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Enhanced Proteolytic Activity of Covalently Bound Enzymes in Photopolymerized Sol Gel

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Trypsin is covalently linked to a photopolymerized sol−gel monolith modified by incorporating poly(ethylene glycol) (PSG-PEG) for on-column digestion of N_α-benzoyl-L-arginine ethyl ester (BAEE) and two peptides, neurotensin and insulin chain B. The coupling of the enzyme to the monolith is via room-temperature Schiff chemistry in which an alkoxysilane reagent (linker) with an aldehyde functional group links to an inactive amine on trypsin to form an imine bond. The proteolytic activity of the immobilized trypsin was measured by monitoring the formation of N_α-benzoyl-L-arginine (BA), the digestion product of BAEE. The BA is separated from BAEE by capillary electrophoresis and detected downstream (18.5 cm from the microreactor) by absorption (254 nm). Using the Bradford assay, we determined that 97 ng of trypsin is bound to the 1-cm microreactor located at the entrance of capillary column. The bioactivity of the trypsin-PSG-PEG microreactor at 20 °C for the digestion of BAEE was found to be 2270 units/mg of immobilized trypsin. The bioactivity of trypsin bound to the capillary wall in the open segment upstream from the monolith was 332 units/mg of immobilized trypsin under the same conditions. In contrast, the activity of free trypsin could not be observed for the digestion of BAEE at 20 °C after 16 h of incubation time.

To determine the amino acid sequence in a polypeptide or protein, one promising strategy is to break the polypeptide or protein into fragments by cleaving at specific amino acid residues.[1] This task may be accomplished by either chemical or enzymatic means. For example, cyanogen bromide cleaves polypeptides only on the carboxyl side of methionine residues whereas the enzyme trypsin specifically hydrolyzes peptide bonds at the carboxyl side of lysine and arginine residues. Increasingly, enzymatic digestion has become the preferred approach. This procedure is usually carried out in free solution or with the enzyme immobilized on some solid support structure, such as in a gel. We report here the preparation of a trypsin microreactor formed by covalently linking trypsin to poly(ethylene glycol)-modified photopolymerized sol−gel (PSG-PEG) inside a fused-silica capillary. Trypsin is a pancreatic serine protease consisting of a 23.8-kDa single-chain polypeptide with 223 amino acid residues. The aspartate residue in the binding site enables trypsin to form a salt bridge with positively charged residues, specifically Lys and Arg side chains of the substrate, thus enabling the cleavage reaction to occur in a highly selective manner. Trypsin can undergo autolysis and lose its activity. Moreover, the presence of autolysis peptide fragments can interfere with subsequent analysis. The immobilization of an enzyme to a solid support minimizes or eliminates unwanted autodigestion.[2−4] Consequently, the immobilization of trypsin offers significant advantages over the use of the same enzyme in free solution.

A variety of different approaches have been developed to immobilize enzymes,[2] in particular, trypsin.[3−5] Two common approaches toward the immobilization of enzymes use either a monolith or a particle-based support in capillaries or microfluidic channels. An enzyme can be entrapped within the pores or cavities of a monolithic material such as sol−gel.[4,6−12] Alternatively, an enzyme can be covalently bound to the surface of a solid support such as organic macroporous monoliths or attached to silica particles.[4,13−19]

Based on our recent success with PSG monoliths,[20−22] we have developed a synthetic procedure for the preparation of an enzyme

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microreactor. Our approach involves a one-step in situ process for the photopolymerization of the parent PSG monolith, followed by room-temperature functionalization of the monolith surface with an aldehyde group for not more than 60 min. We illustrate this enzyme microreactor by performing tryptic digests. Trypsin is covalently bound to the surface of an aldehyde-functionalized PSG-PEG monolith through the formation of an imine bond formed at room temperature between the aldehyde group of the PSG-PEG and a terminal amine group in the enzyme. Capillary electrophoresis (CE) with absorption detection has been integrated as an on-line separation method downstream from the enzyme microreactor for the analysis and detection of undigested substrate and products.

The proteolytic activity of a 1-cm-long trypsin-PSG-PEG monolith in a fused-silica capillary was determined by following the on-column digestion of N_α-benzoyl-L-arginine ethyl ester (BAEE), an artificial substrate. The bioactivity of trypsin is further demonstrated through the digestion of two biologically relevant peptides, neurotensin and insulin chain B. We find that the immobilized trypsin has a bioactivity that is hundreds to thousands of times greater than in free solution. Although the present study utilizes trypsin, the method of preparing an enzyme microreactor is a general one that is expected to have wide applications.

EXPERIMENTAL SECTION

Apparatus. All experiments were carried out on a Beckman P/ACE 2000 or P/ACE 5000 (Beckman Coulter, Inc., Fullerton, CA) each equipped with UV absorbance detectors for on-line detection of substrates and products at 214 nm. We use polyimide-coated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of 75 μm i.d. × 365 μm o.d. and 25.5-cm total length (18.5-cm effective length). The sol−gel reaction solutions are irradiated at 365 nm with a Spectrolinker XL-1500 UV cross-linker (Spectronics Corp., Westbury, NY).

Reagents and Chemicals. All buffers were prepared using a Milli-Q water purification system and degassed by sonication for 5 min prior to use. Methacryloxypropyltrimethoxysilane (MPTMS), poly(ethylene glycol) dimethacrylate with an average MW of 330 (PEG-DM), trimethoxysilylbutyraldehyde (linker), toluene, N_α-benzoyl-L-arginine ethyl ester (BAEE), N_α-benzoyl-L-arginine (BA), bovine trypsin (TPCK treated), bovine insulin chain B oxidized, and phosphate-buffered saline (PBS, pH 7.5) were purchased from Sigma-Aldrich (Milwaukee, WI) and used as received. BAEE, BA, and insulin chain B were all dissolved in 50 mM Tris-HCl (pH 7.4) buffer for analysis. Irgacure 1800 was received from Ciba, Inc. (Tarrytown, NY).

Trypsin-PSG-PEG Monolith Preparation. Two PSG-PEG reaction solutions were prepared in the following manner. Solution A was made by mixing 400 μL of MPTMS, 175 μL of PEG-DM, and 100 μL of 0.12 N HCl at room temperature and in the dark for 15 min. Solution B was made by first dissolving 40 mg of Irgacure 1800 in 320 μL of toluene followed by the addition of 80 μL of solution A, which was stirred at room temperature and in the dark for 5 min. A capillary column with a 1-cm irradiation window near one end of the capillary was flushed with ~200 μL of solution B. An irradiation window was made by removing with a razor blade 1 cm of the polyimide coating ~2 cm from the inlet of the capillary. The ends of the capillary were sealed with Parafilm plugs, and the capillary was irradiated in a UV cross-linker for 5 min. Unreacted starting materials were rinsed from the capillary using an ethanol-filled syringe in a hand-held manual vise. When the PSG-PEG monolith is not in use, it is rinsed and stored in either ethanol or acetonitrile.

Aldehyde Functionalization. A PSG-PEG monolith was derivatized with a butyradehyde linker by first rinsing the column with toluene. Linker solutions of trimethoxysilylbutyraldehyde were prepared as 10, 30, or 50% (v/v) in toluene prior to each derivatization. Approximately 100 μL of the linker solution flowed through the PSG-PEG column continuously for 2 h at room temperature. The reaction was stopped, and unreacted starting material and byproduct were removed from the column by rinsing with toluene. When a butyraldehyde-activated PSG-PEG monolith is not in use, it is rinsed and stored in either ethanol or acetonitrile.

Enzyme Immobilization. Before attaching trypsin to a butyrladehyde-activated PSG-PEG monolith, the monolith was rinsed with 200 μL of 100 mM PBS buffer to remove any organic solvent in the capillary column that might interfere with the trypsin derivatization. A fresh solution of trypsin was prepared by dissolving 10 mg of TPCK-treated trypsin in 1 mL of PBS buffer. The monolithic column was rinsed with ~100 μL of the trypsin/PBS solution before filling the column with the same solution and capping the ends. The trypsin-filled column was placed in a refrigerator (4 °C) for 19 h to allow the trypsin to react with the aldehyde functional groups in the monolith. The reaction was stopped, and unreacted trypsin was removed from the column by rinsing the column with PBS buffer. When the trypsin-PSG-PEG monolithic column is not in use, it is stored in a PBS buffer at 4 °C.

Determination of Bound Trypsin. The amount of trypsin bound to the PSG surface was determined using a Bradford assay,[23] which utilizes Coumassie Blue dye. The resulting trypsin−dye complex is detected and quantitatively measured by absorption spectroscopy. Prior to the assay, the column is chopped to isolate the 1-cm portion near the inlet of the monolith, which contains the bound trypsin. This 1-cm section is then filled with 100 μL of 0.1 N NaOH to cleave bound trypsin. Exposure to NaOH was allowed to proceed for 2 h at room temperature. Trypsin standards were prepared in 0.1 N NaOH at a concentration range of 1−100 μg/mL. Each of the trypsin standards as well as the cleaved trypsin from the 1-cm portion of the column was placed in individual wells of a 96-well microtiter plate. To each solution was added 100 μL of the Bradford reagent. The samples were incubated at room temperature for 5−45 min. The absorbance of the resulting trypsin−dye complex in each of the wells was measured at 595 nm. We find that the protein concentration is linear at the concentration range of 1−10 μg/mL.

Proteolytic Activity. We determined the activity of the trypsin immobilized onto a PSG-PEG monolithic support by observing the on-column digestion of BAEE. BAEE solutions in the concentration range of 2.5−25 mM in the Tris running buffer were injected either electrokinetically or hydrodynamically in a trypsin-PSG-PEG monolithic column. The digestion product, BA, and any

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(23) The Bradford assay used in these experiments to determine the amount of bound trypsin was modified from the protocol that Sigma-Aldrich provides with its commercially available Bradford reagent (product number B6919).

unreacted BAEE were eluted using CE and detected at 214 nm. The activity of trypsin (U) is expressed[23] in terms of micromoles of substrate (BAEE) per minute at 37 °C. In what follows, we will report bioactivities in units of U but specify under what temperature they were obtained.

Sample plug lengths were determined using thiourea prepared in the separation solution. Thiourea was injected into the column by pressure or applied voltage. The sample plug lengths were calculated using the following expression, $(L_d/t_R)t_{inj}$, where L_d is the length of the capillary from the inlet to the detection window, t_R is the elution time of thiourea, and t_{inj} is the injection time of the sample. Electroosomotic flow (EOF) was determined using the following expression, $(L_dL_T)/(Vt_R)$, where L_T is the total length of the column, V is the applied voltage, and t_R is the elution time of thiourea. By knowing the EOF of the microreactor column, we were able to move the sample plug to the microreactor by varying the injection time of the separation solution.

Digestion of Neurotensin and Insulin Chain B. A solution of neurotensin was prepared by dissolving 1 mg of neurotensin or insulin chain B in 1 mL of water. These solutions were stored at −20 °C when not in use. After cooling to room temperature, ~20 μL of the solution was placed in a sample vial and the sample was injected using pressure (0.5 psi). The incubation period at 20 °C varied from 10 s to 15 min in the monolith. A voltage of 8 or 10 kV was applied to the column after the incubation time had been reached, and the substrate and product(s) were eluted from the column using CE and detected at 214 nm.

Capillary Electrophoresis. A trypsin-PSG-PEG capillary was installed in a P/ACE 2000 or 5000 capillary cartridge. The total length of the capillary column was 25.6 cm with the length from the detection window to the outlet being 6.7 cm. The effective length of the column was 18.5 cm. We used 50 mM Tris as the running buffer in all runs. The 1-cm enzyme microreactor region was ~5 mm from the capillary inlet. Prior to operation, the trypsin-PSG-PEG capillary column was conditioned by rinsing with a Tris buffer-filled 0.5-mL disposable syringe in a manual vise followed by electrokinetic conditioning at 5 kV for 30 min.

Data Analysis. Peak integrations were performed using Grams AI software (ThermoGalactic, Salem, NH). Peak areas were corrected for any variations in the bulk flow from run to run.

RESULTS AND DISCUSSION

Trypsin-PSG-PEG Monoliths. The nature of the solid support is important as it should facilitate the binding of the enzyme yet remain inert when the desired chemical reactions are performed. PEG with a molecular weight of 330 was incorporated into the PSG monolith, resulting in a PSG-PEG monolith that is sufficiently hydrophilic to minimize the interactions of peptides and proteins on the monolith surface. Broad and tailing peaks were observed for trypsin-PSG monoliths in the absence of PEG. Peak shapes were narrower and more symmetrical when the parent monolith was prepared with 26% (v/v/v) PEG-DM. At higher volumes of PEG-DM, the reaction solution was biphasic, owing to the decreased solubility of PEG-DM in toluene. It has been reported that modified hydrophilic surfaces can effectively reduce surface-induced denaturation of trypsin as well as reduce attractive protein−surface interactions observed with hydrophobic surfaces.[24−26]

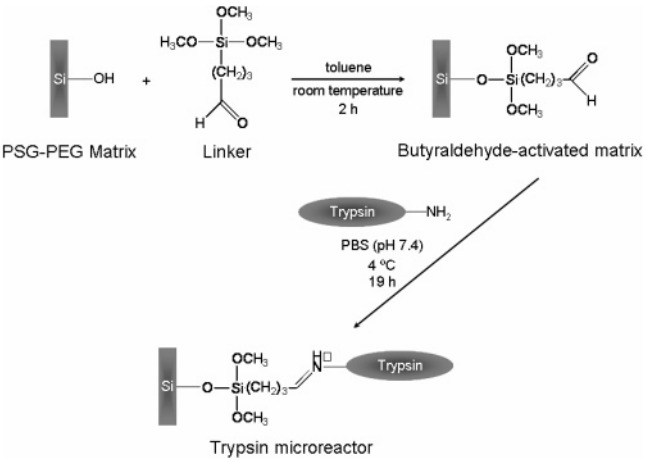

Figure 1. Strategies for functionalization of the PSG-PEG monolith surface with trimethoxysilylbutyraldehyde linker and attachment of trypsin to a butyraldehyde-activated PSG-PEG monolith.

A common procedure for covalent attachment of biomolecules involves the activation of an amine-functionalized surface with glutraldehyde to create aldehyde groups that can react readily with the primary amine groups of biological molecules.[27] Because the bulk PSG monolith contains free silanol (SiOH) groups, an aldehyde functionality can be placed on the monolith using a trimethoxysilylbutyraldehyde linker in one step as illustrated in Figure 1, resulting in a butyraldehyde-activated monolith. Consequently, we avoid the use of glutraldehyde, which is unstable and difficult to purify, to create an aldehyde-functionalized PSG-PEG surface. Figure 1 shows a single-point attachment of the linker to the monolith surface via a Si−O bond. Multipoint attachment of the linker, however, may occur through the formation of two or three Si−O bonds between the linker and the monolith.[22]

The last reaction step in Figure 1 illustrates our strategy for the covalent attachment of trypsin to a butyraldehyde-activated PSG-PEG monolith. The aldehyde group of the bound linker reacted with the terminal primary amine of trypsin to form a trypsin-PSG-PEG monolith. The reaction between trypsin and the aldehyde functional group on the PSG-PEG surface was allowed to proceed in PBS buffer at 4 °C to minimize the autolysis of trypsin in the bulk solution during the immobilization period. At this low temperature, the reaction proceeded overnight for ~19 h. Shorter reaction times failed to produce a monolith with good proteolytic activity. When the reaction was allowed to proceed at room temperature for 3−19 h, the resulting trypsin-PSG-PEG monolith had very little to no proteolytic activity for the substrate BAEE, suggesting that extensive autolysis of the trypsin in solution had occurred.

Any unbound trypsin was removed by rinsing the monolith with PBS buffer, which was subsequently tested for the presence of trypsin by monitoring the formation of BA. We tested the

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Table 1. Proteolytic Activity and Separation Parameters of Trypsin-PSG-PEG Monoliths Prepared with Different Amounts of Linker[a]

% linker (v/v)	% BA (RSD[b])	t_m (min) (RS[b])	R_s (RSD[b])
10 ($n = 2$)	5.1 (5.0)	2.24 (0.13)	14.1 (2.1)
30 ($n = 3$)	20.7 (5.7)	2.56 (1.2)	21.1 (1.6)
50 ($n = 3$)	69.3 (5.7)	2.50 (0.54)	20.1 (3.1)

[a] The percentage BA is based on corrected peak areas. The digestion conditions are 2.5 mM BAEE; 50 mM Tris (pH 7.5) running buffer, 10-kV applied voltage, 20 °C; 2-min incubation time. [b] RSDs are given in percentages.

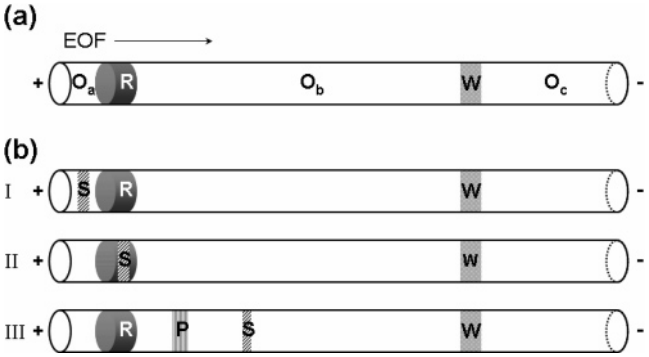

Figure 2. (a) Schematic illustration of a trypsin-PSG-PEG monolithic column where O_a, O_b, and O_c are open segments, R is a 1-cm trypsin microreactor, and W is the detection window. O_a is a 5-mm open segment, O_b is an open segment (~17 cm) between the monolith and W, and O_c is a 6.7-cm open segment. Bulk solution flow (EOF) is from the anodic inlet to the cathodic outlet of the capillary column. (b) On-column enzyme digestion of a substrate (S) and separation of S and product (P). The entire capillary is filled with running buffer. (I) A plug of substrate is introduced into the column by pressure or voltage; (II) the substrate plug is moved into the monolith and then allowed to react with bound enzyme for varying periods of time; (III) the product that is generated during the digestion is separated and eluted from undigested S when the separation voltage is turned on.

monolith for the presence of unbound trypsin. We judged the monolith to be free of unbound trypsin when BA product could no longer be detected at 214 nm using CE for the analysis.

We studied the effect of using different amounts of linker on the proteolytic property of a trypsin-PSG-PEG monolith. The amount (v/v) of butyraldehdye linker was varied in toluene by 10, 30, and 50%. Table 1 shows the effect of these solutions on the formation of BA from the digestion of BAEE during a 2-min incubation period. Increasing the amount of linker bound to the PSG-PEG monolith surface should have the same effect as increasing the concentration of trypsin in solution. The performance of the immobilized enzyme at 20 °C improves with an increase in the percentage of linker in the derivatization solution, resulting in more linker attached to the monolith surface. It follows then that there should be a higher concentration of bound trypsin with an increase in the amount of bound linker on the monolith. Based on corrected peak areas, the monolith prepared with 50% (v/v) linker solution allowed for the highest percentage (69.3%) of BA product to be formed during the incubation period. Even at the lowest concentration of linker (10%) in the derivatization solution, the column exhibited some proteolytic activity, resulting in 5.1% BA formed. The migration times of both BAEE and BA in columns prepared with 30 and 50% linker solutions were comparable and not affected by the different surface coverages of the linker. Faster migration times for BAEE and BA were observed for the 10% linker column, indicating that the EOF in this column was faster than in the other two columns because of the presence of higher free silanol content. The resolution between two analytes is given by the expression, $R_s = 1.177(t_m^B - t_m^A)/(w_h^A + w_h^B)$, where t_m^B and t_m^A are the elution times for analytes B and A, respectively, and w_h^A and w_h^B are the peak widths at half-height for A and B, respectively. As shown in Table 1, R_s values were comparable for the 30 and 50% linker columns but 10 times smaller in the 10% column. The 50% column achieved the best digestion and separation, but all of the subsequent measurements were made on trypsin-PSG-PEG columns with 30% linker solution. When binding the enzyme to the PSG-PEG surface, care was taken to stay within physiological conditions so as not to inadvertently alter or block the active site of the enzyme.

The length of the trypsin-PSG-PEG monolith was varied from 0.5 to 10 cm. Under our current procedure for the removal of the outer polyimide coating of a capillary, we found it to be technically challenging to prepare reproducible monoliths of lengths that were less than 1 cm, but 1-cm monoliths were reproducibly prepared. Furthermore, a capillary column with a 1-cm stripe of its polyimide coating removed is more mechanically robust than a capillary column with a longer stripe of its coating removed from the outside of the capillary. A 1-cm trypsin-PSG-PEG monolith was efficient for the proteolytic digestion of our chosen substrates and downstream CE separation was baseline-resolved. Righetti and co-workers[28] reported that increasing the length of the enzyme microreactor from 1.5 to 10 cm in a capillary column resulted in better peptide mapping and higher resolution of the peptide peaks. Because the substrate is digested as it flows through the microreactor, the contact time between the enzyme and the protein substrate is short. In contrast, we allow for much longer contact times between the immobilized trypsin and a substrate by including an incubation period in the on-line enzyme digestion procedure. When the monolith length approached 10 cm, the separation performance of the column was adversely affected. The method of separation is based on capillary electrochromatography (CEC) rather than on CE, and poorer resolution of the substrate and product was observed when separation was by CEC.

Proteolytic Activity of a Trypsin-PSG-PEG Monolith for the Digestion of BAEE. We evaluated the proteolytic activity of a trypsin-PSG-PEG monolithic column using BAEE, a synthetic amino acid substrate. Trypsin catalyzes the hydrolytic cleavage of the ester linkage in BAEE to form BA. The design of a trypsin-PSG-PEG monolithic column is shown in Figure 2a, where there is a short (5 mm) open segment upstream from the PSG monolith. Figure 2b shows the steps involved in on-column digestion of a substrate (S) in a trypsin-PSG-PEG capillary column. A 0.57-mm sample plug, which is smaller in length than O_a, is introduced into the column (I). On-column digestion of S in the sample plug is performed in the monolith by moving the plug electrokinetically into R (II), where the plug reacts with the monolith for varying lengths of time; during this incubation period, S is digested into product (P). The incubation period is stopped when the separation

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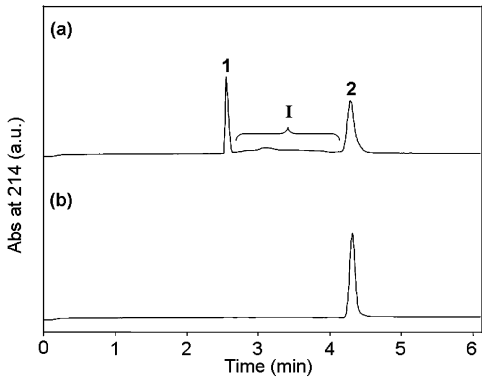

Figure 3. On-column tryptic digest of 25 mM BAEE at digestion times of (a) 0 and (b) 2 min. Peak 1 is undigested BAEE, and peak 2 is BA product. Inset: Region I is an overlap of BA peaks that are generated when the sample plug moves through the remainder of the monolith. A 75 μm i.d. $\times$ 365 μm o.d. $\times$ 25.3 cm long capillary contained a 1-cm trypsin-PSG-PEG monolith placed 5 mm from the inlet. The BAEE sample was injected for 2 s at 2 kV. The running buffer was 50 mM Tris (pH 7.5). The applied voltage was 10 kV, and the temperature was 20 °C.

voltage is applied, sweeping P and any undigested S toward the detection region. Both P and S are separated electrophoretically in segment O_b.

Figure 3 illustrates the digestion of 25 mM BAEE to BA at different incubation times in the monolith. BA product is formed even when the substrate is exposed for only 10 s to the monolith as shown in Figure 3a. As the digestion time is increased to 2 min, BAEE is completely digested, and only a BA peak is detected as shown in Figure 3b. In Figure 3a, region I represents overlapping BA peaks that are generated when the remaining BAEE in the sample plug is digested as it passes through the rest of the trypsin-PSG-PEG monolith during separation. It is not surprising to detect product from the undigested BAEE in the sample plug as it moves through the monolith because even a 10-s residence time of the BAEE results in the formation of some BA as observed in the "0-min" reaction time experiments where the sample plug waits in the monolith for 10 s while the sample and buffer vials move into different positions and the separation voltage ramps up to its target value. As shown in Figure 3b, region I is not observed when all of the BAEE substrate is allowed to react with the trypsin in the monolith.

Two schemes exist for injecting substrate into the immobilized enzyme microreactor. One approach is the "continuous flow" approach, which is carried out by applying an external voltage to the microreactor without interruption during the entire digestion and separation run. It has been reported that the substrate flow velocity can markedly affect the efficiency of the immobilized enzyme activity.[17,29] For an enzyme microreactor integrated into a capillary, a substrate flow velocity of 10.6 mm/s was sufficient for high recovery of the amino acid sequence of a protein substrate.[17] We found that varying the substrate flow velocity in the 0.5−1.14 mm/s range after injection of BAEE in a trypsin-PSG-PEG monolithic column had no effect on the digestion efficiency of the column. A separation flow velocity in a trypsin-PSG-PEG column of 1.14 mm/s was calculated using thiourea as an unretained marker.

Efficient digestion of BAEE by immobilized trypsin was achieved using an alternative substrate mobilization approach that involved incubation of the substrate with the immobilized trypsin. The incubation approach facilitates sufficient interaction between the bound trypsin and the substrate.

Landers and co-workers[29] studied these two substrate injection approaches in a trypsin microreactor integrated into a microfluidic device. They found no significant differences in peak heights and widths of the product and substrate peaks using both approaches.

Trypsin immobilization on the PSG-PEG monolith was analyzed using the Bradford method to measure the total bound enzyme. In a 1-cm-long monolith, 97 ng of trypsin is bound to the monolith surface. The proteolytic activity of a 1-cm trypsin-PSG-PEG monolith integrated into a capillary column was determined to be 2,270 units/mg of trypsin at 20 °C while the bioactivity of trypsin in bulk solution under the same experimental conditions was not observed even after 16 h of incubation time. The RSD for capillary-to-capillary reproducibility with respect to the activity of immobilized trypsin is 7.8% ($n = 4$). This enhancement in the proteolytic efficiency of immobilized trypsin compared to bulk catalysis can be ascribed to (1) minimization or elimination of the autolysis of trypsin[2,4,12,16] and (2) the possible stabilization of the structure of trypsin, resulting in higher accessibility of the substrate to the active site of the enzyme.[24−26,30,31] In some cases,[24,25] the increase in the proteolytic activity of a bound enzyme also arises from the larger enzyme-to-substrate (E/S) ratio, such as 20:1−1:1. Typically, the E/S ratio must be kept low (1:100−1:20) in free solution to minimize the autodigestion of the free enzyme. Surprisingly, the E/S ratio in our experiments is 1:3220. The proteolytic activity of the trypsin-PSG-PEG monolith, however, is at least 2000 times more active than in free solution. The enzyme activity that is observed for trypsin-PSG-PEG monoliths is significantly higher than what has been observed for entrapped trypsin in sol−gel, where the activity is 700 times higher than in free solution.[12] Clearly, covalent attachment of trypsin to the surface of a hydrophilic sol−gel monolith results in higher proteolytic activity of trypsin versus trypsin entrapped in the pores of a sol−gel matrix.[12] In addition to the advantages that covalent attachment offers (no self-destruction of the enzyme and structural stability), the hydrophilic PSG-PEG monolith may provide a favorable microenvironment that further enhances the structural stability of bound trypsin. Yamada and co-workers[25] have reported that water absorptivity of grafted poly(acrylic acid) (PAA) and poly(methacrylic acid) (PMAA) on a polyethylene surface has considerable influence on the activity of immobilized trypsin. Water absorptivity increased for the more hydrophilic surface (PAA) as compared to the less hydrophilic surface (PMAA), which resulted in an increase in the activity of trypsin bound to PAA.

As expected, increasing the digestion temperature leads to an increase in the amount of BA produced. For a BAEE concentration of 2.80 mM and a digestion time of 15 min, there is a 59% increase in BA peak area when the temperature is raised from 20 (corrected peak area, 6.58×10^{-5}) to 30 °C (corrected peak area, 16.0×10^{-5}), indicating that the digestion reaction rate has increased. One of the advantages of our trypsin-PSG-PEG microreactor is

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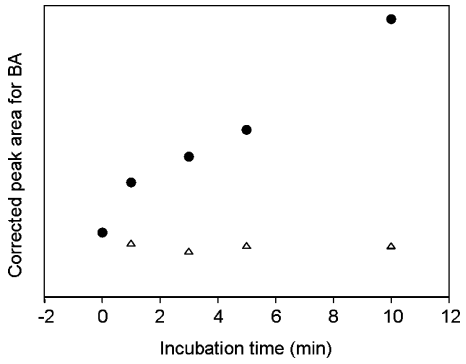

Figure 4. Comparison of the proteolytic activities as a function of incubation time in the open segment of the inlet of the capillary (△) and on the monolith (•). The injection and separation conditions are the same as in Figure 3.

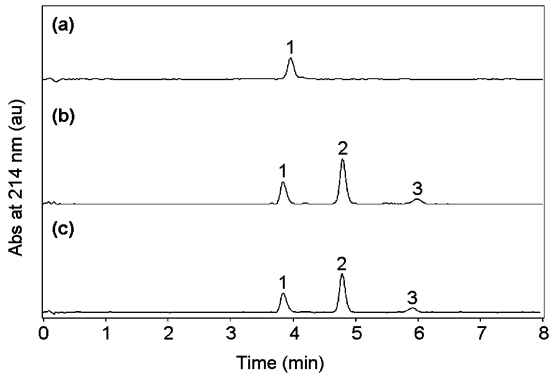

Figure 5. Digestion of neurotensin (a) with no incubation time; (b) on the monolith at the entrance to the 1-cm portion containing bound trypsin (region 1), 5-min incubation time; and (c) on the monolith at the exit to the 1-cm portion containing bound trypsin (region 2), 5-min incubation time. Peak 1 is neurotensin; peak 2 is neurotensin fragment 1−8; peak 3 has not been positively identified but is expected to be neurotensin fragment 9−13. The separation and injection conditions are the same as in Figure 3 except that the applied voltage is 8 kV.

the high proteolytic activity that is observed at room temperature (20 °C). Consequently, all of the digestion reactions that we have described were conducted at 20 °C, which is significantly lower than the temperature of 37 °C at which the maximum rate of protein digestion occurs with free trypsin.

Proteolytic Activity in the Open Segment versus the Monolithic Segment. Because of the presence of free silanol groups on the surface of the capillary wall, the wall itself is also derivatized with the linker. Consequently, trypsin is also bound to the capillary wall. Enzymes, such as alcohol dehydrogenase and lactate dehydrogenase, have been covalently immobilized on the inner surface of capillaries.[32,33] These enzyme-coated capillaries have been shown to be effective at digesting appropriate substrates. The mass transfer in the open segment of the capillary will be slower compared to the mass transfer in the monolith. As a result, tryptic digest in the open segment of the capillary should be slower than in the monolith.

Figure 4 compares the formation of BA product from the digestion of BAEE over a 10-min incubation time in the open and monolith segments. When the digestion is allowed to occur in the open segment of the capillary column, less BA product is formed compared to that in the monolith. The formation of BA product in the open segment of the capillary is independent of the incubation time. The BAEE sample plug actually spends ∼10 s in this open section because this is the length of time needed for us to change the positions of the buffer and sample vials. Approximately 75% more BA product is formed during an incubation period of 10 min in the monolith segment. In the open segment of the capillary, the proteolytic activity of trypsin bound to the capillary wall was determined to be 332 units/mg of bound trypsin. The proteolytic activity of trypsin bound to the monolith is enhanced by 85% compared to trypsin bound only to the capillary wall.

Stability of the Trypsin-PSG-PEG Monolith. Reproducibility from run to run on a single trypsin-PSG-PEG column was determined from 90 runs under identical conditions over the course of 3 days. The RSD values for the migration time and corrected peak areas of BA were 3.22 and 8.55%, respectively.

The bioactivity of the trypsin-PSG-PEG column is preserved when the column is stored at low temperature (4 °C). After 20 days, the trypsin-PSG-PEG column retains ∼90% of its proteolytic activity. The bioactivity, however, decreases when the column is stored at room temperature. We find that a 33% decrease in the bioactivity of the column takes place, based on BA peak areas at the end of a 10-day aging experiment at room temperature.

Hydrolytic Digestion of Peptide Substrates. *Neurotensin.* We demonstrated the proteolytic activity of the enzyme microreactor by cleaving the endogenous neurotransmitter neurotensin, a 13-amino acid peptide (MW 1672.92). Trypsin cleaves at the C-terminal end of the ester bond between amino acid residues 8 and 9, resulting in two fragments, neurotensin fragment 1−8 (NTF 1−8) and neurotensin fragment 9−13 (NTF 9−13). As expected, the residence time of the neurotensin sample plug in the monolith determines how much of the neurotensin will be digested. This behavior is illustrated in Figure 5. The fragment NTF 1−8 (MW 1030.13) was identified by spiking the sample with commercially available NTF 1−8. The other fragment NTF 9−13 was not identified, but there is a new peak that appears (peak 3), which we believe is NTF 9−13.

In Figure 5a, a sample plug of neurotensin is injected into the column and the separation voltage is turned on immediately. The sample plug is in the open segment upstream from the monolith during the 10-s period in which the sample and buffer vials move and the target voltage is reached. No digestion product is observed. When the sample plug is incubated for 5 min in the entrance to the 1-cm section of the monolith near the inlet (region 1) after having been moved from the open segment after injection, NTF 1−8 (peak 2) is detected as illustrated in Figure 5b. Moving the sample plug to another part of the 1-cm portion of the monolith (region 2), which is downstream from region 1, resulted in the production of NTF 1−8 (Figure 5c) with a similar corrected peak area (19.3×10^{-5}) as the fragment produced in region 1 (22.0×10^{-5}). This result suggests that the trypsin is homogeneously distributed in the 1-cm portion of the monolith. Some digestion product (corrected peak area, 8.66×10^{-5}) is produced when the sample plug is allowed to incubate for 5 min in the open segment upstream from the monolith. In terms of peak area, 58% more product forms in the monolith as compared to the open segment of the capillary column.

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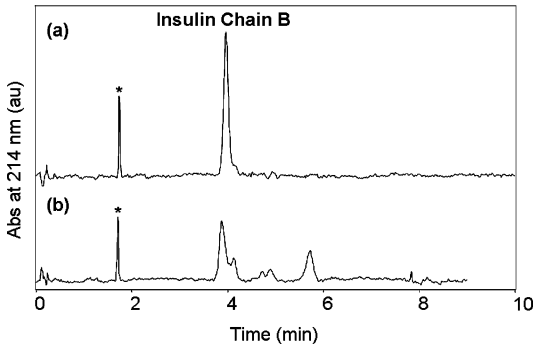

Figure 6. On-column digestion of insulin chain B at 20 °C. Digestion in the (a) open section (inlet); (b) the peak marked with an asterisk (∗) is believed to be a contaminant in the sample solution and not a digestion product. The separation conditions are the same as in Figure 3.

Insulin Chain B. Immobilized trypsin on a PSG monolithic surface can hydrolytically cleave insulin chain B (MW 3495.89), a larger peptide with 30 amino acids, than neurotensin. There are two cleavage sites, one between amino acid residues 22 and 23 and the other between amino acid residues 29 and 30. Three fragments are formed: one with 21 amino acids, another with 7 amino acids, and a third that is alanine. Figure 6 demonstrates the on-column digestion of insulin chain B at 20 °C in different segments of a trypsin-PSG column. No digestion product is observed when a plug of insulin is allowed to react in the open segment where mass transfer to the trypsin bound to the walls is very slow (Figure 6a). On the other hand, the insulin chain B peak area and height are decreased and new peaks are observed when the insulin sample plug is allowed to react in the trypsin-PSG monolith segment of the column downstream from the detection window (Figure 6b) for 15 min, resulting in a 48% decrease of the insulin peak area (RSD = 6%, $n = 3$). These results encourage us to plan future experiments in which the enzyme microreactor is used to digest various different proteins for subsequent analysis of the protein fragments.

CONCLUSIONS

A reactive hydrophilic macroporous PSG-PEG monolith was prepared in situ in a fused-silica capillary by using a combination of sol−gel chemistry and photopolymerization, followed by functionalization of the monolith surface with an alkoxysilane reagent containing an aldehyde group. The activated monolith was transformed into an enzyme microreactor by covalent attachment with the enzyme trypsin. The technique is general, and trypsin was chosen because it is the most highly selective protease. The resulting trypsin-PSG-PEG microreactor has long-term stability and high proteolytic activity for the on-column digestion of large peptides. The proteolytic activity of the bound trypsin was enhanced more than 2000 times that in free solution. The short digestion times at low temperature contrasts with the longer reaction times at higher temperatures necessary in tryptic digests in free solution. The covalently bound enzyme also allowed for easy interfacing downstream to CE, which would facilitate further applications using larger biomolecules such as proteins.

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