith is one of such materials that have been used as an affinity chromatographic medium [27, 28] and enzymatic reactor carrier [17, 26] because of their biocompatibility and easy modification. Furthermore, proteins could be easily coupled to the monolithic supports by the modification of the epoxide groups on the carriers.

After digestion, the separation and detection of peptides by the on-line combination of HPLC and MS is regarded as one of the most powerful techniques [29–31]. We have recently successfully prepared a monolithic tryptic micro-reactor in a capillary on a support of poly(glycidyl methacrylate) using a JEOL JSM-6360LV scanning electron microscope. The capillaries were first rinsed, respectively, by methanol, water, 0.1 mol/L sodium hydroxide, water, 0.1 mol/L HCl, and methanol. They were then dried with a stream of nitrogen gas and methanol was pumped through the capillaries overnight at room temperature, followed by washing with methanol and drying with nitrogen gas. Using such a procedure, the monolithic supports could be covalently anchored to the inner wall of capillaries.

The capillaries were first rinsed, respectively, by methanol, water, 0.1 mol/L sodium hydroxide, water, 0.1 mol/L HCl, and methanol. They were then dried with a stream of nitrogen gas at 70°C. Subsequently, a 50% solution of γ-MAPS in methanol was pumped through the capillaries overnight at room temperature, followed by washing with methanol and drying with nitrogen gas. Using such a procedure, the monolithic supports could be covalently anchored to the inner wall of capillaries.

2.3 Preparation of monolithic support

The capillaries were first rinsed, respectively, by methanol, water, 0.1 mol/L sodium hydroxide, water, 0.1 mol/L HCl, and methanol. They were then dried with a stream of nitrogen gas at 70°C. Subsequently, a 50% solution of γ-MAPS in methanol was pumped through the capillaries overnight at room temperature, followed by washing with methanol and drying with nitrogen gas. Using such a procedure, the monolithic supports could be covalently anchored to the inner wall of capillaries.

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phosphate buffer pH 8.0 was pumped through the columns for 5 h at room temperature. Trypsin was then coupled to the support by continuously introducing 2.5 mg/mL trypsin in a 100 mM phosphate buffer pH 8.0 containing 0.05 mol/L benzamidine for 12 h at 4°C. Later, the microreactor was washed by 5 mg/mL sodium borohydride solution overnight. It was filled with 50 mM Tris-HCl buffer pH 7.0 containing 10 mM CaCl2 and 0.02% NaN3, and stored in the freezer at 4°C before use.

### 2.5 Assay of enzymatic activity

Activity of the free trypsin in aqueous solution was determined by a UV-visible recording spectrophotometer UV-9100 (Ruili, Beijing, China) with 1 mM or 2 mM BAEE as the substrate [32]. Activity of the immobilized enzyme was determined by 20 mM BAEE hydrolysis at volumetric flow rates from 450 nL/min to 812 nL/min at 25°C. The generated product was measured by CE separation and determination at 214 nm and 253 nm using a 50 mM Tris-HCl pH 7.5 buffer.

### 2.6 Protein digestion

The performance of the microreactor was evaluated by digesting 1 mg/mL cytochrome c, dissolved in a 100 mM NH4HCO3 buffer pH 8.0 containing 20% ACN.

A comparative study of protein digestion in aqueous buffer and a mixture of water and ACN was performed. In the former situation, 0.1 mg/mL cytochrome c was dissolved in a 100 mM NH4HCO3 buffer pH 8.0, while in the latter, the same buffer was used expect that 20% ACN was added. The protein solutions were pumped through the enzyme reactor using a syringe at 37°C. The products were collected in vials and maintained under refrigeration throughout the chromatographic analysis.

### 2.7 CE experiments

The CE experiments for the analysis of protein digests were performed in 10% acetic acid aqueous buffer pH 2.2. Samples were injected hydrodynamically for 30 s at 2.0 psi. The temperature was kept at 25°C, and the UV detection wavelength was set at 214 nm. The applied voltage was ramped from 0 kV to 20 kV in 10 s.

### 2.8 HPLC-MS/MS experiments

The digest of cytochrome c was separated by HPLC using buffer A (95% ACN containing 0.8% formic acid) and buffer B (5% ACN containing 0.8% formic acid). The gradient was from 10% A to 20% A in 5 min, to 45% A in 3 min, kept at 45% A for 32 min, to 100% A in 5 min, and then 100% A for 15 min at a flow rate of 250 μL/min.

For the analysis with pneumatically assisted ESI, an electrospray voltage of 4.5 kV and a nitrogen sheath gas flow of 40 U were employed. The temperature of the heated capillary was set to 220°C. Three types of scanning were used to obtain data. In the full-scan mode, ions were collected under three microscans with the maximum ion injection time of 100 ms, covering the mass range from m/z 300–2000. The zoom scan and MS/MS were performed in a data-dependent mode. The MS/MS collision energy was 35%. Ten microscans with the maximum ion injection time of 100 ms were performed in both zoom scan and MS/MS mode.

### 2.9 Protein identification using SEQUEST search program

Protein identification was performed using the BioWorks software with SEQUEST search program. The MS/MS raw data obtained from the LC-MS/MS analysis were used for database search. Trypsin was selected because of its protein cleavage specificity. Both b-ions and y-ions were included in the database search. The searched database was horse.

### 3 Results and discussion

#### 3.1 Preparation of the microreactors

Although soft gels, such as a polyacrylamide monolith, are often used for enzyme immobilization because of the excellent accessibility of reaction sites, such supports can not be used at high pressure due to the ease of deformation at high flow rates. In contrast, the mechanic strength of polymethacrylate-based monolithic materials with a heavily cross-linked macroporous structure is sufficient to withstand relatively high pressure. Therefore, to obtain such rigid materials, the polymerization mixture must contain large amounts of cross-linking monomer and porogen.

In our study, a mixture of dodecanol and cyclohexanol was chosen as the optimal porogenic reagent. With the polymerization temperature set at 55°C, a monolith with high surface area and good permeability was readily achieved using high content of cyclohexanol. The percentage of cyclohexanol in the porogen mixture was optimized as 85%.

In the preparation of a monolith, the effect of acrylamide as the inert hydrophilic co-monomer on the enzymatic activity of the support was studied through experiments of BAEE digestion under a pressure of 4 MPa. Only less than 2 mM BAEE was converted into BA through the microreactor without added acrylamide. In contrast, nearly 10 mM BAEE was hydrolyzed with the reactor containing a high content of acrylamide. These results showed that the monolithic reactor using acrylamide as the co-monomer had a higher activity against BAEE than the one without it.

Epoxy-activated carriers are the most often used enzyme supports, because of their easy fabrication and modification as well as relatively low cost [33]. They can be used directly for
enzyme coupling primarily via protein amino groups in buffer, or the epoxy groups can be modified by a variety of different reagents. However, compared to glutaraldehyde-mediated coupling, the direct reactions between oxirane groups and amino groups are slower. Therefore, to increase the amount of enzyme immobilized on the supports, high temperature and long reaction time have to be used, leading to the impairment of enzymatic activity. To overcome such problems, in our study, the monolithic supports were prepared by copolymerization of GMA with EDMA, which offered high mechanical strength and chemical stability. Even after high concentration ammonia solution treatment, there was no breakage of any part of the whole monolithic materials.

In the modification process, glutaraldehyde was used to couple the support with enzyme. Its reactions with protein amino groups could be performed easily with fast speed even under an ambient condition. Reducing the C=N bonds to C-N by sodium borohydride improved the stability of immobilized enzyme. Furthermore, a suitable distance of enzyme to the carrier backbone was formed, allowing optimal enzymatic flexibility.

In our experiments, after introducing glutaraldehyde into the column, the color of monolithic support was turned yellow because of the formation of C=N. The more C=N bonds formed, the deeper the color of the support. In our experiments, with a constant porogen composition and monomer to porogen ratio, and the more acrylamide in the polymerization mixture, a deep yellow color of the supports was observed. Therefore, the sites for trypsin immobilization were increased, resulting in a faster digestion reaction. However, since the solubility of acrylamide in the polymerization solution is limited, we decided to optimize its content. The optimal content of acrylamide was finally set as 6.0% of the final polymerization solution.

**3.2 Characterization of the monoliths**

Figure 1 shows the scanning electron microscopy images of the internal morphologies of monolith, which demonstrates that the porous polymer was homogeneous across the entire monolith. The uniform and small sizes of the particles provided a high surface area for the immobilization of enzyme, and the macroporous structure resulted in low backpressure and high throughput. These images also reveal that the monolith was completely anchored to the inner wall of the capillary so that the leakage of proteins before digestion could be avoided.

In our experiments, pressure created by pump was required to deliver protein solutions through the monolith. The pump was operated under constant pressure and the flow rate through the monolith was measured by weighting the effluent from the monolithic capillary column. Our results showed that a good linear relationship between the flow rate and the backpressure was achieved in the pressure range of 4–10 MPa, corresponding to the flow rate of 449–812 nL/min. Further increasing the pressure to 20 MPa resulted in no obvious blockage, demonstrating the excellent stability and feasibility of the microreactor under high pressure.

With BAEE as the substrate, a comparative study of the hydrolytic activity of the microreactor and trypsin solution was carried out. The enzymatic activity for this microreactor was determined to be 2.8 AU/min, compared to 0.011 AU/min for the 0.01 mg/mL free trypsin solution, which showed that the enzymatic activity of the former one was about 255 times higher than the latter. Unfortunately, our current instrumentation can not measure the amount of enzyme immobilized on the monolith directly, but the approximate activity of the microreactor could be calculated by comparing with that of free trypsin solution using UV detection. In our work, the activity of immobilized trypsin was calculated to be equivalent to that of 2.6 mg/mL free trypsin solution.

Furthermore, the effect of flow rate on the activity of microreactor was evaluated with BAEE as substrate. The effluent was collected and analyzed by CE, and the percentage of reacted BAEE was chosen as the parameter to evaluate the hydrolysis activities of immobilized trypsin. We found that with an increase of the flow rate from 449 to 812 nL/min, the percentage of reacted BAEE was decreased from 52.2% to 32.2%, similar to that seen previously [22]. Therefore, a relatively low flow rate was preferred to increase the interaction between the enzyme and the substrate.

![Figure 1. Scanning electron microscopy of internal structure of monolith. Magnification, × 1000, × 2000, × 5000, from left to right, respectively. Conditions: monomers, GMA:EDMA:acrylamide 3:5:2; porogenic solvent, cyclo-dextran:1-dodeconol 85:15; monomer:porogen 30:70; at 55°C, for 12 h.](www.proteomics-journal.com)
To evaluate the stability of the enzymatic reactor, a monolithic reactor was stored at an ambient temperature in a 50 mM Tris-HCl pH 7.0 without any other additives. Figure 2 shows that the enzymatic activity was only reduced a little after a month, demonstrating that when the column was kept, even in a harsher situation, for a long time, the monolithic reactor could still display high activity and stability. The reproducibility of the microreactor was also evaluated using BAEE as substrate. Similar hydrolysis activities were obtained with different microreactors, demonstrating the good reproducibility.

3.3 Digestion and identification of protein

Cytochrome c is a standard protein for testing proteolytic digestion, and contains 104 amino acids and 21 cleavage sites for trypsin digestion. To evaluate the performance of our prepared microreactor, 1 mg/mL cytochrome c solution containing 20% ACN was pumped through the monolith prepared with immobilized trypsin at the flow rate of 450 nL/min, affording a residence time of 1 min 13 s. The effluent from the microreactor was collected, and analyzed by CE-UV and HPLC-MS/MS. Figure 3.1 shows that there was only a single peak for cytochrome c, and that the digest contained a complex mixture of numerous peptides, as shown in Fig. 3.2, demonstrating the high throughput and high efficiency of the monolith based microreactor.

The tryptic digests were further analyzed by HPLC-MS/MS on a Finnigan LCQ IT mass spectrometer. Peptides separated by HPLC were detected using ESI-MS with a full mass scan mode, followed by a high resolution zoom scan of the ion with strongest signal in the first MS scan, and an MS/MS analysis on the ion selected for the zoom scan, which can resolve the isotopic distribution of the selected ion and thus determine the charge state of the ion. The SEQUEST program uses uninterpreted MS/MS data to search a sequence database, and the cross-correlation function is used to evaluate the comparison of the reconstructed MS/MS spectrum with the observed MS/MS spectrum. The magnitude of the cross-correlation value indicates the quality of the match between the sequence and the spectrum, and the difference between the normalized cross-correlation score and the second ranked sequence shows the quality of the match versus all the other top ranking sequences in the database. The base peak chromatogram of digests is presented in Fig. 4. It can be seen that more than 20 peaks were resolved. After database searching of the MS/MS spectrums, nine peptide peaks were identified to match the digestion of cytochrome c with search thresholds with Xcorr $\geq$ 1.5 and DelCn $\geq$ 0.1 in the molecular weight range of 300–3500, which resulted in the sequence coverage about 57.7%. When protein-sequencing grade enzyme was used, the number of unmatched peaks was eliminated or reduced.

The strategy of PMF using MALDI TOF or ESI-TOF MS has found significant application for the rapid identification of protein. A level of uncertainly in the identification can be observed when searching large databases with this technique with only masses of peptides provided. Methods for protein sequence analysis using MS/MS have become powerful for protein identification. A typical “triple-play” on an IT mass spectrometer provides not only the masses of peptides but also a pattern of fragment or sequence ions that is fairly unique to a given sequence. By combining sequence information with the mass of the overall peptide or the masses of fragment ions, a higher level of specificity is created [2]. In our study, plenty of doubly charged precursor ions were observed, which generally provide more sequence information than that of the corresponding singly charged ions.
Complete precursor peptide sequences can be confirmed from the corresponding CID spectra. Such sequence information is useful in a database search to confirm the correct identification, even if it is a partial sequence of a peptide. Figure 5 showed the CID spectrum of peptide eluting as peak 8, which was doubly charged and had an m/z of 856.79. The spectrum revealed that most of the b- and y-ions were produced from this precursor ion. The peptide sequence was confirmed by the presence of y-ions together with b-ions. Thus, the absence of several fragment ions had no influence on the determination of the peptide sequence.

3.4 Effect of hydrolysis solution

Organic solvents can affect the overall structure of protein, making it more accessible to trypsin proteolysis. In our study, the digestion of cytochrome c in ACN solution was compared with that in aqueous solution by infusing the protein solution through immobilized trypsin microreactor. We found that digestion in 20% ACN was more effective than in aqueous buffer. The new strategy of digestion in the mixed solution means that no other pretreatments of protein, except for the incubation in the organic-aqueous solvent, are necessary, which can reduce the presence of contaminants or a high background, which impede the effective identification of peptides ions in MS/MS analysis.

Table 1 shows the analysis of peptides obtained by on-column and in-solution digestion of cytochrome c in both aqueous and aqueous-organic solutions by HPLC-MS/MS. It can be seen that similar peptide fragments and sequence coverage were obtained, while using the on-column digestion, the digestion time could be clearly shortened. Another interesting result was that more peptides from the trypsin were found with higher Xcorr values in the sample solutions containing ACN than in the aqueous solutions since three peptides were found in the digestion solution containing 40% acetonitrile with Xcorr values at 1.35, 2.11, and 3.39, respectively, while there was just one trypsin peptide found in aqueous digestion with a Xcorr value of 1.13 (threshold of Xcorr \(\geq 1.0\)). In contrast, for the digestion performed in the monolithic reactor with immobilized trypsin, no trypsin peptides were found in either the acetonitrile-containing or aqueous solutions. Thus, we conclude that the autolysis of trypsin was eliminated or reduced by enzyme immobilization.

4 Concluding remarks

Tryptic microreactors were prepared on a porous poly(GMA-co-EDMA-co-acrylamide) monolith in capillaries by the immobilization of trypsin. These microreactors were successfully applied to digestion of proteins. We has demonstrated that much shorter digestion time can be achieved on the microreactor compared to that used with digestion in solution. With a fast reaction rate and low backpressure, the monolithic enzymatic microreactor may potentially become a powerful tool for rapid and high throughput proteome re-
Figure 5. MS/MS spectrum of the doubly charged precursor ion with \( m/z \) 856.79 at the retention time 22.24 min. A sequence is confirmed from the labeled b- and y-ions in the spectrum. Fragments observed in the spectrum are underlined and assigned.

Table 1. Peptide fragments obtained from HPLC-MS analysis of the tryptic digestions of cytochrome c

<table>
<thead>
<tr>
<th>Solvent</th>
<th>On-column digestion</th>
<th>In-solution digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent time</td>
<td>7 min 51 s</td>
<td>31 h</td>
</tr>
<tr>
<td>Sequence</td>
<td>EDLIAYLK</td>
<td>GITWKEETLMEYLENPK</td>
</tr>
<tr>
<td></td>
<td>GITWKEETLMEYLENPK</td>
<td>GITWKEETLMEYLENPK</td>
</tr>
<tr>
<td></td>
<td>IFVQK</td>
<td>IFVQK</td>
</tr>
<tr>
<td></td>
<td>IFVQKCAQCHTVEK</td>
<td>KANTNE</td>
</tr>
<tr>
<td></td>
<td>KTEREDLIAYLK</td>
<td>KTEREDLIAYLK</td>
</tr>
<tr>
<td></td>
<td>KTGGAPGFTYTDANK</td>
<td>KTGGAPGFTYTDANK</td>
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<td></td>
<td>KTGGAPGFTYTDANK</td>
<td>KTGGAPGFTYTDANKNNK</td>
</tr>
<tr>
<td></td>
<td>KYIPGTK</td>
<td>KYIPGTK</td>
</tr>
<tr>
<td></td>
<td>TGEREDLIAYLKK</td>
<td>TGEREDLIAYLKK</td>
</tr>
<tr>
<td></td>
<td>TGPNLHGLFGRK</td>
<td>TGPNLHGLFGRK</td>
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<tr>
<td></td>
<td>TGQAPGFTYTDANK</td>
<td>TGQAPGFTYTDANK</td>
</tr>
<tr>
<td></td>
<td>TGQAPGFTYTDANK</td>
<td>TGQAPGFTYTDANKNNK</td>
</tr>
<tr>
<td>Coverage</td>
<td>50.0%</td>
<td>18.3%</td>
</tr>
</tbody>
</table>

a) Molecular weight range: 300–3500, thresholds: \( X_{corr} \geq 1.5, DelCN \geq 0.1. 

b) 100 mM \( \text{NH}_4\text{CO}_3 \) pH 8.0.

c) 100 mM \( \text{NH}_4\text{CO}_3 \) pH 8.0 containing 20% ACN.

d) 100 mM \( \text{NH}_4\text{CO}_3 \) pH 8.0 containing 40% ACN.

In addition, the digestion of proteins in solutions containing ACN was proved more compatible with that in both on-column digestion and in-solution reaction. Furthermore, the methacrylate ester-based monolithic microreactor, fabricated successfully with a cross-linked macroporous structure and excellent mechanical stability, also demon-
strates the potential of on-line digestion of protein in an HPLC system. Further investigation of our monolithic enzymatic reactor coupled with micro- or nano-HPLC for on-line protein digestion, separation and identification is being carried out in our laboratory.

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5 References