Mesoporous Silica Nanoreactors for Highly Efficient Proteolysis

Jie Fan,[a] Wenqing Shui,[a] Pengyuan Yang,[a] Xiaoyan Wang,[a] Yunmin Xu,[b] Honghai Wang,[b] Xian Chen,*[c] and Dongyuan Zhao*[a]

Abstract: Protein digestion inside the nanoreactor channels of mesoporous silica (SBA-15) is reported, and evaluated by using peptide-mass mapping. Both proteases and substrates were efficiently captured within these biocompatible nanoreactors. After 10 minutes, the mass spectrum of the protein digests released from the mesoporous-silica-based nanoreactors revealed the presence of eight peptides covering 58% of the protein sequence with an intense signal (signal/noise ratio >70). In comparison, the conventional overnight in-solution digestion of proteins under otherwise identical conditions generated only three peptides (27% sequence coverage). We propose that this order-of-magnitude increase in the proteolytic reaction rate is mainly attributed to two factors: substrate enrichment within mesoporous silica channels and enzyme immobilization. The surface properties and macrostructure of the mesoporous silica were studied to reveal their significant influence on proteolytic reactions.

Keywords: mass spectrometry · mesoporous materials · nanoreactors · peptides · proteolysis

Introduction

Over the last decade, mesoporous silicas (MPSs) have emerged as a new type of solid host for enzyme immobilization/catalysis due to their open pore structure, uniform pore size distribution, and large pore volume.[1–6] The stability and reusability of entrapped enzymes are enhanced for enzymatic hydrolysis of small organic molecules, however, the reaction efficiency is generally lower than that of free enzymes in solution.[1,3,7–9] Additionally, MPS-based nanoreactors are expected to have a significant effect on reactions involving proteins, as the MPS pore size is comparable to protein size and the internal surface may show a specific affinity for the reactants.

Proteolysis by trypsin is an essential step for generating peptide fingerprints for protein identification in proteomic analysis.[10] The conventional time-consuming digestion process suffers from incomplete digestion and low protein-sequence coverage.[11] Efforts to increase tryptic digestion efficiency by immobilizing enzymes in confined zones of a capillary,[12,13] packed beads,[14] porous silicon,[15] and porous polymer monoliths[16] have been reported. These microreactors, varying in size from several hundred nanometers[16] to millimeters,[12,13,17] could offer the advantages of rapid analysis, reduced sample consumption, and reduced cost. Very recently, we have developed a new device for protein digestion that is based on a highly ordered mesoporous silica, FDU-12.[18] Interestingly, this class of nanoreactors was found to simultaneously achieve highly efficient substrate entrapping and unfolding, enzyme flashing, and protein digestion, distinguishing them from microscale fluidic devices.[12,13,17]

Herein, we investigate the unique influence of MPS on the enzymatic digestion of biomolecules. The widely studied MPS SBA-15 was employed as a nanoreactor for tryptic digestion of myoglobin because both its surface character and macrostructure can be independently modified to allow the exhaustive study of each individual effect.

[a] Dr. J. Fan,[+] W. Shui,[+] Prof. P. Yang, X. Wang, Prof. D. Zhao
Department of Chemistry, Shanghai Key Laboratory of Molecular Catalysis and Innovative Materials
Fudan University, Shanghai 200433 (P.R. China)
Fax: (+86) 21-6564-1740
E-mail: dyzhao@fudan.edu.cn

[b] Dr. Y. Xu, H. Wang
Institute of Genetics
Fudan University Shanghai 200433 (P.R. China)

[c] Prof. X. Chen
BN-2, Biosciences Division
Los Alamos National Laboratory
Los Alamos, New Mexico 87545 (USA)
Fax: (+1) 505-665-3024
E-mail: chen_xian@lanl.gov

[+] These authors contributed equally to this work.

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Results and Discussion

Mesoporous silica SBA-15[19] consists of one-dimensional mesochannels packed in a two-dimensional hexagonal structure. SBA-15 materials of different particle size and surface character were synthesized to construct the silica-based nanoreactors for accommodation and reaction of the protease (trypsin, $M_r = 25400$ Da, pI is this the isoelectric point (IEP) $= 8.23$) and the substrate (myoglobin, $M_r = 16900$ Da, pI $= 7.36$). Transmission electron microscopy (TEM) images, X-ray diffraction (XRD) patterns, and N_2-sorption isotherms (Figure 1; see Supporting Information SI-1 and SI-2) were used to confirm that the SBA-15 materials consisted of highly ordered, hexagonally arranged mesochannels with a pore diameter of ~8 nm. These different materials were employed as nanoreactors for protein adsorption and tryptic digestion.

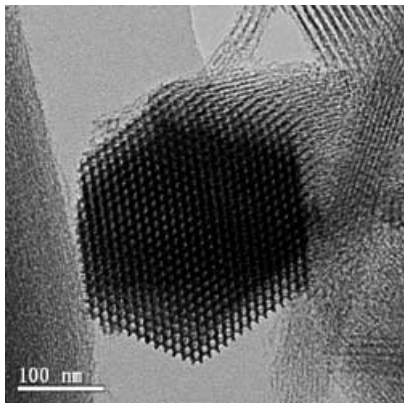

Figure 1. Typical TEM image of a mesoporous silica SBA-15 sample (S-2-SH) with thiol functionalization after removal of the block copolymer by solvent extraction.

At pH 6.5, trypsin and myoglobin are positively charged and were rapidly (less than 1 min) captured into the negatively charged mesochannels of SBA-15. After entrapment, no protein was detected in the supernatant with UV-visible spectrometry. It is widely accepted that when the mesopore diameter is sufficiently large for "comfortable" entrapment of biomolecules, proteins penetrate deep into mesoporous networks rather than adsorb onto the external surface.[1,4,20] In our case, the pore diameter (~8 nm) of SBA-15 is twice the globular diameter of proteins (~4 nm); therefore, we believe that the majority of the proteases and substrates were completely entrapped within the mesochannels. Moreover, the rapid entrapment of 15 wt% myoglobin or trypsin within SBA-15 was also observed. This rapid and high-capacity absorption is caused by in-pore entrapment instead of external site binding.[21]

The subsequent proteolysis of the entrapped myoglobin was triggered by adjusting the pH of the buffer from acidic (pH 6.5) to basic (pH 8.2). The electrostatic interaction between the proteins and the negatively charged silica walls limits the motion of entrapped enzymes and proteins, however, the high-capacity (up to 50 wt%) adsorption of proteins within the mesochannels of SBA-15 suggested that the rational translational and rotational motions of entrapped proteins had been preserved to achieve such a crowded "packing".[21] This is mainly due to the open pore structure of mesoporous silica and the noncovalent interaction between the silica surface and the protein. The motions of entrapped biomolecules may allow them to move inside the mesochannels to meet each other and achieve the desired digestion. During the rapid buffer pH alteration, no protein was released from the mesochannels into solution, as monitored by means of UV-visible spectrometry. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry[22] was used to analyze the digestion products generated from different nanoreactors. Proteolysis efficiency was evaluated based on protein identification results using peptide-mass fingerprinting (PMF) and the peptide peak intensity in the PMF spectra (Figure 2).[23]

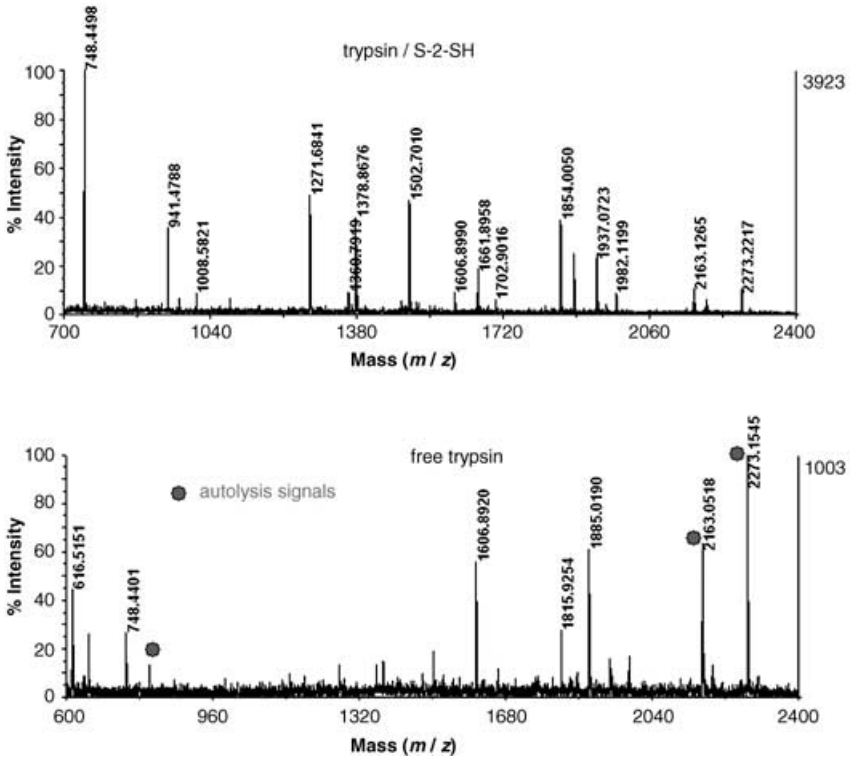

Figure 2. PMF spectra of myoglobin proteolysis products from protein digestion in SBA-15 (S-2-SH) nanoreactors (top) and in solution (12 h; bottom).

Substrate-enrichment effect: Surprisingly, the mass spectrum of the product obtained from the 10 minute proteolysis of myoglobin inside the mesochannels of SBA-15 (sample S-2-SH) yielded intense peaks (signal/noise ratio (S/N) > 70). This mass spectrum provided the confident identification of 8 peptides (Table 1, Figure 2, and Supporting Information SI-3), with a MOlecular Weight SEarch (MOWSE) score of 164 (a score of 61 is the identification threshold value for peptide peaks of S/N > 3). Thus, highly efficient proteolysis was achieved in SBA-15 nanochannels. In contrast, even after a 12 h incubation period of predenatured myoglobin in solution in the presence of trypsin, its PMF failed to be identified (MOWSE score of 57, below the threshold for identification). In fact, in-solution digestion generated only two peptides at the similar S/N level after a 6 h reaction and three peptides after overnight incubation (based on analysis of the mass spectrum). The difficulty in digesting this rigid-structure protein by free enzymes has been reported in other studies.[11] It is noteworthy that in our case, we applied the same enzyme/substrate ratio (E/S = 1:2) for both in-solution and in-nanoreactor digestion. For the in-solution measurements, the autolysis signals from the free enzyme significantly complicated the mass spectra and interfered with protein identification. But, as shown in Figure 2, enzyme entrapment in nanoreactors minimized undesired autodigestion while maintaining a remarkably high proteolytic efficiency, one advantage of enzyme immobilization. The isoelectric points of trypsin and myoglobin are 8.23 and 7.36, respectively. It suggests that in the buffer solution of pH 7.5–8.0, the trypsin can be tightly attached to the silica surface, and its motion and collision at high concentration can be well restrained. This could partially explain why autolysis of the trypsin is avoided in our system and the digestion of substrate protein was preferred predominantly. Moreover, the processing of the proteolytic reaction also depends on the accessibility of the tryptic cleavage sites of the protein. We have found that the myoglobin entrapped in mesopores undergoes an in situ unfolding, which makes its cleavage sites more exposed;[18] whereas, the trypsin is able to resist to some extent the denaturing force exerted by mesoporous silica and remains active in the same way that it does in the partially denaturing environment of 2 M urea.[18,29]

Table 1. Comparison of the proteolytic efficiency of trypsin/MPS biocatalysts and free enzyme.

SBA-15 samples[a]	Protein score	Peptide matches[b]	Sequence coverage
S-26-Ext	52	3	13%
S-6-Ext	51	3	20%
S-2-Ext	97	5	44%
S-2-SH	164	8	58%
S-2-MW	78	4	23%
S-2-Cal	0	0	0%
free trypsin (6 h)	40	2	20%
free trypsin (12 h)	57	3	27%

[a] SBA-15 samples are denoted as S-X-Ext, -SH, -MW, and -Cal. X represents the particle size measured by a laser-light-scattering spectrometer; -Ext, -MW, and -Cal indicate that templates of the sample were removed by ethanol extraction, microwave digestion, and calcinations, respectively; -SH signifies that the sample was thiol-functionalized (2 wt%) by an in situ synthesis,[10] and templates were removed by ethanol extraction. [b] Theoretically, myoglobin can generate 18 peptides (containing at least 2 amino acids) without missed cleavages, but some of them are too small to be detected by MALDI-TOF, such as YK, HK, or FK. Generally, sequence coverage is a preferred parameter for evaluating protein identification and it can reflect digestion efficiency when other experimental conditions are optimized. Apparently, the ideal sequence coverage is 100%, which is rarely achieved due to the limitation of instrumental sensitivity, detection bias, and so forth, and it significantly depends on the S/N cutoff defined in a database search. In our case, the best hit for a specific SBA-15-based nanoreactor is 79% using peptide peaks with S/N > 20, or 58% with S/N > 70. The values are high enough for confident identification given that the common threshold is around 20–25%.

Accordingly, the increased number of identified peptides obtained from protein digestion in the MPS-based nanoreactors resulted in a higher sequence coverage compared to those obtained from in-solution proteolysis (58% vs 27%). Furthermore, a close comparison of the mass spectrum intensity due to a single peptide ($m/z = 748.4$, Figure 2) generated from digestion in the silica-based nanoreactors and from digestion using typical solution methods, revealed that the S/N ratio was increased 15-fold by digestion in the nanoreactors using the identical instrumental settings. Although the peak signal of MALDI spectra is generally only semi-quantitative, the reproducible PMF from five pairs of solution and nanoreactor samples, and the large difference in peak intensity between in-nanoreactor and in-solution samples, strongly indicated that proteolysis within nanoreactors generates far more abundant peptides in much less time than the in-solution reaction. Prolonged digestion in SBA-15 nanoreactors did not produce additional peptide peaks or higher sequence coverages, suggesting that the proteolytic reaction within the nanoreactors rapidly reached its equilibrium status within the initial 10 minutes. In contrast, in-solution proteolysis produces additional peptides during the course of several hours (sequence coverage increased from 20% at 6 h to 27% at 12 h).

We have discussed the interesting discovery of substrate unfolding (which can substantially facilitate proteolysis) in MPS-based nanoreactors elsewhere:[18] in this paper, we mainly emphasized the substrate enrichment within the regular arrays of mesochannels in SBA-15, which we suggest is responsible for the remarkable acceleration of the proteolytic reaction. According to previous studies,[24] the reaction rate of proteolysis is dependent on the concentration of the enzyme and substrate, the pH, the temperature, and the existence of inhibitors or activators. In our case, all these factors were kept constant for both in-solution and in-nanoreactor proteolysis. However, the local concentration of the protease and substrate (trypsin and myoglobin) was dramatically increased to ~100 times that of those in solution by their entrapment within the SBA-15 nanoreactor (according to the pore volume of SBA-15 and the solution volume; Figure 3).

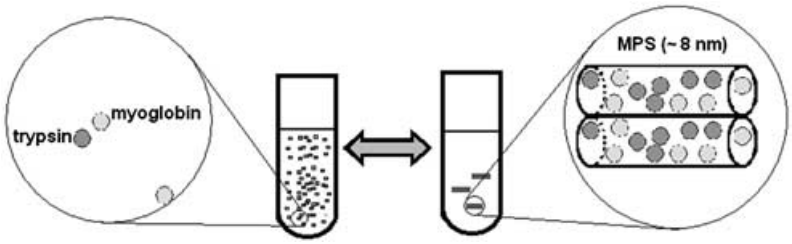

Figure 3. Schematic illustration of proteolysis occurring in solution (left) and in mesoporous silica SBA-15 nanoreactors (right).

Because we used a large E/S ratio (1:2), increasing the enzyme concentration was expected to have little influence on the overall reaction rate.[24] Therefore, we expected at least a 100-fold increase in the reaction rate obtained within SBA-15 nanoreactors, compared with the in-solution reaction, as a result of the increased substrate concentration (if we assume that the reaction follows first-order kinetics). In our experiments, the proteolysis rate in the mesochannels of SBA-15 was estimated to be 300–400 times faster than that for in-solution digestion according to PMF signal intensity and reaction time to reach equilibrium. The order-of-magnitude agreement between the observed kinetics and the kinetics predicted by simple concentration arguments supports our model of substrate enrichment within the nanoreactor.

Surface-character effect: Different surface properties of SBA-15 resulted in drastically different performances as solid hosts for enzymatic catalysis. It has been reported that specific surface functionalization can modify the activity of enzymes entrapped within MPSs for small-molecule hydrolysis compared with the free enzyme.[5,7]

In the present study, placing trypsin inside thiol-functionalized SBA-15 (S-2-SH) demonstrated the highest catalytic efficiency, while the enzyme entrapped in the S-2-Cal sample showed the lowest activity (S-2-SH > S-2-Ext > S-2-MW ≫ S-2-Cal, see Table 1 and the Experimental Section) when all other factors were held constant. Ethanol extraction was found to be effective in both removing the organic templates (block copolymers) from mesoporous silica and providing an optimized mesoscopic environment for highly efficient biocatalysis. In comparison, removal of the block copolymer templates by calcination resulted in poor performance (no peptides detected for sample S-2-Cal). We suspect that the mesoporous silica treated by ethanol extraction features abundant hydroxyl groups on the silica wall. The strong interaction between protein substrates and silanol groups (Si–OH) abundant on the mesochannel walls would likely accelerate the unfolding process and expose more cleavage sites to be accessed by proteases.[18,25] The channel-wall interactions with trypsin do not appear to cause conformational changes sufficient enough to deactivate the catalytic properties of the enzyme. However, the complicated effect of chemical treatment of the MPS inner wall on biomolecule interactions requires further study to elucidate the underlying mechanisms.

Macrostructure effect: The macrostructure of MPS has not been previously recognized as an important factor for enzymatic catalysis in nanoreactors. However, when we prepared SBA-15 solids with different particle sizes (Figure 4) and tested their performance as hosts for proteases, macrostructure was found to play a significant role. Our results indicate that a decrease in the particle size of mesoporous silica produces an increase in the proteolytic efficiency of SBA-15 nanoreactors (S-2-Ext > S-6-Ext > S-26-Ext), as evaluated by the number of identified peptides and sequence coverage (Table 1). Because the pore size, pore volume, surface area, and the surface character of each SBA-15 varied only slightly (see Supporting Information SI-2), the differences in proteolytic efficiency are attributed to the effect of SBA-15 particle size. By reducing the particle size, the particle outer surface area is increased leading to a higher number of surface-accessible channels. We believe that this facilitates the release of peptide digestion products back into solution.[21]

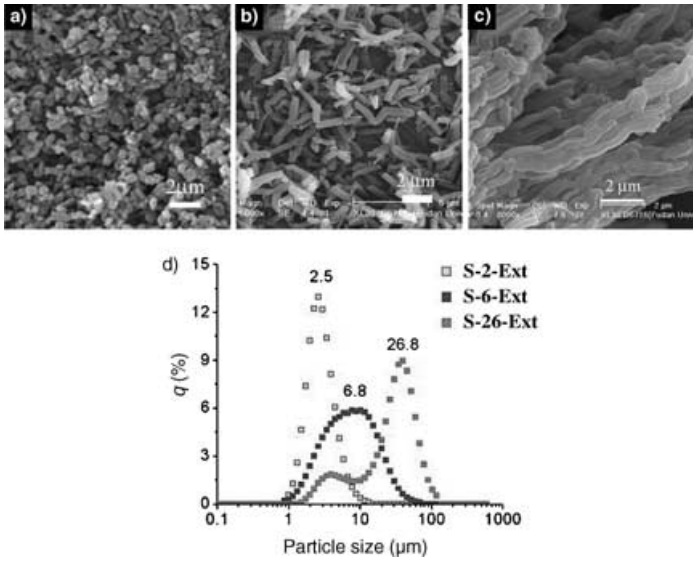

Figure 4. SEM images of mesoporous silica SBA-15 samples: a) S-2-Ext, b) S-6-Ext, and c) S-26-Ext. d) Particle size distribution measured by a laser-light-scattering spectrometer (Horiba LA-300).

Conclusion

It has been previously discovered that molecular crowding in biological cells can induce order-of-magnitude enhancements in catalysis reaction rates for large molecules and pro-

teins, compared with in-solution reactions.[26,27] In our case, the remarkable enhancement of proteolytic efficiency within molecular-scale nanoreactors based on mesoporous silica suggests a new strategy for designing biomimetic nanosystems. However, it is likely that additional research is necessary (e.g., kinetic studies) to truly understand why mesoporous-silica-based nanoreactors work so efficiently. The complexity of our reaction system and relative crudeness of our kinetic experiments limited our attempt to establish a reliable kinetic model. However, we have uncovered several new factors (compared with classic kinetic factors: enzyme concentration, substrate concentration, pH, ionic strength, and temperature)[28] affecting the rate of the proteolytic reaction and final performance of protein identification, such as surface characteristics and macrostructure. The detailed analysis of these factors should lead to a deep understanding of the nature of proteolysis in silica-based nanoreactors, and is the focus of our continued work.

Experimental Section

Synthesis of mesoporous silica: Mesoporous silica SBA-15 materials were prepared by using the block copolymer $EO_{20}PO_{70}EO_{20}$ (Pluronic P123) as the structure-directing agent according to the established procedure (ref. [19]). For a typical synthesis, P123 (2.0 g, 0.4 mmol) and potassium chloride (6.7 g, 90 mmol) were dissolved in HCl (2.0 M, 60 mL) at 40 °C. To this solution, tetraethyl orthosilicate (TEOS, 4.2 g, 20 mmol) was added under vigorous stirring. After stirring for 24 h, the mixture was transferred into an autoclave and heated at 100 °C for another 24 h. The solid products were collected by filtration, washed with water, and dried at room temperature in air. The final product was assigned the name S-2-X, in which X designates the postsynthesis treatment parameters.

The SBA-15 samples (S-2-, S-6-, and S-26-) with different particle sizes of 2, 6, and 26 μm, respectively (−2, −6, and −26, respectively, represent the particle size measured by a laser-light-scattering spectrometer) were synthesized according to the above procedure with different KCl concentrations of 1.5, 0.75, and 0.0 M, respectively.

The as-synthesized SBA-15 sample (S-2-) was calcined by slowly increasing the temperature from room temperature to 500 °C at a rate of 8 °C min^{-1} and heating at 500 °C in air for 6 h. The final product was assigned the name S-2-Cal. The as-made SBA-15 samples (S-2-, S-6-, S-26-) were also exposed to ethanol extraction (Ext) and microwave (MW) digestion to remove the block copolymer species. These samples were assigned the names S-2-Ext, S-6-Ext, S-26-Ext, and S-2-MW.

The S-2-SH sample was mesoporous silica SBA-15 (S-2-) functionalized with thiol (2 wt %) and was prepared by a one-step method according to the above procedure, except that a mixture of TEOS (98 wt %) and 3-mercaptopropyltrimethoxysilane (MPTS: 2 wt %) was used as the silica source instead of pure TEOS. The block copolymer templates were removed by ethanol extraction.

The mesostructures of the samples were characterized by analysis of XRD patterns and TEM images (see Supporting Information SI-1, SI-2).

Process of proteolysis: For the proteolysis in the MPS samples, the MPS solid (2.5 mg) was first placed in a 2.0 mL vial and incubated with protein solution (0.4 mL) containing myoglobin (50 μg) in sodium phosphate buffer (pH 6.5). The mixture was then agitated on an Eppendorf Thermomixer at 8 °C for 10 min for complete protein adsorption in the MPS. The MPS beads were pelleted by centrifugation and washed twice with millipore water. Then, the trypsin solution (10 μL, 2.5 μg $μL^{-1}$ in 0.1 % trifluoroacetic acid (TFA)) was added to the vial containing the MPS beads and the same buffer and then placed under vortex conditions for 10 min. The beads containing the protein/trypsin/SBA-15 composite were then quickly washed and pelleted, as described previously, before removing the supernatant. There was no absorption at $\lambda = 280$ nm for any supernatant or wash-off, as analyzed by means of UV/Vis spectrometry. Finally, the mesoporous silica beads were incubated with aqueous NH_4HCO_3 solution (0.2 mL, 25 mM) and agitated for 10 min at 37 °C. The digestion products in the supernatant were obtained by centrifugation. For in-solution proteolysis without MPS beads, myoglobin (50 μg, previously denatured at 100 °C for 5 min) in NH_4HCO_3 buffer was mixed with the trypsin solution (10 μL, 2.5 μg $μL^{-1}$) to reach a total volume of 0.25 mL, and the mixture was incubated at 37 °C for 6 or 12 h. The protease/substrate ratio was fixed as 1:2 in both cases, rather than entrapping a large amount of enzyme to digest trace proteins, as is often carried out for sol–gels and silica supports.

The digestion products were diluted fivefold with 0.1 wt % TFA in water and mixed with a matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile, 0.1 % TFA) in a 1:1 ratio. The mixture (0.5 μL) was spotted on the MALDI sample plate. All tryptic digests were analyzed by PMF on an Applied Biosystems 4700 proteomics Analyzer. Each peptide product released from different trypsin/SBA-15 samples was loaded onto 5 spots on a MALDI target. The PMF spectrum of each spot was obtained by accumulation of 2000 laser shots. The method was optimized to achieve spot-to-spot reproducibility. The acquired PMF data were then submitted to the Swissprot database for protein identification using GPS Explorer Software with a mass tolerance of 80 ppm, a S/N threshold of 70, and a maximum miss cleavage of 1. The identification results for proteolysis within different SBA-15 materials is represented by the average of the five spectra of each peptide sample.

Acknowledgements

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