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Immobilized metal-ion chelating capillary microreactor for peptide mapping analysis of proteins by matrix assisted laser desorption/ionization-time of flight-mass spectrometry

Peptide mass mapping analysis, utilizing a regenerable enzyme microreactor with metal-ion chelated adsorption of enzyme, combined with matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) was developed. Different procedures from the conventional approaches were adopted to immobilize the chelator onto the silica supports, that is, the metal chelating agent of iminodiacetic acid (IDA) was reacted with glycidoxypropyltrimethoxysilane (GLYMO) before its immobilization onto the inner wall of the fused-silica capillary pretreated with NH_4HF_2 . The metal ion of copper and subsequently enzyme was specifically adsorbed onto the surface to form the immobilized enzyme capillary microreactor, which was combined with MALDI-TOF-MS to apply for the mass mapping analysis of nL amounts of protein samples. The results revealed that the peptide mapping could routinely be generated from 0.5 pmol protein sample in 15 min at 50°C, even 20 fmol cytochrome *c* could be well digested and detected.

Keywords: Capillary microreactor / Immobilized enzyme / Matrix assisted laser desorption/ionization-time of flight-mass spectrometry / Metal-ion chelated adsorption / Miniaturization / Peptide mapping analysis
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1 Introduction

MALDI-TOF-MS for peptide mapping analysis has emerged as a powerful tool for identification and characterization of proteins [1, 2]. The technique involves enzymatic cleavage of the intact proteins by proteases, followed by mass determination of the generated peptides, and subsequent identification of the protein by matching the set of measured peptide masses with the calculated masses of the theoretical digestion, based on protein or cDNA sequence database. Enzymatic proteolysis requires times ranging from several minutes to overnight, depending on the reaction conditions, and the properties of the enzyme. For the digestion of proteins with low amounts of free enzyme, several hours or more is required, and enzyme autoproteolysis would impair signal interpretation, especially for low amounts of analytes.

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Abbreviations: **BAEE**, *N*_ε-benzoyl-L-arginine ethylester hydrochloride; **CHCA**, α-cyano-4-hydroxycinnamic acid; **GLYMO**, glycidoxypropyltrimethoxysilane; **IDA**, iminodiacetic acid; **TPCK**, L-1-(tosylamido)-2-phenyl-ethyl chloromethylketone; **Z-arg**, *N*_ε-benzoyloxycarbonyl-L-arginine

If a high concentration of free enzyme is used, the digestion proceeds quicker but autolysis products become more abundant [3]. On the contrary, the immobilized enzyme has been developed for characterization of proteins with benefits from the reusability and stability of enzyme, the higher digestion efficiency of analyte proteins, and no autolysis products [4–10].

Nelson [4, 5] *et al.* immobilized trypsin onto the MALDI-TOF-MS probe tips, and then enzymatically active probe tips were used for the tryptic mapping of chicken egg lysozyme. The digestion is found to be rapid and convenient. But the bioreactive probes are critical to reuse because of the loss of the enzymatic activity due to the rigorous washing tip process or the occupation of the active site of the proteases by the excessive matrix. Most reported microreactors are based on the format of microcolumns, that is, the enzyme is immobilized on support media [6, 7], and then packed into the microcolumn, or onto the surface of silicon-based microfluidic chips [8, 9] or on the fused-silica capillary directly by the covalent bonding method [10–12]. Compared with digestion of protein by the immobilized enzyme on probe tips, less analyte is needed for the mass mapping and the enzyme is reusable. Licklider *et al.* [10] reported the use of a capillary microreactor for protein digestion, followed by on-line ESI and off-line MALDI analysis. The microreactor is

vibrated with an electric engraving tool to enhance reaction rates and reduce the digestion time to about 30 min [11]. Similarly, a thermal denaturation procedure can effectively accelerate the digestion of a protein by a capillary micro-reactor; by this method 9 fmol horse heart cytochrome *c* could be well characterized within 5 min [12].

The enzyme can be reused by its irreversible immobilization enzyme onto the supports by covalent bonding, but the regeneration of the prepared reactor is limited when the activity of the bound enzyme is damaged or destroyed [13]. Metal-ion chelated immobilization of the enzyme is quite different from conventional approaches, and the enzyme is bound to the support media based on the Lewis acid-base interaction through the divalent cation chelators such as iminodiacetic acid (IDA), which is chemically bound onto the matrix. Thus, the enzymes could be removed with EDTA elution for regeneration of the support media. The conventional procedures for the immobilization of chelator on silica matrix would be (i) the derivatization of silica with the silane agent, and (ii) the chemical linkage of the metal chelator to the silane-modified silica material. Unfortunately, this immobilization approach results in low ligand densities because the secondary reaction has to be performed below pH 8 for protecting the siloxane bonds between silane and silica matrix, yet the optimum pH at 10–12 [14].

In this study, we prepared such a regenerable capillary microreactor by synthesis of the chelator silane reagent and then reaction onto the capillary wall following the immobilization of the metal ion and enzyme, respectively. The capillary microreactor was successfully used for the rapid digestion of nL amounts of protein sample for peptide mapping analysis with MALDI-TOF-MS.

2 Materials and methods

2.1 Materials

3-Glycidoxypropyltrimethoxysilane (3-GLYMO), IDA, horse heart cytochrome *c*, α -cyano-4-hydroxycinnamic acid (CHCA), and L-1-(tosylamido)-2-phenyl-ethyl chloromethylketone (TPCK)-treated trypsin (EC 2.4.21.4, from bovine pancreas) were purchased from Sigma Chemical (St. Louis, MO, USA). HPLC-grade trifluoroacetic acid (TFA) was obtained from Merck (Schuchardt, Germany). *N*_ε-Benzoyl-L-arginine ethylester hydrochloride (BAEE), *N*_ε-benzoyl-L-arginine and *N*_ε-benzoyloxycarbonyl-L-arginine (Z-arg) were from Fluka (Buchs, Switzerland). Analytical reagent grade ammonium hydrogen difluoride (NH₄HF₂) was bought from Dandong 4th Chemical Plant (Liaoning, China). All samples were analyzed by MALDI-TOF-MS using CHCA as the matrix. CHCA was recrystallized from hot methanol and stored in the dark. Underiva-

tized fused-silica capillaries (50 μm ID, 365 μm OD) were obtained from Yongnian Optic Fiber Plant (Hebei, China). All the other chemicals were of analytical reagent grade. The deionized water used in all procedures was purified with Milli-Q water system (Millipore, Milford, MA, USA). Proteins were dissolved in 20 mM ammonium bicarbonate solution at a proper concentration. Aliquots from protein solutions were thermally denatured by incubating at 90°C for 20 min in airtight microcentrifuge tubes. Following incubation, the proteins were transferred to an ice-water bath to quench the denaturation process. 0.02 M Na₂HPO₄ and NaH₂PO₄ solution were mixed at ratio of 18:1, and adjusted to pH 8.0 with 0.02 M NaH₂PO₄ solution.

2.2 Synthesis of GLYMO-IDA-silane

IDA (4.25 g) was dissolved in 50 mL of deionized water, and the obtained solution was adjusted to pH 11.0 with 10 M NaOH. Then the solution was transferred into a flask bottle placed in the ice-bath at 0°C, and 1.4 mL of GLYMO was slowly added under stirring. The mixed solution was heated to 65°C for reaction 6 h with stirring, subsequently placed into an ice-bath for 5 min to decrease the temperature to 0°C again, and 1.6 mL of GLYMO was added and mixed. Then the solution was raised to 65°C for reaction of another 6 h under stirring. Similarly, another 1.7 mL of GLYMO was added to the solution, which was further reacted at 65°C overnight under stirring. Finally, the prepared GLYMO-IDA-silane solution was adjusted to pH 6 with concentrated HCl and stored in a refrigerator by sealing the vessel for usage.

2.3 Immobilization of trypsin on the surface of the capillary wall

100 cm of bare fused-silica capillary was first cleaned with concentrated HNO₃ at 80°C for 30 min. The capillary was filled with saturated NH₄HF₂ solution and then sealed at both ends for chemical etching at 200°C overnight. The saturated NH₄HF₂ solution was obtained from the supernatant solution by dissolving 1.5 g NH₄HF₂ in 2 mL water. After that, the sealed ends of the capillary were cut off and the capillary was activated with concentrated HCl for 1 h. The prepared GLYMO-IDA-silane solution (pH 6) was injected into the capillary for reaction at 95°C for 2 h. 50 mM Cu²⁺ solution was circulated through the capillary and allowed to react at room temperature for 1 h. Thereafter, a 0.02 M phosphate solution (pH 8.00) containing TPCK-treated trypsin (0.5 mg/mL) was circulated through the activated capillary for 1 h. Finally, the unbound trypsin was rinsed from the capillary with ~10 column volumes of 0.02 M phosphate buffer (pH 8.00) and stored in the refrigerator at 4°C until use.

2.4 Characterization of the enzyme microreactor

The activity of the immobilized enzyme was determined with MS [15] using BAEE as substrate and Z-arg as internal standard. Briefly, a series of substrate BAEE solutions ranging from 5 to 50 μM by keeping concentration of Z-arg at 20 μM were injected into capillary tubes for enzymatic reaction at 40°C for 2 min, then the reaction solution was deposited onto the probe tip for MS analysis with CHCA as matrix.

2.5 Sample preparation and analysis

Sample was prepared with the sandwich method [15] with CHCA as matrix. For the matrix solution CHCA was dissolved in (I) acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) (70:30 v/v) and (II) acetone/water (99:1 v/v), respectively, by keeping CHCA at 20 $\mu\text{g}/\mu\text{L}$. The procedures for the sample preparation were as follows. First, a thin layer of matrix crystals was deposited on the probe tip by applying 0.5 μL CHCA solution (II) and allowing the droplet to spread and dry quickly. Subsequently, droplets of 1 μL of aqueous 0.1% TFA solution, sample solution from the capillary with syringe, and 0.5 μL of CHCA solution (I) were deposited onto the surface of the first layer of matrix and then the mixture was allowed to dry at ambient temperature. After that, the layer of sample crystal was washed with droplets of 0.5 μL TFA solution on the probe tip, then incubated for a few seconds and evaporated using a stream of pressurized air for MS analysis. The rinsing could be repeated 1–3 more times to improve the spectra quality.

2.6 TOF-MS

MS was performed on a Bruker Biflex™ II (Bruker, Bremen, Germany), equipped with a delayed ion extraction device. All mass spectra reported were obtained in the positive-ion linear mode and calibrated using an external calibration equation generated from the ion signal of bovine insulin oxide B and lysozyme. Typically, 40–200 laser shots were added per spectrum.

3 Results and discussion

3.1 Capillary microreactor with immobilization of enzyme by metal-chelated adsorption

Various methods for the immobilization of IDA onto silica matrix have been reported in the literature [16, 17]. Most of them proceed from GLYMO-activated silica by immobi-

lization of GLYMO onto the silica and then the chemical linkage of IDA through an epoxy group on GLYMO molecule with the similar reaction as described for soft gels. It is reported [14] that the optimum pH for the immobilization of IDA onto epoxy-activated supports is 10–12, while $>$ pH 8.3 considerable amounts of siloxane bonds between silane and silica matrix would be hydrolyzed. Thus, a relatively low pH value (about pH 8) has to be used for the reaction of IDA with the GLYMO-activated silica, which may result in a low ligand density of IDA because only partially epoxy groups on the activated silica will take part in the reaction with IDA under the unfavorable reaction conditions at low pH. Such a disadvantage can be solved by synthesis of the GLYMO-IDA-silane [18] before GLYMO is immobilized onto the silica. IDA can react with GLYMO in solution under stirring at pH 11. The resulting silane solution is stable, and no precipitation or polymerization occurs in solution at high pH during reaction. The prepared solution of GLYMO-IDA-silane is adjusted to pH 6 for the immobilization onto the silica, or could be stored at room temperature for at least 1 month.

On the other hand, fused-silica capillary is pretreated with NH_4HF_2 , in order to increase the surface area and wettability permitting the homogeneous spreading of the hydrophilic carboxylsilyl layer [19]. The pretreated capillary with surface roughening by NH_4HF_2 at high temperature has been used to prepare the immobilized trypsin capillary microreactor in our lab [12] and proved to be more efficient for protein digestion, probably due to the fact that the immobilized amount of trypsin is increased because of the increased surface area. The silanol groups on the roughened surface of capillary are then activated using HCl accompanied by removal of the metal ions from the capillary inner surface. After that, GLYMO-IDA-silane is introduced to react with the capillary wall, and then Cu^{2+} solution and the enzyme trypsin buffer solution are introduced to form an immobilized enzyme capillary microreactor. The whole procedure for immobilization of trypsin onto the inner wall of the capillary is shown in Fig. 1.

3.2 Characterization of Cu-IDA-trypsin microreactor

Usually, the activity of the immobilized enzyme is characterized by the values of the Michaelis constant (K_m) and maximum velocity (V_{max}), commonly determined by spectrophotometrical methods. However, such a conventional method is difficult to evaluate the property of the immobilized enzymes, because only nL amounts of enzymatic reaction solution can be obtained in our case. Recently, MALDI-TOF-MS was applied for the determination of

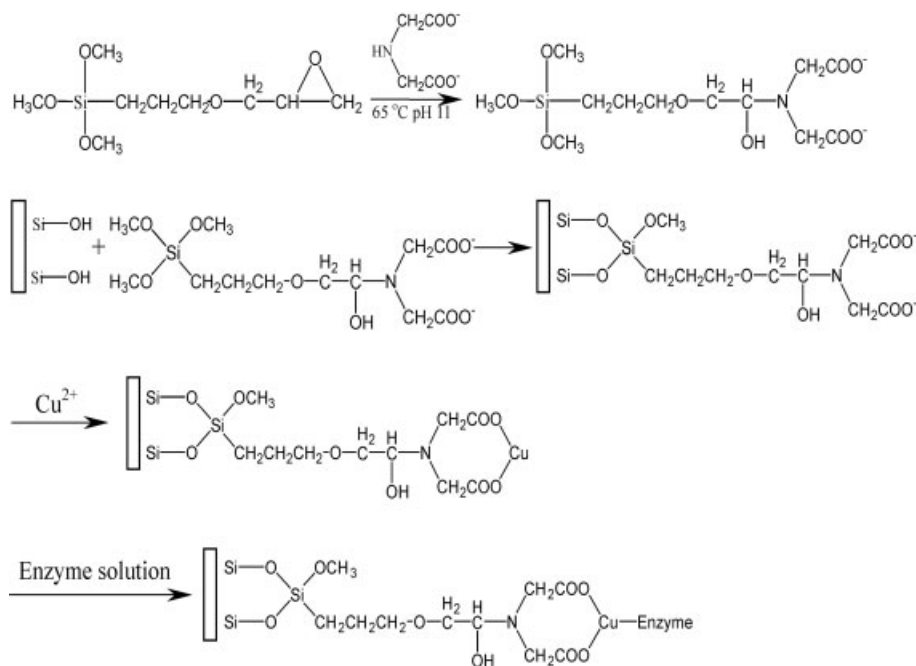


Figure 1. Scheme for preparation of the metal ion immobilized enzyme capillary microreactor. GLYMO-IDA-silane is synthesized before reacted onto the wall of silica capillary.

enzymatic reaction constants (Guo *et al.*, in preparation; [20]), and the internal standard, which is structurally related to the reaction product, had to be added into the reaction solution to calibrate concentration of the enzymatic reaction product. Once the product concentration is calculated, then the reaction velocity at any specific substrate concentration could be calculated, thus K_m and V_{max} could be derived from a linearized form of the Michaelis-Menten equation using a Lineweaver-Burke plot. Figure 2 shows the plot for the enzymatic conversion of BAEE in the immobilized trypsin capillary microreactor; the obtained reaction constants for the immobilized trypsin are $V_{max} = 0.83 \mu\text{M/s}$ and $K_m = 0.056 \text{ mM}$, respectively. Similarly, the enzymatic reaction constants of the free trypsin in solution were measured by keeping a trypsin concentration at 0.03 mg/mL and substrate concentrations varying from 5 to $50 \mu\text{M}$ (Fig. 2). For the free trypsin, the measured enzymatic reaction constants are $V_{max} = 1.50 \mu\text{M/s}$ and $K_m = 0.070 \text{ mM}$, respectively. It can be seen that the V_{max} value of the immobilized enzyme is lower than that of the free enzyme due to the fact that a portion of the enzyme was denatured during the immobilization process, and also active sites of the immobilized enzyme may be blocked due to the different orientations on the capillary wall. The K_m of the immobilized enzyme is also lower than that of the free enzyme. The value of the K_m reflects the concentration of substrate required so that the reaction velocity is half of its maximum value. The interactions between the solid support and substrate could increase the concentration of

substrate around the enzyme, and the lower concentration of substrate is needed to achieve half of the maximum value.

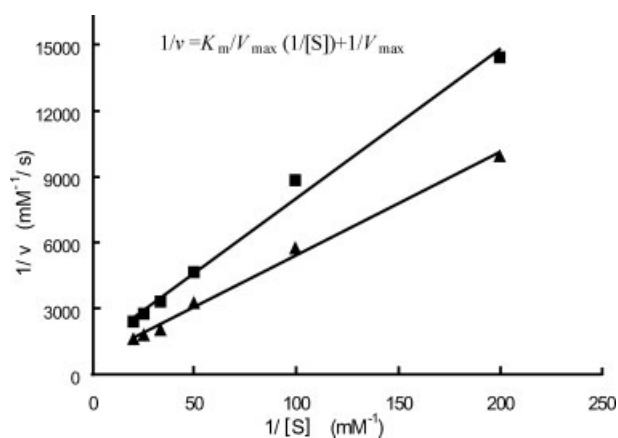


Figure 2. Lineweaver-Burk plots of the reciprocal initial reaction rates, $1/v$, versus the reciprocal of substrate concentration, $1/[S]$, for (■) free and (▲) immobilized enzyme of trypsin.

3.3 Cu-IDA-trypsin microreactor application in MALDI mass mapping

Protein of cytochrome c (0.5 pmol) is digested with the prepared microreactor for 15 min at 50°C . The resulting peptide fragments are characterized with MALDI-TOF-MS, and the obtained mass spectrum is shown in

Table 1. Identified peptide residues digested from horse heart cytochrome c for 15 min with the metal ion immobilized trypsin capillary microreactor

Mass (calculated) ^{a)}	Mass (Δ) ^{b)}	Mass (Δ) ^{c)}	Residues
634.8	634.0 (0.8)	634.1 (0.7)	9–13
732.8	732.4 (0.4)	732.3 (0.5)	1–7
807.0	806.8 (0.2)		73–79
965.1	964.8 (0.3)	965.8 (–0.7)	92–99
1019.1	1019.1 (0.0)		14–22
1036.4	1036.6 (–0.2)		80–88
1169.3	1169.1 (0.2)		28–38
1261.5	1261.9 (–0.4)	1261.5 (0.0)	14–25
1297.5	1297.9 (–0.4)		28–39
1351.6	1351.7 (–0.1)		89–99
1508.7	1509.1 (–0.4)	1508.3 (0.4)	92–104
1599.7	1599.8 (–0.1)	1599.6 (0.1)	39–53
1634.9	1634.8 (0.1)	1635.7 (–0.8)	9–22
1736.0	1736.7 (–0.7)	1734.8 (1.2)	87–100
1763.1	1763.0 (0.1)		8–22
1805.1	1805.2 (–0.1)		23–39
1948.3	1948.3 (0.0)		6–22
2210.6	2210.8 (–0.2)		56–73
2324.6	2323.2 (1.4)		54–72

a) Average chemical masses in Da, for protonated $[(M+H)^+]$ tryptic fragments

b) Observed m/z values in Da of 0.5 pmol cytochrome c, refer to Fig. 3a

c) Observed m/z values in Da of 20 fmol cytochrome c, refer to Fig. 3b

Fig. 3a. Ion signals of 19 distinct species are observed, corresponding to digest products of cytochrome c. Table 1 shows the correlation between the masses calculated for the enzymatically cleaved fragments and those observed in the mass spectrum. The average mass error over the entire series is less than 1 Da, with the greatest contribution arising from signals of weak intensity (due to presumably a less precise peak-centroid determination).

The prepared microreactor is also applied to digest as low as 20 fmol of cytochrome c and the obtained mass spectrum with MALDI-TOF-MS is shown in Fig. 3b. Still 8 peaks responding to the peptide fragments from cytochrome c are observed, which are enough to identify a protein. Also as expected, a sacrifice in S/N is observed. Additionally, the resolution of mass spectrum is decreased as slightly higher laser irradiance was used to acquire the spectrum. The correlation between masses calculated for the enzymatic cleavage fragments and those observed in the mass spectrum is also listed in Table 1.

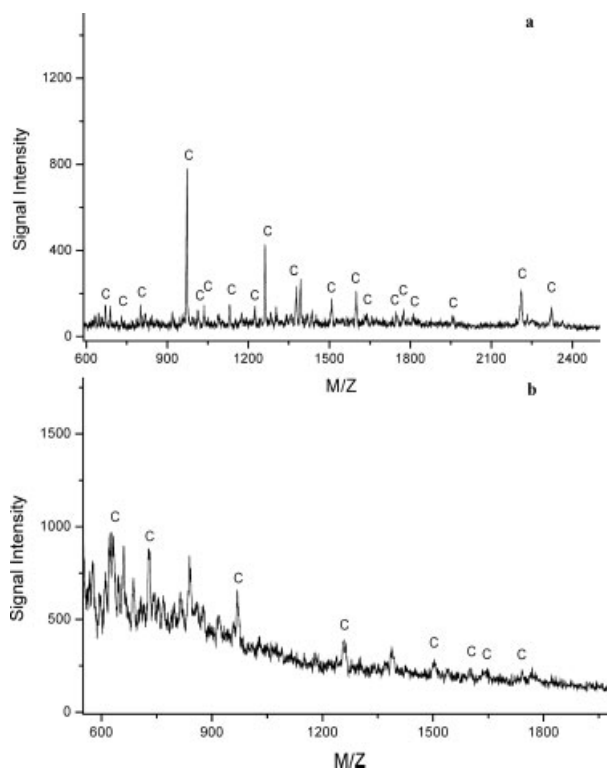


Figure 3. MALDI mass spectra for the digestion products of (a) 0.5 pmol and (b) 20 fmol cytochrome c by the metal ion immobilized trypsin capillary microreactor. MS experimental conditions: (a) value of laser attenuation at 40%, and the number of shots at 60; (b) value of laser attenuation at 35%, and number of shots at 150. Cytochrome c is digested at 50°C for 15 min, and (a) 19 peaks and (b) 8 peaks are determined to the corresponding peptide fragments from cytochrome c, respectively. C, peptide from cytochrome c.

3.4 Regeneration of the Cu-IDA-trypsin microreactor

Compared with the covalently immobilized enzyme microreactor, the major advantage of the prepared microreactor by Cu-IDA chelated adsorption of enzyme over covalently immobilization of enzyme is that the enzyme can easily and simply be regenerated in former case. The prepared microreactor should be regenerated after 4–6 times of usage for digestion of proteins. The regeneration process includes three steps: elution of the metal-ion and enzyme from the capillary wall with EDTA solution and then rechelating of the metal-ion such as copper onto the surface following the immobilization of the enzyme. The regeneration ability is evaluated with the digestion of cytochrome c under the same conditions before and after the regeneration of the prepared microreactor. The digested products are detected with MALDI-TOF-MS.

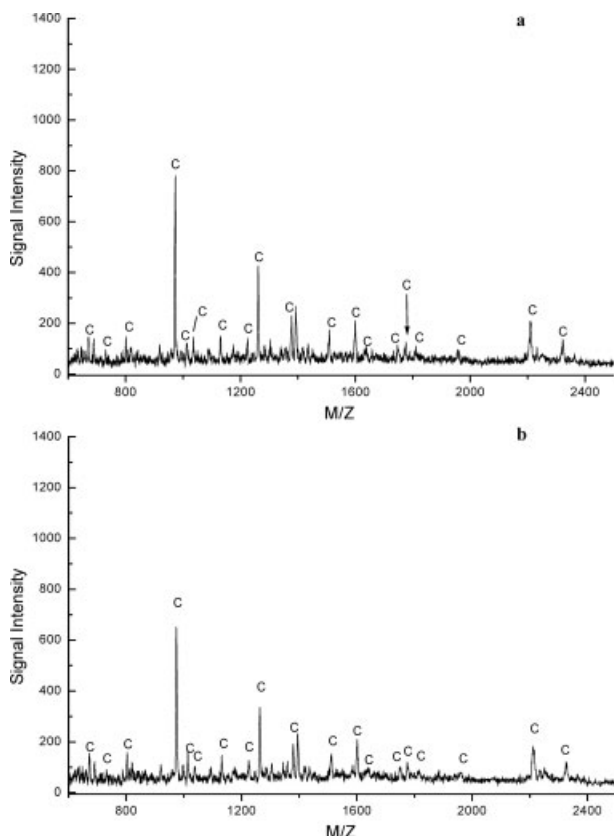


Figure 4. MALDI mass spectrum for digestion product of the cytochrome *c* by the metal ion immobilized trypsin capillary microreactor (a) before and (b) after regeneration. MS experimental conditions: (a), (b) value of laser attenuation at 40%, and number of shots at 60. 0.5 pmol cytochrome *c* is digested at 50°C for 15 min. Almost the same fragmentation pattern is observed with almost the same mass accuracy and intensity. C, peptide from cytochrome *c*.

Figure 4 shows the mass spectra for digestion products of 0.5 pmol cytochrome *c* by the prepared microreactor before and after regeneration. It can be seen that the pattern of two mass spectra is almost the same, which indicates a good reproducibility of the microreactor after regeneration process.

Another advantage of the prepared microreactor is convenient to change the types of the immobilized enzymes, just by applying different enzyme solution as the same procedures for the regeneration process. Since each type of enzyme has the specificity for hydrolysis of proteins, enzymatic cleavage using different enzymes could give complementary structure information of a protein [21]. Figure 5 shows a mass spectrum for the digestion products of cytochrome *c* with the immobilized chymotrypsin capillary microreactor; ten peaks are assigned to the digested fragments of cytochrome *c*.

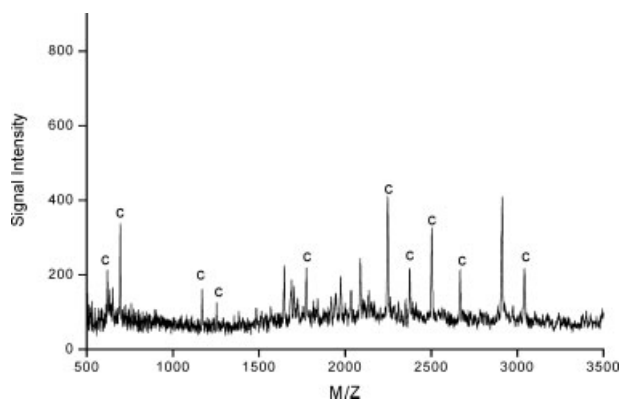


Figure 5. MALDI mass spectrum for digestion product of cytochrome *c* by the metal ion immobilized chymotrypsin capillary microreactor. MS experimental conditions: value of laser attenuation at 40%, number of shots at 100. 0.5 pmol cytochrome *c* is digested at 50°C for 15 min, and ten peaks are determined to the corresponding peptide fragments. C, peptide from cytochrome *c*.

4 Concluding remarks

We have reported the preparation of a regenerable capillary microreactor by metal-ion chelated adsorption of enzyme for peptide mapping analysis. The chelator of IDA was first reacted with GLYMO to form GLYMO-IDA-silane under optimum reaction conditions at pH 11, and then the GLYMO-IDA-silane is immobilized onto the inner capillary wall to increase the density of immobilized IDA. The main advantages of the prepared microreactor with this approach include its ability of regeneration and good reproducibility of microreactor before and after regeneration, and less consumption of sample solution. On the other hand, the prepared microreactor can easily be constructed by immobilizing the different types of enzymes for the peptide mapping analysis, which can provide complementary structure information of a protein.

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