Talanta 155 (2016) 78–86

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fast and efficient proteolysis by reusable pepsin-encapsulated magnetic sol-gel material for mass spectrometry-based proteomics applications

H. Mehmet Kayili [a,b], Bekir Salih [a,*]

[a] Hacettepe University, Department of Chemistry, 06800 Ankara, Turkey
[b] Çankırı Karatekin University, Department of Chemistry, 18100 Çankırı, Turkey

ARTICLE INFO

Article history:
Received 28 December 2015
Received in revised form
30 March 2016
Accepted 5 April 2016
Available online 7 April 2016

Keywords:
Sol-gel
Encapsulation
Pepsin
Proteolysis
Mass spectrometry
Proteomics

ABSTRACT

Hydrophobic silicon-based material having magnetic properties was fairly synthesized by a classical sol-gel approach. Pepsin enzyme was encapsulated in the sol-gel material and the enzyme activity was evaluated in consequence of the digestion of some common proteins such as α- and β-casein, cytochrome c, myoglobin, and bovine serum albumin (BSA) both in a single protein batch and in the protein mixture. The optimum digestion time of the studied proteins using pepsin-encapsulated magnetic sol-gel material was found to be 20 min. To produce the magnetic sol-gel material for convenient and easy proteomics applications, Fe_3O_4 was doped inside sol-gel material during the gelation step. It was observed that the activity of encapsulated pepsin was not affected by the amount of Fe_3O_4. Poly(ethylene glycol) was also inserted in sol-gel bulk to obtain suitable roughness and increase the hydrophilicity of the material surface to let protein molecules reach to the sol-gel material easily. The digestion of the protein mixture and non-fat bovine milk was performed with the pepsin-encapsulated magnetic sol-gel material and the digested solutions were analyzed using SDS-PAGE, MALDI-TOF-MS and LC-MS/MS for the protein identification. Reusability of the pepsin-encapsulated sol-gel material was examined and it was determined that they could be used at least 20 times. Finally, IgG digestions with a fast incubation time period were carried out using pepsin-encapsulated sol-gel material for generation of $(Fab)_2$ product to evaluate the kinetic performance of the material.

1. Introduction

Proteomics studies are needed to detect sequences of peptides and proteins to determine the structure, especially translational modifications, and the function of proteins. Digestion of proteins using a proteolytic enzyme is one of the most significant step of bottom-up-based proteomics studies [1–4]. The conventional digestion procedure requires a time-consuming incubation step to form a sufficient amount of peptides. Also, numerous auto-digestion products may be formed since the proteolytic enzyme is in a solution together with the protein [3,5]. For the elimination of these problems, fast, efficient and easy procedures are needed, and a reusable material is required to reduce the cost of the procedure for high-grade proteolysis.

In recent years, proteolytic enzymes have been immobilized on different substrates such as polymer [6], membrane [7], gel beads [8], glass [9], sol-gel supports [10], porous silicon matrix [11], and porous monolithic materials [12,13]. The immobilization of enzymes is very valuable in minimizing enzyme-autolysis fragments and reducing the cost of the procedure for proteolytic digestion. One of the facile and affordable method in enzyme encapsulation is sol-gel process, which is widely used in encapsulation of biomolecules [14–21]. It has many advantages such as thermal and mechanical strengths, encapsulation of large amount of enzymes, easy preparation without any covalent modification, and flexibility of controlling pore size and geometry [15,22]. The sol-gel process includes the hydrolysis and polycondensation of alkoxysilane precursors. The enzymes are combined into the matrix throughout this process by the encapsulation [22, 23].

In many enzyme encapsulated applications, enzyme encapsulated materials are separated from experimental media using some techniques such as filtration, and centrifugation [5,24]. However, these procedures depend on the particulate size and size distribution of the materials. In many cases, particulate size and size distribution of the materials can cause some problems in the applications of proteomics. The advantage of magnetic particulates for biocatalysis applications is the ease of specific separation, recovery and speed [25,26]. On the other hand, magnetic

* Corresponding author.
E-mail address: bekirsal@gmail.com (B. Salih).

http://dx.doi.org/10.1016/j.talanta.2016.04.014
0039-9140/

nanoparticulates have been used widely in bioseparation [27], drug delivery [28], and biomolecular sensors [29], and they have provided fast and easy separation by affecting the magnetic field.

Pepsin is a nonspecific protease that cleaves generally at the C-end of phenylalanine (Phe) and leucine (Leu) residues at pH 2. It is commonly used for the digestion of antibodies to enable generation and purification of antigen-binding fragments $(Fab)_2$ [30–32]. The enzymatic process of this application is very crucial for therapeutic use of polyclonal antibody fragments. Pepsin can be also used for the characterization of disulfide bonds generating disulfide-linked peptides [33]. In addition, it is useful to identify the specific proteins and it could be applied for the trace analysis of proteins [34, 35]. Besides these applications, the hydrogen-deuterium exchange process occurs in acidic conditions and preferably low temperature for the exploration of protein function. Unlike trypsin, pepsin is quite tolerant of such conditions and pepsin digestion in this process is a crucial step [36,37]. Due to common usage of the pepsin in these important applications, it was chosen as a target enzyme in the study.

Here, we synthesized a unique pepsin encapsulated sol-gel material usable in a facile and economical process for mass spectrometry-based proteomics applications. Magnetic Fe_3O_4 nanoparticulates were doped inside pepsin encapsulated sol-gel material for fast and easy separation. The digestion efficiency and reusability of this sol-gel material was examined in the digestions of several proteins including myoglobin, α- and β-casein, bovine serum albumin, cytochrome c, and IgG. Real-world complex sample such as non-fat milk was also tested to examine the matrix effects and the whole protein digestion in a real sample.

2. Materials and methods

2.1. Materials

Tetramethyl orthosilicate (TMOS, 96%, w/w), trimethoxy (octadecyl) silane (98%, w/w), DL-1,4-dithiothreitol (DTT), iodoacetamide (IAA), poly(ethylene glycol) (PEG) (Mr∼1000), α-cyano-4-hydroxycinnamic acid (α-CHCA), sinapinic acid, and the proteins used in this study such as cytochrome-c (horse heart, C2506), myoglobin (horse heart, M1882), α-casein (bovine milk, C6780), β-casein (bovine milk, C6905), albumin (bovine serum, B4287), IgG (human serum reagent grade, ≥95%, I4506), and porcine stomach mucosa pepsin (P7000, 632 U/mg solid and 1470 U/mg protein) were purchased from Sigma (St. Louis, MO) and used without further purification. The non-fat bovine milk was purchased from a local store. Water used in all experiments was purified using an Expe-Ultrapure Water System (Mirae St Co., Korea).

2.2. Methods

2.2.1. Preparation of pepsin-encapsulated magnetic sol-gel material

Pepsin-immobilized magnetic sol-gel material was prepared via the acid-catalyzed sol-gel reaction. Pepsin (50 mg) was dissolved in 1.44 mL water and then the magnetic Fe_3O_4 nanoparticulates (9.5 mg; 0.25% w/w, Fe_3O_4/dried sol-gel particulates) were added in the enzyme solution. To this solution, aqueous poly (ethylene glycol) (1%, v/v) (144 μL) and methanol (1 mL) were added and homogenized using a Vortex-Mixer. Trimethoxy (octadecyl) silane (2.69 mL, 6 mmol), tetramethyl orthosilicate (1.52 mL, 10 mmol), and fuming nitric acid (28 μL) were finally added to the mixture. Gelation was generally occurred within 10 min. while slightly shaking the reaction vessel. After drying overnight, the synthesized sol–gel particulates were washed with water and methanol to remove residuals. Different types of sol–gel materials such as pristine, Fe_3O_4 doped, pepsin-encapsulated, and pepsin-encapsulated and Fe_3O_4 doped nanoparticulates, were prepared by the same procedure.

2.2.2. Protein denaturation

Myoglobin, α-casein, and β-casein were prepared directly in 5% formic acid solution without any denaturation, however BSA and cytochrome C were subjected to denaturation step due to having disulfide bonds. The disulfide bonds were reduced by the incubation of proteins with 25 mM of 1,4-dithiothreitol (DTT) at 56 °C for 1 h. The reduced cysteine residues were alkylated by addition of 25 mM iodoacetamide (IAA) solution. The alkylation occurred in the dark at room temperature for 30 min. Twenty-five microliter of non-fat milk was diluted ten times with water prior to digestion. Denaturation and the reduction of milk proteins were performed using the same method as described above. The resulting samples were lyophilized using a freeze dryer (Christ, Alpha 1-2B, GERMANY) and dissolved in 5% formic acid solution prior to enzymatic digestion.

2.2.3. In-solution digestion

In this study, 0.5 mg of each protein and pepsin enzyme were dissolved in 0.5 mL of 5% formic acid solution (pH 1.5–2.0), separately. For all proteins, digestion was carried out using pepsin (enzyme-to-protein ratio of 1:50, v/v) at 37 °C for 20 h. The digestion was terminated by keeping the mixture in a fridge at −25 °C.

2.2.4. Digestion by pepsin-encapsulated magnetic sol-gel material

Pepsin-encapsulated magnetic sol-gel particulates (10 mg) were placed in protein solutions prepared in 0.5 mL of 5% formic acid solution (pH 1.5–2) dissolving 0.5 mg protein. The ratio of sol-gel material to protein was 20:1, (w/w) and the digestion was carried out at 37 °C for 30 min. The digestions were terminated by removing pepsin-encapsulated sol-gel material by a magnet. The digestion solutions were then stored in a fridge prior to their mass spectrometric analysis. To remove all adsorbed peptide fragments and non-digested proteins, pepsin-immobilized magnetic sol-gel material was washed with 250 μL of ethanol and water mixture (1:1, v/v) three times.

IgG was digested by the pepsin-encapsulated magnetic sol-gel material to generate its $(Fab)_2$ fragment. To achieve this, 10 mg of the material was incubated by 0.2 mg of IgG containing 0.1 mL solutions prepared in 20 mM sodium acetate buffer at pH:4.3. IgG samples were incubated at 37 °C for different time periods and collected at Eppendorf tubes for SDS-PAGE and MALDI-MS analysis.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The collected IgG digests (30 μg) were diluted in water and mixed with 4 × Laemmli buffer. The analysis was performed under non-reducing conditions. The samples and Bio-Rad precision plus protein standards were loaded onto a Mini-PROTEAN TGX Stain-Free Precast Gels and run with TGS buffer. Bands were visualized by Coomassie Brilliant Blue R-250 staining solution.

2.4. Characterization of pepsin-encapsulated magnetic sol-gel material

2.4.1. Fourier Transform-Infrared Spectroscopy (FT-IR)

FT-IR was used to characterize the chemical structure of pepsin, pristine sol-gel material, sol-gel material doped with Fe_3O_4 nanoparticulates, pepsin-encapsulated sol-gel material, pepsin-encapsulated sol-gel material containing PEG, and pepsin-encapsulated sol-gel material containing PEG doped with magnetic