RESEARCH ARTICLE

Amino-functionalized macroporous silica for efficient tryptic digestion in acidic solutions

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Amino-functionalized macroporous silica foam (NH_2-MOSF) has been developed as a host reactor to realize highly efficient proteolysis in acidic solutions where normal tryptic reactions cannot occur. The digestion protocol consists simply of adding the functionalized NH_2-MOSF into the protein and trypsin solutions without altering the bulk pH or preloading the enzymes on the materials. With this protocol, digestion of sample fractions from LC can be efficiently realized in the acidic solutions directly. Digestion of a protein fraction extracted from rat liver tissue after LC separation was performed to illustrate this principle, where 103 proteins were successfully identified at pH 3 after 1.5 h of tryptic digestion.

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1 Introduction

Modern proteome research at the levels of organelles, cells, tissues, organs, and organisms relies on a wide variety of techniques to detect proteins and elucidate their functions either in a healthy or disease state [1, 2]. Among all these techniques, MS-based analyses are extensively used both in laboratory-based and clinical experiments for the discovery of diagnostic, prognostic, and therapeutic protein biomarkers [3, 4]. To date, comprehensive proteome information can be obtained by analyzing fragmental peptides from protein digests, making proteolysis an essential procedure in many MS-based proteomics methods [5, 6]. Among various proteases, trypsin is most widely used, which cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine. However, trypsin shows the highest biological activity at pH 8 and 37°C, and the tryptic digestions take place rather slowly in neutral solutions and hardly in acidic conditions. This is a limitation for high-throughput analyses that involve coupling proteolysis with separation techniques.

LC-MS/MS is universally used to perform high-throughput surveys of proteomes from a biological sample through MS/MS sequencing [7–9]. Basically, the complex biological samples are separated by LC, where fractions are collected, digested, and detected by LC-MS/MS. Considering the optimum conditions for proteolysis, the acidic LC fractions have to be lyophilized and redissolved in a weak basic buffer (pH 8), which requires several steps in the workflow. To circumvent the multistep pretreatment for adjusting pH, the development of novel methods capable of retaining enzymatic activity in different pH conditions especially in acids and increasing proteolytic efficiency are vital for large-scale proteome and peptidome research [5, 6]. In order to retain enzymatic activity and accelerate enzymatic

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Abbreviations: APTS, aminopropyltriethoxysilane; **SEM**, scanning electron microscopy; **TEM**, transmission electron micrographs

reactions, various microfluidic reactors and nanoreactors have been developed in recent proteomic protocols [10–21]. Benefiting from the ordered pore structure, high surface area and high pore volumes, porous materials have been demonstrated to be good candidates for nanoreactors [16–21]. Additionally, with the surface modification of silica materials, selective enrichment and isolation of posttranslational modified peptides or proteins can be obtained [22–24].

In this study, we have developed a nanoreactor for tryptic digestion of proteins in acidic solutions, based on the newly designed amino-functionalized macroporous silica foams (MOSFs) [25], ~100 nm in pore diameters, denoted NH_2-MOSF. NH_2-MOSF was added into the fractions of LC systems to carry out digestions directly following separation, even when the bulk solutions are acidic. This NH_2-MOSF-based protocol was applied to the digestion of biological samples separated by RPLC. Successful digestion of one protein fraction from rat liver was achieved in a solution at pH 3. The present approach greatly simplifies the digestion step during analysis of proteomes.

2 Materials and methods

2.1 Chemicals

Triblock copolymer $EO_{20}PO_{70}EO_{20}$ (denoted P123, where EO is poly(ethylene oxide) and PO is poly(propylene oxide)), potassium chloride (KCl, 99.5%, AnalaR, Australia), fuming hydrochloric acid (37%, Lab-Scan, Analytical Science, Thailand), ethanol (99.5%, Asia Pacific Specialty, Australia), ammonium bicarbonate, dry toluene, 3-aminopropyltriethoxysilane (APTS), and tetramethylorthosilicate (99%) were purchased from Aldrich. Trypsin (from bovine pancreas) and myoglobin (horse heart) were obtained from Sigma. 2,5-DHB (99.9%), CHCA (99%), ACN (99.9%), and TFA (99.8%) were purchased from Merck. The normal rat liver cytoplasm sample was obtained from the Liver Cancer Institute of Zhongshan Hospital, Fudan University. Deionized water (18.4 MΩ·cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Synthesis and characterization of macroporous silica and the amino-functionalized materials

The macroporous silica (MOSF) used in this study were prepared according to the method previously reported [25]. The synthesis of MOSF was conducted at 35°C in pH 5.0 NaAc-HAc buffer solution, using tetramethylorthosilicate as a silica source and P123 as a template agent. For standard modification [26], APTS was used as the coupling agent. MOSF materials were firstly dried and degassed at 110°C, and then dispersed in dry toluene (0.1 g MOSF in 30 g toluene). An excess of APTS (3 mL) was added under stirring and the mixture was stirred and refluxed for 24 h at 110°C. The resulting solid was filtered and washed sequentially by toluene, dichloromethane, and ethanol three times. The final NH_2-MOSF products were obtained after drying at 70°C overnight. A zeta-potential meter (Malvern Zetasizer Nano) was used to measure the zeta potentials of the materials in an ammonium bicarbonate buffer solution (pH ~ 8) at 25°C. The infrared spectra were obtained with a FT-IR360 (Nicolet). Nitrogen sorption isotherms were obtained using Quantachrome's Quadrasorb SI analyzer at 77 K. Prior to measurement, the samples were degassed at 120°C for at least 8 h under vacuum. Scanning electron microscopy (SEM) images were recorded on a JEOL Philips XL30 microscope operating at 20 kV. The samples were coated with gold before observation. Transmission electron microscopy (TEM) images were obtained on a JEOL 2011 microscope operated at 200 kV.

2.3 Digestion of standard proteins in solutions of different pH values

In-solution digestion was performed according to the standard procedures but under different pH conditions. Proteins were dissolved (20 ng/μL) in various solutions (pH ~2.5–8, different concentrations of TFA were applied to achieve solutions with pH ~ 2.5–6 and ammonium bicarbonate buffer (25 mM) was used to obtain solution with pH 8) and incubated at 37°C with trypsin. Considering that the trypsin used in this work is not sequencing grade, an enzyme/substrate ratio was kept at 1:30 w/w for digestion as normally used cases. For NH_2-MOSF-mediated digestion, the microparticles were directly dispersed in various solutions (pH ~2.5–8, different concentrations of TFA were applied to achieve solutions with pH ~ 2.5–6 and ammonium bicarbonate buffer (25 mM) was used to obtain solution with pH 8) with a final concentration of 0.4 mg/mL before addition of the enzyme (except that 2 mg/mL NH_2-MOSF was used when the pH value of the solution is 2.5–3). The digestion products were analyzed after 10 min of proteolysis by an Applied Biosystems 5800 proteomics analyzer (MALDI-TOF MS), using CHCA (99%) as matrix. All mass spectra were obtained in the positive ion reflection mode. Mass spectrometric data analysis was performed with the GPS Explorer software from Applied Biosystems with Mascot as a search engine and NCBInr as a database with one missed cleavage site accepted and peptide mass tolerance of 80 ppm.

2.4 Analysis of biological samples

The samples were prepared according to published literature [19]. Rat liver tissues were obtained from the Liver Cancer Institute in Zhongshan Hospital, Fudan University. The samples were dissolved in buffer containing a urea (7 M) and sulfourea (2 M) mixture with protease inhibitors and phosphatase inhibitors (1 mM PMSF, 0.2 mM Na_2VO_3, and

1 mM NaF). The tissue samples were subsequently homogenized (Handheld rotor–stator homogenizer, TissueRuptor, QIAGEN) in an ice bath and vortexed for 30 min. The suspensions were centrifuged at 18 000 × *g* for 1 h at 4°C, after which the supernatants were collected. The protein concentrations of the extracted samples were determined according to the modified Bradford method [27]. The extracted proteins were reduced with DTT (20 mM) at 37°C for 30 min and then alkylated with iodoacetamide (25 mM) for another 30 min at room temperature in darkness. The extracted and denatured sample from rat liver was taken for RPLC separation on a Shimadzu LC-20AD capillary pumping system by using a column of Agilent ZORBAX SB-C8 (4.6 × 25 mm, 5 μm, 300 Å, C8, Hypersil, EliteHPLC, China) for 90 min at 25°C. A total of 0.05% v/v TFA in water was used as mobile phase A and 0.05% v/v TFA in ACN was used as mobile phase B.

A fraction of the separated rat liver samples (retention time from 35 to 38 min) in the chromatographic solution was divided into three equal parts. Two fractions, with a final protein concentration of 150 μg/mL at pH 3, were directly used for digestion with or without NH_2-MOSF at an enzyme/substrate ratio of 1:30 w/w for 1.5 h. The third part, initially lyophilized and redissolved in the ammonium bicarbonate buffer (25 mM, pH ~8) with a final protein concentration of 150 μg/mL, was then digested by trypsin under the optimized conditions at 37°C for 12 h with an enzyme/substrate ratio of 1:30 w/w.

The digested peptides were analyzed by RPLC/ESI-MS/MS using the Thermo-Fisher LTQ Orbitrap mass spectrometer. The mass spectra were analyzed using the Bioworks software (Version 3.3.1, Thermo Scientific) based on the SEQUEST algorithm. The database used was the rat UniProtKB/Swiss-Prot database. To reduce false-positive identification ratios, a decoy database containing the reverse sequences of the proteins in the rat UniProtKB/Swiss-Prot database was appended. The searching parameters were setup as follows: partial trypsin (KR) cleavage with two missed cleavages; fixed carbamidomethyl modification of 57.02 Da for cysteine; oxidation of methionine as a variable modification; the peptide mass tolerance of 50 ppm; and the fragment ion tolerance of 1.0 Da. The peptide identification criteria were setup based on ΔCN (≥0.1) and Xcorr (double charges, ≥2.3; triple charges, ≥2.8) with false discovery rate less than 1.0%. Proteins that identified at least from two effective peptides were reserved as validated identifications.

3 Results and discussion

3.1 Characterization of NH_2-functionalized macroporous silica

NH_2-MOSF has been successfully prepared by modifying the surfaces of macroporous silica with amino groups. Fourier transform infrared spectroscopy was utilized to characterize the surface modification, shown in Fig. 1A. The dominant

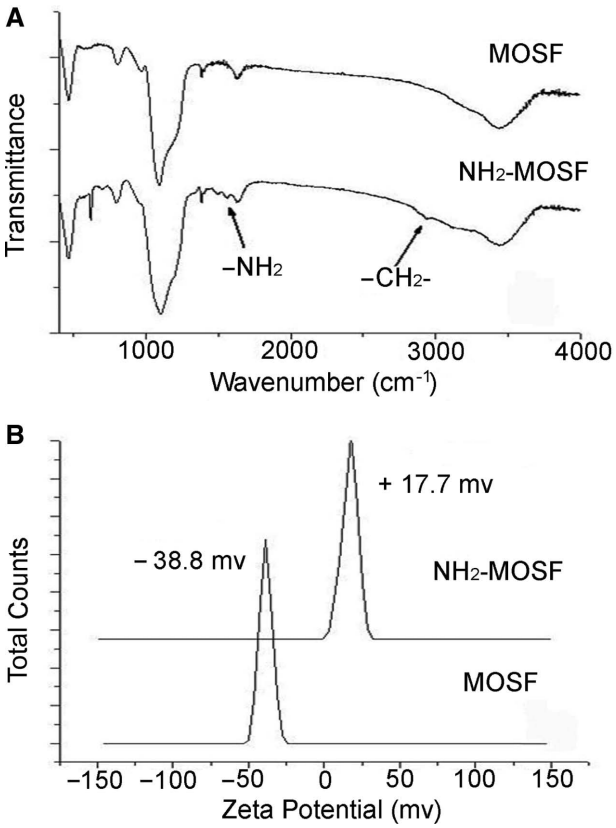

Figure 1. (A) Fourier transform infrared spectra and (B) zeta potential distributions of MOSF and NH_2-MOSF.

multipeaks at 1000–1100 cm^{-1} are typically assigned to the Si-O bond and Si-OH groups in the bare MOSF material. In the case of NH_2-MOSF, an obvious additional single peak rises around 1580 cm^{-1}. This peak may be attributed to grafted -NH_2 groups demonstrating the successful modification of MOSF with amino groups. The -CH_2- groups of the silica coupling agent (APTS) result in the double peaks around 2920 cm^{-1} [28]. Zeta potentials of MOSF and NH_2-MOSF were also examined as shown in Supporting Information Table 1. Figure 1B displays the zeta potential distributions of the two materials. MOSF has a zeta potential of −39 mV due to the presence of silanol groups in abundance on the surface [29]. In comparison, for NH_2-MOSF a new peak appears at +18 mV while the former recognized peak is absent, indicating modification of the surfaces by amino groups.

SEM and TEM were carried out to further investigate the morphologies and structures of the materials before/after functionalization (Fig. 2). Compared to the bare siliceous foams as shown in Supporting Information Fig. 1, the arrays of macropores (~100 nm in diameter) are well retained in NH_2-MOSF. The TEM images also reveal the foam structure of the NH_2-MOSF with hexagonal column assemblies, Fig. 2B. From both SEM and TEM images, no large aggregate was observed for NH_2-MOSF, suggesting the well-dispersed state of the amino species. In-depth characterization of the

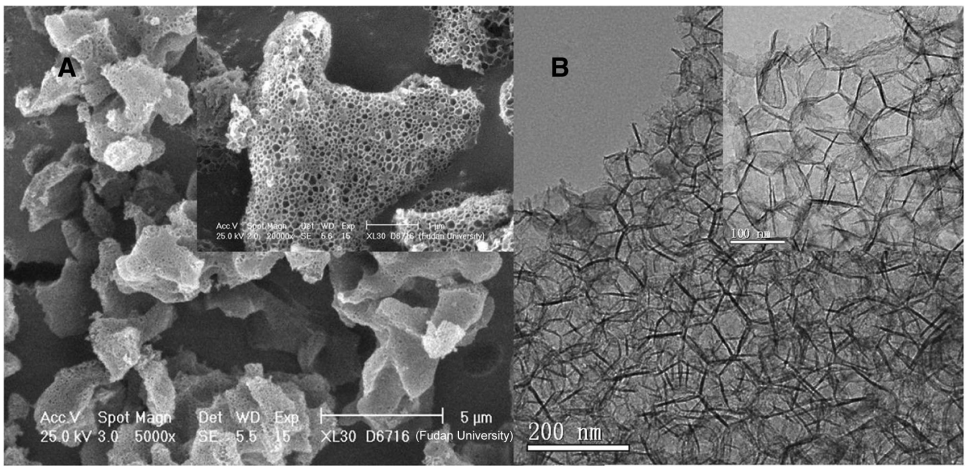

Figure 2. (A) SEM and (B) TEM images of NH_2-MOSF, the inset, are partially zoomed images of the material.

structural properties of MOSF and NH_2-MOSF was achieved by nitrogen sorption analysis and summarized in Supporting Information Table 1. As shown in Supporting Information Fig. 2, the MOSF material has a surface area of 344 m^2/g and a pore volume of 1.31 cm^3/g, while the NH_2-MOSF material still possesses a surface area of 300 m^2/g and a pore volume of 0.79 cm^3/g after functionalization. The incorporation of amino groups onto the macroporous structure contributes to the decrease in surface area and pore volume of NH_2-MOSF. On consideration of each characterization technique, it can be concluded that the macroporous structure is well preserved and the amino groups are homogeneously distributed in the foams.

3.2 Tryptic digestion assisted by NH_2-MOSF in acidic solutions

To demonstrate the feasibility of using NH_2-MOSF to assist tryptic digestion under acidic conditions, myoglobin (20 ng/µL) was selected as a model substrate. The proteolysis was performed in a series of solutions with pH values ranging from 2.5 to 8 by directly adding the macroporous NH_2-MOSF and enzyme into the protein solutions, while MOSF assistance and in-solution digestion were carried out for comparison. The resulting products were analyzed by MALDI-TOF MS. Proteolysis efficiency was evaluated based on the results of generated peptides using PMF method. As shown in Figs. 3 and 4, the NH_2-MOSF-assisted tryptic digestions at different bulk pHs are effective. For example, nine peptide peaks from myoglobin were observed with a high S/N when the proteolysis was performed at pH ~3 in the presence of NH_2-MOSF for 10 min with an enzyme/substrate ratio of 1:30 w/w, Fig. 3A. To show a better application of this protocol in such an acid condition, myoglobin digestion was also carried out at an enzyme/substrate ratio of 1:3 w/w. There were 19 highly resolved peptides (S/N > 10) observed on the mass spectrum with the amino acid sequence coverage of 95% and the molecular weight search (MOWSE) score of 214, Fig. 3B. In comparison, when the digestion was performed at pH ~3 with either MOSF assistance or in bulk solution, no peptide was identified even after overnight reaction, with an enzyme/substrate ratio of 1:30 or 1:3 w/w, Fig. 3C, Supporting Information Fig. 3A. Even in a very acidic system at pH ~2.5, the digestion mediated by NH_2-MOSF could still be conducted in 10 min as shown in Fig. 3D and E. There were 5 and 13 peptides (S/N > 10) observed on the mass spectrum of myoglobin digest with the enzyme/substrate ratio of 1:30 w/w and 1:3 w/w, respectively. On the contrary, both conventional in-solution digestion and MOSF-assisted digestion at such pH conditions remained infeasible as shown in the Fig. 3F and Supporting Information Fig. 3B. These results clearly indicate that the good performance of the tryptic digestions in acidic solutions is mainly achieved in the presence of NH_2-MOSF.

Additionally, 15, 16, and 17 peptides were identified by MS from myoglobin digests after 10 min of proteolysis assisted by NH_2-MOSF at pH ~4, 5, and 6, respectively (Fig. 4A–C). In contrast, in-solution proteolysis without NH_2-MOSF yielded only three, four, and six peptides (Supporting Information Fig. 4A, C, and E) identified by MS after 10 min of reaction and 11, 11, and 15 peptides observed after overnight reaction at pH ~4, 5, and 6, respectively (Supporting Information Fig. 4B, D, and F). At pH ~8, the optimum pH for tryptic digestion, the resulting identification of myoglobin after a 10 min proteolysis assisted by NH_2-MOSF was better than that for a 10 min in-solution digestion without NH_2-MOSF and comparable to that for a 12 h in-solution proteolysis, Fig. 4D and Supporting Information Fig. 4G and H. There were 17 and 18 peptides peaks from myoglobin observed with sequence coverage of 95 and 96% after NH_2-MOSF assistance and overnight in-solution reactions, respectively.

3.3 Analysis of biological samples

In order to examine the practical feasibility of the proposed system, the macroporous NH_2-MOSF has been used to

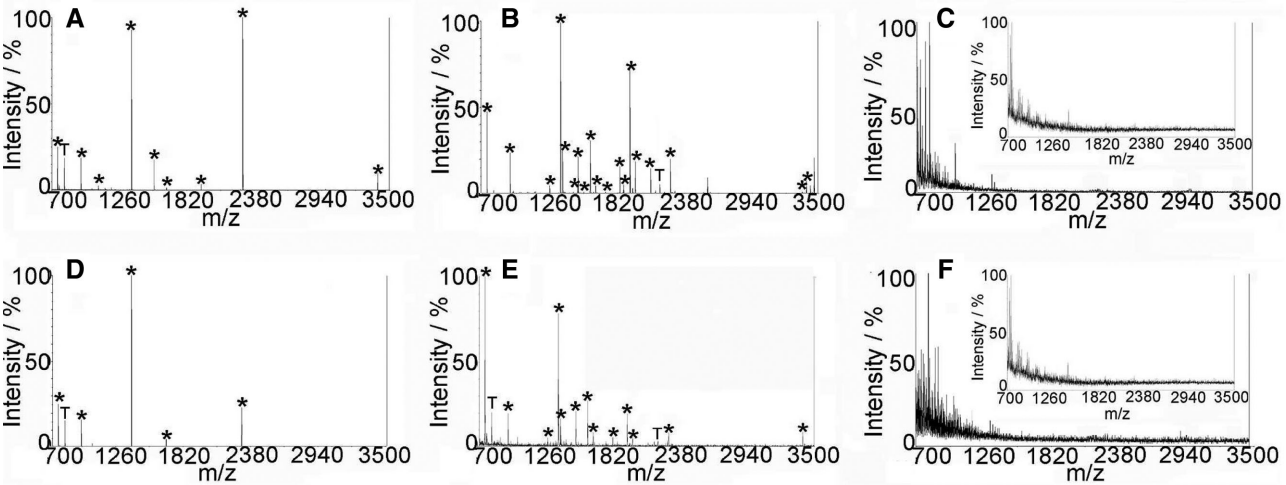

Figure 3. PMF spectra of the digested products after 10 min of proteolysis of myoglobin with the assistance of NH_2-MOSF at the enzyme/substrate ratio of (A, D) 1:30 w/w and (B, E) 1:3 w/w at (A, B) pH 3 and (D, E) pH 2.5, respectively. PMF spectra of the digested products after 10 min of proteolysis of myoglobin with the assistance of MOSF or without assistance (inset) at (E) pH 3 and (F) pH 2.5 with an enzyme/substrate ratio of 1:3 w/w. The identified peptides are marked by the asterisk and tryptic autolysis peaks are labeled with T.

assist the tryptic digestion of a complex biological sample, that is proteins extracted from the cytoplasm of rat liver tissue. The sample was separated by RPLC, and two fractions of proteins were digested by trypsin with and without the assistance of NH_2-MOSF. The resulting digests were subjected to an RPLC-ESI-MS/MS system measured by the ThermoFisher LTQ Orbitrap mass spectrometer. The mass spectra were analyzed by Bioworks software (Version 3.3.1, Thermo Scientific) based on the SEQUEST algorithm. As shown in Fig. 5 and Supporting Information Table 2, 103 proteins were identified based on at least two significant peptides matched after 1.5 h of digestion in the presence of NH_2-MOSF in the chromatographic solution at pH 3. The molecular weight values of the proteins identified with the NH_2-MOSF strategy were mainly between 10 and 100 kDa (~96%, 99 of 103), and the p*I* values ranged from 4 to 12. To illustrate the RPLC-ESI-MS/MS process, the chromatography of digestion products, a mass spectrum of peptides from a fraction eluted at retention time (RT) = 39.51 min, and the MS/MS spectrum of a parent peak with m/z 806.91 are shown in Fig. 5A–C, respectively. In contrast, only six proteins were identified when proteolysis was performed for 1.5 h under the same conditions as in Fig. 5 but without NH_2-MOSF, Supporting Information Table 3. However, by lyophilizing and redissolving the same fraction of proteins in ammonium bicarbonate buffer solution (pH ~8), 107 proteins were identified after 12 h of proteolysis in the absence of NH_2-MOSF at 37°C. More than 80% of these proteins were overlapped by those identified by the NH_2-MOSF strategy, Supporting Information Table 4. These results clearly show that tryptic digestion

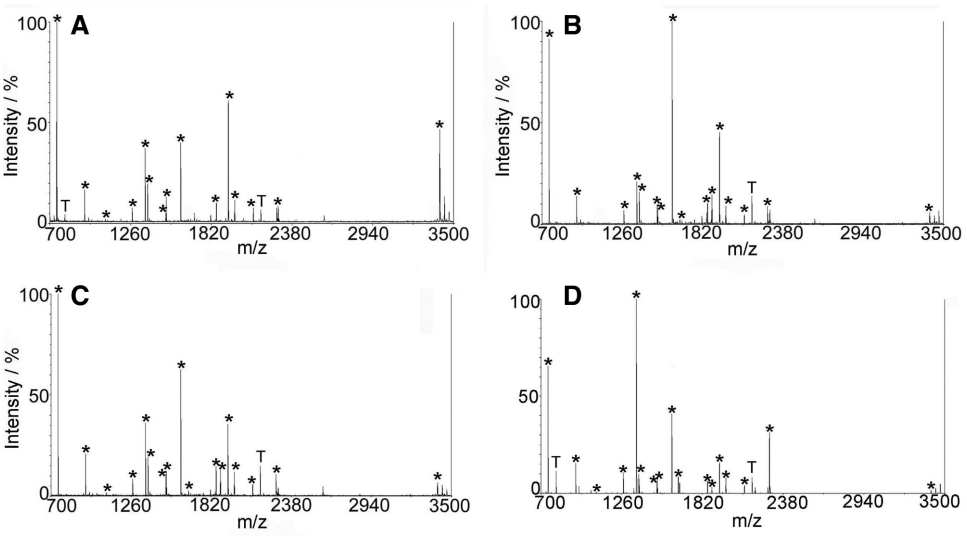

Figure 4. PMF spectra of the digested products after 10 min of proteolysis with the assistance of NH_2-MOSF at (A) pH 4, (B) pH 5, (C) pH 6, and (D) pH 8, respectively. The identified peptides are marked by the asterisk and tryptic autolysis peaks are labeled with T.

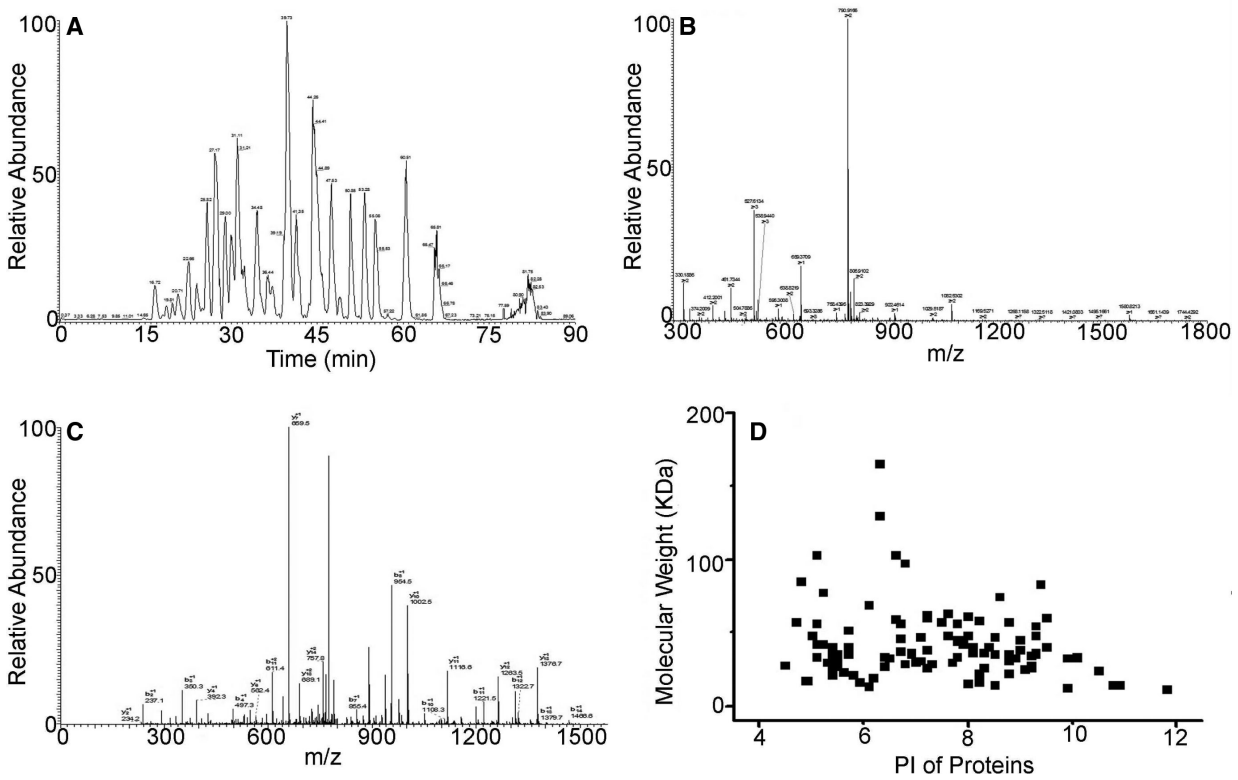

Figure 5. (A) Base peak chromatography of the digest of rat liver tissue sample obtained with the NH_2-MOSF-assisted digestion strategy; (B) the mass spectrum of peptides from a fraction eluted at retention time (RT) = 39.51 min according to Fig. 5A; (C) the MS/MS spectrum of a parent peak at $m/z = 806.91$ according to Fig. 5B (one peptide from sp|Q4KM49|SYYC_RAT Tyrosyl-tRNA synthetase with the sequence of VHLM*NPM*VPGLTGSK); (D) plot of p*I* values of the proteins identified with the NH_2-MOSF strategy as a function of molecular weight values.

in acidic solutions with the assistance of NH_2-MOSF is very efficient and comparable to conventional digestion in optimum buffer conditions, while the incubation time is reduced and operation procedures are simplified.

4 Conclusion

In summary, we have developed a functional NH_2-MOSF-mediated digestion protocol applicable to a wide range of pH systems, even in the very acidic solutions. NH_2-MOSF-assisted proteolysis has been applied to the analysis of protein fractions obtained by LC separation of biological samples from human or rat liver at acid condition. The NH_2-MOSF-assisted protocol is extremely simple to use, and circumvents the time-consuming aspects of protein digestion before MS analysis. The large-scale analysis of proteomes and peptidomes stands to benefit substantially from incorporation of such a protocol.

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The authors have declared no conflict of interest.

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