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Integration of On-Line Protein Digestion, Peptide Separation, and Protein Identification Using Pepsin-Coated Photopolymerized Sol−Gel Columns and Capillary Electrophoresis/Mass Spectrometry

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A miniaturized pepsin reactor was prepared inside a fused-silica capillary (i.d. 75 μm) by coating a pepsin-containing gel on a photopolymerized porous silica monolith. The pepsin-encapsulated film was prepared by a sol−gel method. The sol−gel reaction was optimized so that the sol solution containing pepsin forms a thin film on the photopolymerized sol−gel (PSG) monolith that was initially fabricated at the inlet of the capillary. Pepsin was encapsulated into the gel matrix without losing its activity. The large surface area of the PSG monolith enabled the immobilized pepsin to achieve a high catalytic turnover rate, and the porous nature of the PSG promotes penetration of large molecular proteins into the column. The immobilized pepsin-digested peptides and proteins, and the resulting mixture of peptide fragments, could be directly separated in the portion of the capillary where no PSG monolith exists. The durability and repeatability of the fabricated pepsin-coated column was tested and found to be satisfactory. An acidic solution consisting of 0.5 M formic acid was used as the running buffer, because it suppresses the adsorption of proteins or peptides on the inner surface of the capillary as well as enables direct connection of the output of the capillary electrophoresis column to a mass spectrometer. The on-line digestion of insulin chain β and lysozyme provides identification of the proteolytic peptides. Recovery was achieved for 100% of the insulin chain β amino acid sequence and 73% of the lysozyme amino acid sequence.

With the recent completion of several genome sequences, attention has been directed toward deciphering their protein complements, or proteomes. This work holds the promise of identifying proteins that may correlate to various disease states.[1] The rapid acceleration of research in the area of proteomics requires the development of new technologies. Peptide mass fingerprinting (PMF) is one of the methods that appears ideally suited to both the identification of proteins and the determination of posttranslational modifications.[2−6] The protein of interest is digested by proteolytic enzymes, followed by separation of the peptide fragments and identification by electrospray ionization mass spectrometry (ESI-MS) or matrix-assisted laser desorption/ionization mass spectrometry. As a consequence of the recent increasing need for new high-throughput and automated identification methods, capillary electrophoresis (CE)−MS coupling has become an important tool in this field of research.[7] The combination of high efficiencies given by CE with the compound identification capability of MS makes this pair of methods a powerful analytical tool for the investigation of these biological compounds. Unfortunately, peptide analysis by CE/MS is still subject to some important limitations. The separation performances in analytical CE can be impaired by the adsorption of proteins on the surfaces of the fused-silica capillary wall.[8] Proteins are macromolecules with a highly ordered structures that are capable of diverse interactions, such as hydrophobic interaction, electrostatic interaction, hydrogen bonding, and van der Waals interaction. Therefore, the separation of these proteins or peptides by CE is sometimes especially difficult. The electrostatic interactions between the positively charged proteins or peptides with the negatively charged silanol groups on the fused-silica capillary surface causes peak tailing, band broadening, and adsorption of peptides or proteins onto the capillary surface. These factors may reduce the resolution

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to an unacceptable level. The use of a low-pH buffer sometimes with an organic modifier is one of the useful methods for the separation of cationic analytes.[9−12] The acidic pH suppresses the dissociation of silanol groups on the inner surface of the capillary and allows peptide or protein separations with minimum wall interactions. Another challenge in implementing CE/MS is that the running buffer must bring about an adequate CE separation of peptides without disturbing the MS signal. The conditions required for CE and MS are in part antagonistic. In CE, a high ionic strength of the running buffer and low volatilities of its components are required, whereas efficient electrospray conditions can only be achieved with a buffer having a relatively low salt concentration.[7] In cases where a low pH is used, volatile acids such as formic acid or acetic acid are commonly chosen.

To facilitate the speed of the analysis of proteins, it is desirable to integrate and automate protein digestion, peptide fragment separation, and identification.[13−15] As such, the on-line digestion of proteins in a capillary will enable faster and more automated protein identification via PMF. Some researchers have previously reported success in on-line protein digestion in a capillary. Many of them involve the immobilization of proteolytic enzymes, most often trypsin, via covalent bonding with supports[16,17] or the inner wall of a capillary,[18] physically adsorption,[19] and encapsulation in gel matrixes.[20] Frèchet et al.[21] modified monolithic columns made of an organic polymer prepared by in situ polymerization with covalently bonded trypsin.

The third method, which we recently developed, is based on the protein encapsulation technique using sol−gel chemistry.[22−25] The reaction involves the hydrolysis and polycondensation of alkoxysilane monomers. During the process, the proteins are incorporated into the matrix. The encapsulation process proceeded in one step under mild conditions so that the biomolecules can retain their structures[26,27] and biological activity.[28] Furthermore, the in situ gelation process makes it easy to fabricate and interface the materials in a variety of forms such as a film,[29] bulk form,[26,28,30,31] or particles.[32] Trypsin was encapsulated into the gel matrix for preparation of the on-line enzyme reactor. The enzymatic reaction and separation of unreacted substrates and products were simultaneously performed in a capillary. The reactor had excellent enzymatic activity, which was ∼700 times higher than that in free solution. Although peptides can penetrate the gel network, which has a nanometer size, larger proteins were excluded and could not be digested. To overcome this problem, it was anticipated that coating the surface of the highly permeable monolithic column with the enzyme-containing gel would allow penetration of larger molecules.

Recently, we prepared monolithic photopolymerized sol−gel (PSG) columns in a single step from methacryloxypropyltrimethoxysilane (MPTMS),[33−35] which contains both methacrylate and alkoxysilane groups. This monolithic column composed of a porous rigid solid with small-sized skeletons and relatively large through-pores provides a high surface area. This unique morphology provides a highly permeable support of the enzymes.

In this paper, we report the development of a coating procedure for preparing a pepsin-encapsulated sol−gel as a thin film on the PSG monolith. We chose to use pepsin because it is a proteolytic enzyme that acts under acidic conditions, which are known to suppress protein binding. Pepsin cleaves peptides at sites of hydrophobic amino acids, such as Phe or Leu, under acidic conditions.[36] Using the column we prepared, we integrated on-line protein digestion, peptide fragment separation, and protein identification by ESI-MS.

EXPERIMENTAL SECTION

Materials and Chemicals. UV-transparent-coated and polyimide-coated fused-silica capillaries (75-μm inside diameter × 375-μm outside diameter) were purchased from Polymicro Technologies (Phoenix, AZ). MPTMS and tetramethoxysilane (TMOS) were purchased from Tokyo Kasei (Tokyo, Japan). 3,5-Diiodo-L-(+)-tyrosine dihydrate, pepsin from porcine stomach mucosa, hemoglobin from bovine, bovine serum albumin, and (±)-dithiothreitol were from Wako Pure Chemical (Osaka, Japan). Phe, lysozyme from chicken egg white, carbonic anhydrase, and insulin chain β (insulin) oxidized from bovine pancreas were from Sigma-Aldrich (Milwaukee, WI). *N*-Acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine (APDT) was from ICN Biochemicals, Inc. (Aurora, OH). The water was purified by a Milli-Q system (Nippon Millipore, Tokyo, Japan).

Preparation of PSG Monolithic Column. The PSG column was prepared from MPTMS using the same procedure described in our previous reports.[33−35] Polyimide Scotch tape (Sumitomo 3M,

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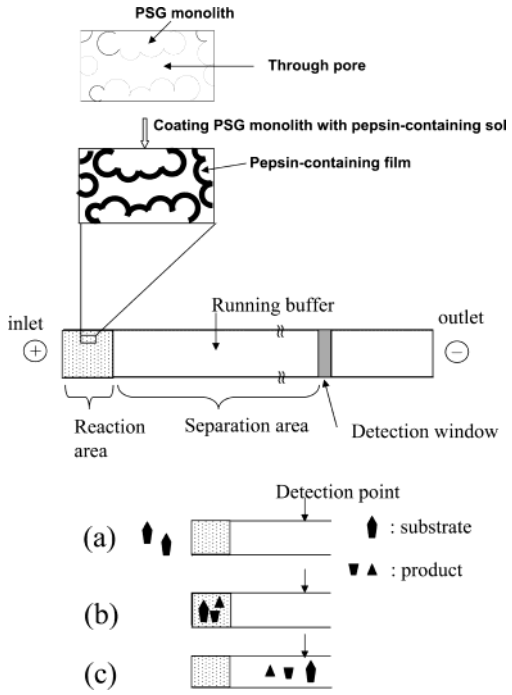

Figure 1. Schematic illustration of an on-line enzyme reactor integrated into CE. (a) Substrates are introduced into the pepsin-coated PSG column. (b)Substrates are catalyzed into products while they flow through the pepsin-coated PSG monolith by electrophoresis. (c) The products and unreacted substrates were separated in the separation section of the capillary by electrophoresis.

Tokyo, Japan) was used to protect the UV-transparent capillary from the photoinduced polymerization reaction. The polymerization reaction took place only in the untaped section (5 cm) of the UV-transparent capillary. After the column was washed with methanol to remove any unreacted reagents, it was dried in an oven at 110 °C overnight.

Coating of the PSG Column with Pepsin-Encapsulated Gel. The sol−gel reaction basically followed the procedures described in our previous report.[22] The monomer solution was obtained by mixing the following reagents just prior to use: (1) 761 μL of TMOS, (2) 169 μL of water, and (3) 11 μL of 0.04 N HCl. This monomer solution was stirred for 20 min so that hydrolysis forms a fully or partially hydrolyzed silane, $SiOH_{4-n}(OMe)_n$.

A total of 45 μL of pepsin solution (25% (w/v)) in 150 mM phosphate buffer (pH 5.5) was added to 15 μL of the hydrolyzed solution. After mixing and ultrasonication for 5 s, the mixture solution was carefully introduced into the capillary from the inlet of the capillary using a 1.0-mL disposable syringe until the pepsin solution soaked through the PSG monolith. After 5−10 min, the excess solution was removed, and the capillary was held at 4 °C for 2 days, so that the remaining sol solution covering the PSG monolith was gelled and became a thin film covering the PSG monolith. The same coating procedure was repeated again.

Figure 1 is a schematic illustration of the fabricated capillary enzyme reactor. At the inlet of the capillary, pepsin is immobilized on the PSG monolith. The substrates are introduced from the inlet of the capillary (Figure 1a). The substrates are digested into peptides by encapsulated pepsin (Figure 1b). Finally, the unreacted substrates and products are separated by electrophoresis (Figure 1c).

Assay of Pepsin Activity. The activity of the free pepsin was determined with APDT and hemoglobin using a standard procedure.[36] The enzymatic activity of the encapsulated pepsin was determined with APDT, insulin, and lysozyme as substrates. The substrates were introduced at the inlet of the capillary. The enzymatic reactions were carried out on-line by allowing a thin plug of the substrates to flow electrophoretically through a pepsin-coated PSG monolith. The substrates were converted to products at the pepsin-encapsulated gel, followed by separation and detection downstream of the pepsin-coated PSG monolith. Pepsin activity for APDT was evaluated using the peak area ratio of the product to the internal standard (IS), Phe. The calibration curve was prepared using 3,5-diiodo-L-tyrosine and Phe.

Sample Preparation. Samples were diluted in the running buffer. Before use, all solutions were filtered through a 0.22-μm membrane (Millipore, Bedford, MA) and degassed by ultrasonication. Lysozyme was reduced for 30 min using dithiothreitol at 37 °C under a nitrogen atmosphere.[37]

Apparatus. All the CE experiments were performed using a capillary electrophoresis system (Beckman model P/ACE System 5510, Fullerton, CA) equipped with a UV detector and a Hewlett-Packard ^{3D}CE system (Palo Alto, CA) equipped with a diode-array detector. An RPR-100 photochemical reactor (Ultraviolet Co., Branford, CT) was used for the photopolymerization reactions. The running buffer was 0.5 M formic acid.

All the CE/ESI-MS experiments were performed using an Agilent CE capillary electrophoresis system equipped with an air pressure pump, an Agilent 1100 series MSD mass spectrometer, an Agilent 1100 series HPLC pump, a G1603A Agilent CE/MS adapter kit, and a G1607A Agilent CE/ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). All the system control, data acquisition, and MSD data evaluation were performed uing a G2201AA Agilent ChemStation software for CE/MSD. The CE/MS adapter kit includes a capillary cassette, which facilitates thermostating of the capillary, and the CE/ESI-MS sprayer kit, which simplifies coupling the CE system with the sprayer. Scanning electron microscopy (SEM) analyses were performed on a Hitachi S-2500 scanning electron microscope (Tokyo, Japan). SEM was used to study the morphological changes of the PSG after coating with pepsin-containing gel.

CE/ESI-MS Conditions. The capillary length was 90 cm. Before each injection, the capillary was equilibrated for 10 min by flushing with the 0.5 M formic acid running buffer. The sample was injected at 20 kV for 20 s. The applied voltage was set at 15 kV, and a pressure of 50 mbar was applied to the inlet capillary during the run. The capillary temperature was thermostated to 25 °C, and the sample tray was cooled below 8 °C. The Agilent 1100 series pump equipped with a 1:100 splitter was used to deliver 10 μL/min of 5 mM ammonium acetate in 50% (v/v) methanol/water to the CE interface, where it was used as a sheath liquid around the outside of the capillary to provide a stable electrical connection between the tip of the capillary and the grounded electrospray needle.

ESI-MS was conducted in the positive ion mode, and the capillary voltage was set at 3500 V. The flow rate of heated dry

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nitrogen gas (heater temperature 300 °C) was maintained at 10 L/min. The spectrometer was scanned from m/z 200 to 1200 at 0.5 s/scan during the separation.

RESULTS AND DISCUSSION

Running Buffer Condition. The buffer conditions are very critical to the enzymatic activity. Because the enzymatic reaction and the separation were performed in the same capillary column, the buffer condition was considered from both standpoints. Because pepsin has its activity under acid conditions and the adsorption of the analytes onto the capillary wall can be suppressed under this condition, 0.5 M formic acid was selected for the enzymatic reaction and separation.[38] Although the reaction buffer of pepsin is generally hydrochloric acid, the volatile characteristics of formic acid were more favorable, considering the on-line connection of CE with MS detection. With this buffer, the proteolytic activity for hemoglobin in free solution was 3055 units/mg of protein, which was close to the labeled value, 3000 units/mg of protein with 0.5 M hydrochloric acid (pH 2.0) as the reaction buffer. This result shows that trypsin has almost the same proteolytic activity in formic acid as in hydrochloric acid.

Development of Enzyme-Coated Monolith. In our previous works,[22−25] we described the preparation and use of protein-immobilized reactors in hydrogels made by sol−gel chemistry. Although we demonstrated the usefulness of these materials for the digestion of small molecules, such as peptides, much larger molecules such as proteins were not able to penetrate the nanopores of the hydrogel network. To overcome this problem, macroporous photopolymerized sol−gel was used as a supporting matrix for enzyme immobilization. This technique is advantageous for the preparation of protein-immobilized reactors because (1) its micrometer-sized pores allow for the penetration of large proteins, (2) its large surface area allows for an increase in the number of immobilized pepsin molecules, and (3) the in situ gelation process makes it easy to fabricate the materials in a variety of forms such as thin films,[29] bulk,[26,28,30,31] or particulate.[32]

Several reaction parameters were studied in order to optimize the performance of the pepsin-immobilized monolith. When the protein is incorporated into the sol−gel matrix, the pH value of the buffer containing pepsin is very important in terms of gelation speed and protein stability.[22] An optimum pH of 5.5 was required for gelation to occur while at the same time maintaining the stability of pepsin in the solution.

Buffer concentration significantly affects the performance of a pepsin-immobilized material. Phosphate buffer concentrations of 100, 200, and 400 mM were studied at a fixed concentration of pepsin (20% (w/v)). An increase in the buffer concentration increased the gelation speed. These coincided with the results given in our previous report.[22] To coat the PSG monolith with the pepsin-containing sol, the excess sol solution should be removed from the capillary before gelation. Therefore, it is desirable that gelation occurs in ∼1 h. We control the gelation conditions so that gelation occurs in ∼1 h by changing the volume ratio of the buffer and TMOS-hydrolyzed solution. When 100 mM phosphate buffer was used, the volume ratio of phosphate buffer and TMOS-hydrolyzed solution was 3:1. With 200 mM phosphate buffer, the ratio was 3:1 or 4:1. And with 400 mM phosphate buffer, the ratio was 4:1. Monolithic columns were prepared using four different coating conditions. The performance of these columns was evaluated using APDT and Phe as a substrate and internal standard, respectively. Because formic acid was used as the running buffer, with a pH of ∼2, carboxyl groups did not dissociate, while amino groups dissociated. Under this condition, the substrate and one of the products, *N*-acetyl-L-phenylalanine, were not charged, whereas the other product, 3,5-diiodo-L-tyrosine, and the internal standard, Phe, were positively charged. Because EOF was not generated, substrate was injected by pressure, and positively charged product, 3,5-diiodo-L-tyrosine, and Phe migrated and were separated by electrophoresis. Figure 2 demonstrates the on-line digestion of APDT substrate using a pepsin-immobilized column prepared by the following coating condition: 100 mM phosphate buffer:TMOS-hydrolyzed solution = 3:1. APDT was successfully hydrolyzed, and the separation of the internal standard and product were satisfactory. The columns prepared using other coating conditions (i.e., phosphate buffer concentrations greater than 200 mM) resulted in columns that could not sustain stable currents. These results show that the concentration of phosphate buffer for gelation should be less than 200 mM.

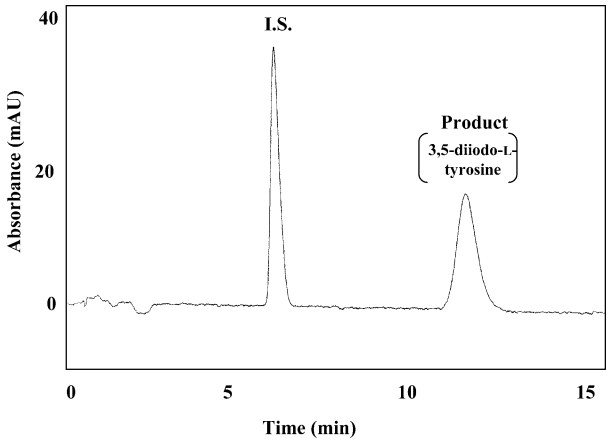

Figure 2. Electropherogram of the on-line pepsin digestion using APDT as the substrate. Conditions: sample, 0.5 mM APDT and 1 mM Phe as internal standard; injection, pressure 20 s; PSG monolith, 5 cm; total length, 30 cm; running buffer, 0.5 M formic acid; applied voltage, 10 kV; detection, 214 nm.

Table 1. Proteolytic Activity of Pepsin-Coated PSG Columns Prepared under Different Conditions[a]

pepsin (%)	phosphate (mM)	product (mM)
20	100	0.06
20	150	0.12
25	100	0.18
25	150	0.38

[a] Conditions: sample, 0.5 mM APDT; 1 mM IS (Phe).

Based on the results, the effect of phosphate buffer concentration on the performance of a pepsin-immobilized material was further investigated. Phosphate buffer concentrations of 100 and 150 mM were studied. Table 1 illustrates the effect that buffer concentration has on the enzymatic activity of these materials. A column made using 150 mM phosphate buffer shows two times more enzymatic activity than a column made using 100 mM phosphate buffer. An increase in buffer concentration in the

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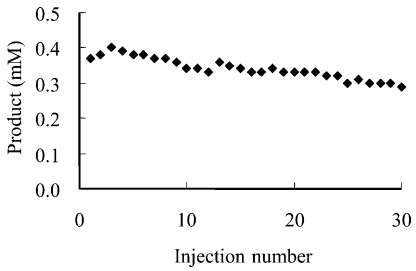

Figure 3. Stability of pepsin-coated PSG monolithic column. Conditions are the same as in Figure 2.

Table 2. Run-to-Run Repeatability of Migration Time[a]

	migration time (min)	
	IS	product
average (min)	5.33	7.43
SD	0.043	0.088
RSD (%)	0.80	1.18

[a] Conditions: sample, 0.5 mM APDT; 1 mM IS (Phe).

reaction solution results in an increase in the rate of gelation, which allows for more pepsin to be incorporated into the gel network and leads to the formation of a more rigid gel network on the PSG monolith surface. A rigid gel is more likely to adhere strongly to the monolith surface than one that is less rigid.

The concentration of pepsin in the reaction solution played an important role in the preparation of a pepsin-immobilized material. At a high concentration of pepsin, 30% (w/v), the TMOS-hydrolyzed solution gelled immediately, making it difficult to introduce the solution into a capillary. Gelation was not observed to occur rapidly at lower pepsin concentrations of 20 and 25% (w/v). Table 1 shows that at 25% (w/v) pepsin in the reaction solution, the enzymatic activity of the material was three times higher than a similar column made with 20% (w/v) pepsin.

An increase in pepsin concentration in the reaction solution alone, however, could not account for the observed increase in enzymatic activity of the pepsin-immobilized material.

It is believed that increasing the pepsin concentration leads to an increase in the gelation speed, which has already been observed when the phosphate buffer concentration is increased. From these results, the coating condition was finally optimized as follows: 25% (w/v) pepsin in 150 mM phosphate buffer (pH 5.5):TMOS-hydrolyzed solution = 3:1.

The optimized pepsin-immobilized material was found to be robust for 30 runs in one column. Figure 3 and Table 2 both illustrate the repeatability of the enzymatic activity and retention times for digestion product and internal standard using a single column. Although the activity slightly decreased, it was efficiently maintained after 30 analyses (Figure 3), and the column was available for more than a week. The repeatability of the retention times was also satisfactory, and the RSD was ~1% (Table 2). The column-to-column repeatability of the enzymatic activity was found to have an RSD of 4%.

Morphological Study. Figure 4 showed the SEM images of PSG material before (left side) and after (right side) pepsin coating. Magnifications of upper and lower micrographs were 1.3 and 5.0K, respectively. The images with magnification of 5.0K showed that the size of macropores was not significantly changed

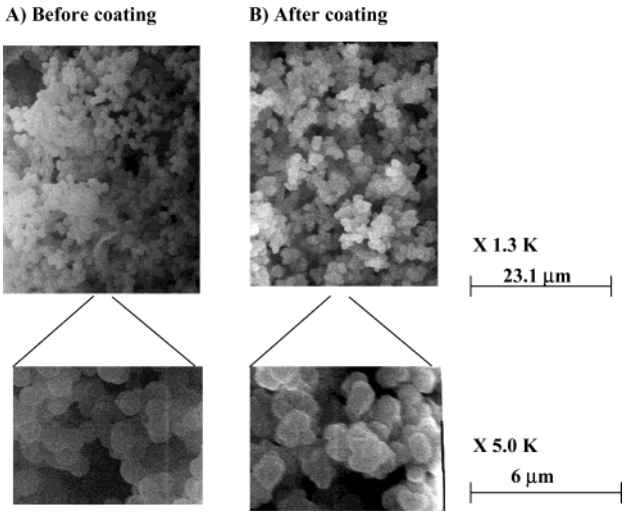

Figure 4. Scanning electron micrographs of the PSG material before (A) and after (B) coating.

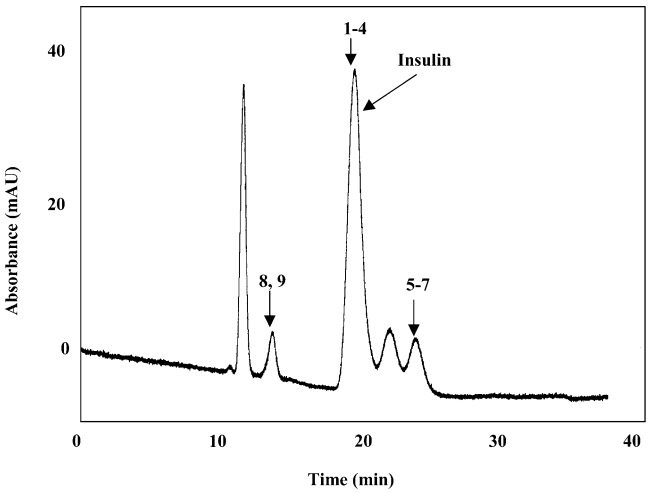

Figure 5. Electropherogram of the on-line pepsin digestion using insulin as the substrate. Conditions: sample, 0.4 mM insulin; injection, 5 kV, 20 s; PSG monolith, 5 cm; total length, 35 cm; running buffer, 0.5 M formic acid; applied voltage, 5 kV; detection, 214 nm. Peak numbers correspond to the peptides listed in Table 3.

after coating with the sol−gel film. This indicates that the sol−gel film is very thin and the coating with pepsin-containing gel gives almost no effect on the structures of the PSG skeletons or macropores.

Digestion of Peptide and Protein. The developed pepsin-coated column was applied to the on-line digestion of peptide and protein. Figures 5 and 6 show the separations of peptide fragments obtained by on-line proteolytic digestion of insulin (MW 3495) and lysozyme (14 300), respectively. The use of an acidic running buffer (pH 2) prevented the adsorption of lysozyme, a basic protein.

The pepsin-coated column was integrated into a CE/ESI-MS system in order to identify each digested peak. Constant liquid flow was applied to the capillary by applying air pressure to the capillary inlet during electrophoresis in order to maintain a conductive liquid junction between the capillary outlet and the electrospray needle. The CE/ESI-MS total ion electropherogram and its mass spectrum of the on-line digestion of insulin are shown in Figure 7 and Figure 8. Each of the peptide fragments was

Table 3. List of Peptides from the On-Line Digestion of Insulin[a]

peak	peptide	sequence	MH^+ (MH^+ calc), Da
1	1−11	(−)FVNQHLC(SO_3H)GSHL	1302.7 (1302.6)
2	1−15	(−)FVNQHLC (SO_3H)GSHLVEAL	1715.2 (1714.8)
3	12−14	(L)VEA	318.2 (318.2)
4	12−15	(L)VEAL	431.2 (431.3)
5	15−25	(A)LYLVC (SO_3H)GERGFF	1351.4 (1351.6)
6	16−24	(L)YLVC (SO_3H)GERGF	1091.6 (1091.5)
7	16−25 or 17−26	(L)YLVC (SO_3H)GERGFF or (Y)LVC (SO_3H)GERGFFY	1238.6 (1238.6)
8	25−30	(F)FYTPKA	726.4 (726.4)
9	26−30	(F)YTPKA	579.3 (579.3)

[a] Conditions: sample, 0.4 mM insulin; injection, 20 kV, 20 s; PSG monolith. 5 cm; total length, 90 cm; applied voltage, 15 kV with 50 mbar pressure.

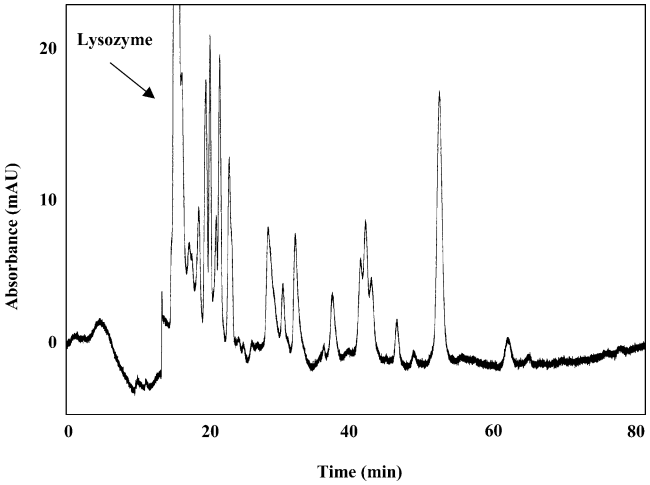

Figure 6. Electropherogram of the on-line pepsin digestion using lysozyme as the substrate. Conditions: sample, 0.25 mM lysozyme; injection, 20 kV, 20 s; PSG monolith, 5 cm; total length, 90 cm; running buffer, 0.5 M formic acid; applied voltage, 20 kV; detection, 214 nm.

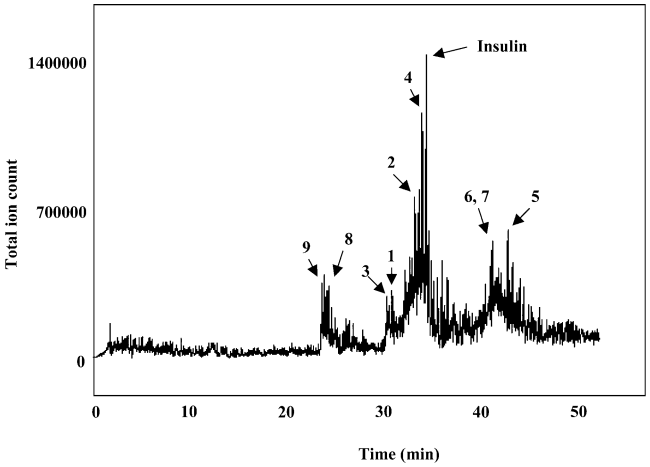

Figure 7. CE/ESI-MS total ion electropherogram of on-line pepsin digestion using insulin as the substrate. Conditions: sample, insulin; injection, 20 kV, 20 s; PSG monolith, 5 cm; total length, 90 cm; running buffer, 0.5 M formic acid; applied voltage, 15 kV; applied pressure, 50 mbar (inlet side); detection, *m*/*z* 200−1200 (0.5 s/scan). Peak numbers correspond to the peptides listed in Table 3.

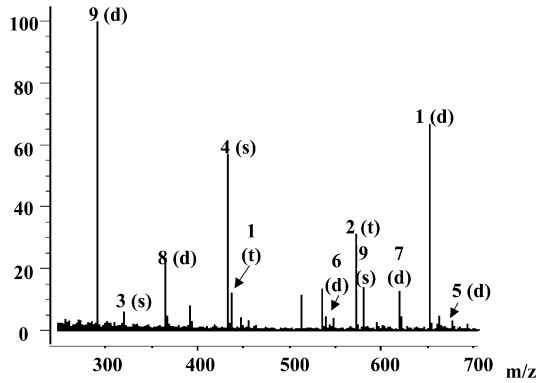

Figure 8. Mass spectrum of insulin digest. Peak numbers correspond to the peptides listed in Table 3. (s), (d), and (t) indicate the singly, doubly, or triply protonated species, respectively.

directly identified on the basis of the mass spectrum taken from the scans under the peaks. Table 3 lists the peptides identified from an on-line capillary digestion of insulin. These peptide fragments were identified using the software Protein Prospector 4.0.5 program (the University of California San Francisco, http://prospector.ucsf.edu). The monoisotopic masses were deduced from the singly, doubly, or triply protonated species, which were obtained with their highest relative abundances. Because the insulin with oxidized cysteine residues from SH to SO_3H was used as a sample, peptide fragments were identified by considering the change of cysteine molecular weight. From the on-line digestion and separation of insulin, nine peptides were identified (Table 3) and 100% of the insulin amino acid sequence was recovered. Peaks at *m*/*z* value of 1238.6 could have several origins, which is an ambiguity that could be solved by performing MS/MS experiments. All peptides corresponded to the cleavages of the C-terminal side of the following amino acids: Ala, Leu, Phe, and Tyr. This indicates the site specificity of the on-line pepsin digestion, which is required for reliable peptide identification. The lists of proteolytic peptide monoisotopic masses were used to match possible proteins using the MS-Fit software, part of the Protein Prospector program, and the insulin precursor was hit with nine identified peptides.

On the other hand, the on-line digestion of lysozyme provides eight identified proteolytic peptides (Table 4). As in the previous case of insulin, the site specificity of on-line pepsin digestion was shown. Ninety-four of 129 amino acids could be identified, which results in a sequence coverage of 73%. Because the molecular mass of lysozyme was larger than that of insulin, the sequence coverage was low. MS-Fit software and the identified peptide fragments, however, indicated that the protein is the lysozyme C precursor. This column also allowed the digestion of carbonic anhydrase (29 kDa), bovine serum albumin (69 kDa), which are larger than lysozyme, and peptide fragments were generated (data

Table 4. List of Peptides from the On-Line Digestion of Lysozyme

peptide	sequence	MH^+ (MH^+ calc), Da
1−8	()KVFGRCEL	951.7(951.5)
9−20	(L)AAAMKRHGLDNY	1346.9(1346.7)
32−38	(A)AKFESNF	842.6(842.4)
39−56	(F)NTQATNRNTDGSTDYGIL	1941.1(1940.9)
57−62	(L)QINSRW	803.6(803.4)
63−83	(W)WCNDGRTPGSRNLCNIPCSAL	2279.4(2279.6)
108−122	(A)WVAWRNRCKGTDVQA	1790.1(1789.9)
123−129	(A)WIRGCRL	903.7(903.5)

not shown). Although we used the known peptide or protein in this study, the sequence data obtained from the MS/MS fragment ions are expected to provide a complementary way for identification of unknown proteins, apart from the peptide masses deduced from the mass spectra.

CONCLUSION

A pepsin-coated PSG monolithic column was fabricated in a fused-silica capillary for integration of on-line protein digestion, peptide separation, and identification by CE/MS. By optimizing the preparation conditions, the pepsin-containing sol formed a thin film on the surface of the PSG monolith without losing the enzymatic activity. Because the highly permeable PSG monolith was employed as the enzymatic reaction support, not only substrates with large molecules such as proteins can access the enzyme and be digested but also fast reaction kinetics were obtained. Furthermore, the use of formic acid as a running buffer suppressed the adsorption of analytes onto the inner surface of the capillary. This also showed that the resultant proteolytic digests can be directly analyzed using ESI-MS. The immobilized pepsin provided reliable and reproducible mass spectrometric results on the sequence data of insulin and lysozyme.

Because CE allows the separation of a protein mixture by various separation modes as well as sample concentration, this on-line system we have developed would facilitate further application for proteomic research by combination of other functions with enzymatic reaction in a single capillary.

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