Analytica Chimica Acta 845 (2014) 77–84

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Immobilization of trypsin on miniature incandescent bulbs for infrared-assisted proteolysis

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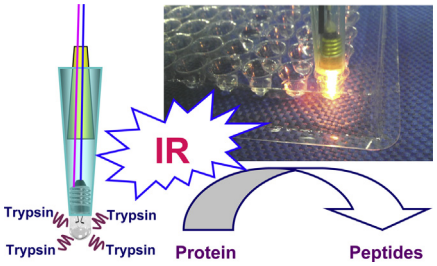
HIGHLIGHTS

- Trypsin was immobilized on miniature incandescent bulbs via chitosan coating.
- The bulbs acted as enzymatic reactors and the generators of infrared radiation.
- The bulb bioreactors were successfully employed in infrared-assisted proteolysis.
- The proteolysis could accomplish within 5 min with high sequence coverages.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 28 July 2014
Accepted 31 July 2014
Available online 2 August 2014

Keywords:
Proteolysis
Peptide mass mapping
Mass spectra
Infrared
Miniature incandescent bulb

ABSTRACT

A novel efficient proteolysis approach was developed based on trypsin-immobilized miniature incandescent bulbs and infrared (IR) radiation. Trypsin was covalently immobilized in the chitosan coating on the outer surface of miniature incandescent bulbs with the aid of glutaraldehyde. When an illuminated enzyme-immobilized bulb was immersed in protein solution, the emitted IR radiation could trigger and accelerate heterogeneous protein digestion. The feasibility and performance of the novel proteolysis approach were demonstrated by the digestion of hemoglobin (HEM), cytochrome *c* (Cyt-*c*), lysozyme (LYS), and ovalbumin (OVA) and the digestion time was significantly reduced to 5 min. The obtained digests were identified by MALDI-TOF-MS with the sequence coverages of 91%, 77%, 80%, and 52% for HEM, Cyt-*c*, LYS, and OVA (200 ng μL^{-1} each), respectively. The suitability of the prepared bulb bioreactors to complex proteins was demonstrated by digesting human serum.

1. Introduction

Nowadays, proteomics has become one of the fastest developing research fields in chemical biology because it gives a much better understanding of an organism than genomics [1–3]. One of its most important tasks is to develop efficient and rapid approaches to identifying a large number of proteins from a wide variety of biological sources. Peptide mapping is a commonly used strategy in proteome research. Proteins are usually digested into peptides that are subsequently identified by mass spectrometry (MS). Because conventional in-solution digestion of proteins is time-consuming and the autolysis of protease may generate interfering fragments, the development of highly efficient proteolysis approaches is of high importance for MS-based peptide mapping [4,5].

To date, a variety of novel methods have been developed for efficient proteolysis. Recent efforts have been made to immobilize proteases on various substrates [6–12]. The digestion time was

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http://dx.doi.org/10.1016/j.aca.2014.07.044
0003-2670/

significantly reduced to several minutes or even to several seconds while the digestion results were comparable to those obtained by using conventional in-solution digestion. The advantages of the immobilized protease for the identification of proteins include higher proteolysis efficiency, the enhanced stability of the enzymes, reduced protease autolysis, and the reusability of the enzymes. Protease, usually trypsin, has been immobilized on the channel walls in microchips or capillaries to fabricate microfluidic bioreactors for efficient proteolysis [6–9]. Besides microchips and capillaries, trypsin was also immobilized on the surface of magnetic particles covalently for efficient protein digestion with the aids of heats and microwaves [10,11]. The heterogeneous proteolysis could be accomplished within 5 min. In addition, various electromagnetic waves have been used to enhance the efficiency of in-solution proteolysis [11–14]. Electromagnetic waves are forms of energy exhibiting wave-like behaviors as they travels through space. Based on the frequency of the waves, they consist of radio wave, microwave, infrared (IR) radiation, visible light, ultraviolet (UV) radiation, X-rays and gamma rays. Among them, microwave, IR ray, and UV light have been employed as energy sources to enhance the efficiency of proteolysis. The digestion time was significantly reduced from 12 to 24 h for the conventional in-solution digestion to several minutes or several seconds for the electromagnetic wave-assisted proteolysis [15].

In 2008, we used IR radiation to accelerate in-solution proteolysis for the first time and the digestion time was significantly reduced to 5 min [14]. However, trypsin could not be reused because it directly mixed with protein solutions. The enzyme-autolysis products might interfere with the identification of proteins. It is interesting to immobilize protease on solid substrates for IR-assisted proteolysis. Miniature incandescent bulbs are usually employed as the light sources of handheld flashlights and the power lamps of instruments. They consist of air-tight glass enclosures and tungsten filaments inside the bulb. The glass bulbs are filled with inert gases to prevent the evaporation and oxidation of the filaments. When an electric current passes through the tungsten wires, it heats the filaments to a high temperature so that they emit electromagnetic waves mainly in the form of IR radiation [16–18].

In this work, trypsin was covalently immobilized in the chitosan (CTS) coating on the outer surface of miniature incandescent bulbs to perform heterogeneous IR-assisted proteolysis. The bulbs acted as not only the substrates for trypsin immobilization, but also as the generators of IR radiation. To the best of our knowledge, there was no early report on such a digestion strategy. It has been coupled with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) for the digestion and peptide mapping of several proteins and human serum. The fabrication details, characterization, feasibility, and application of the novel trypsin-immobilized miniature incandescent bulbs are reported in the following sections.

2. Experimental

2.1. Reagent and solutions

Ammonium bicarbonate (NH_4HCO_4), acetonitrile (ACN), acetic acid, glutaraldhyde, and sodium hydroxide were all purchased from SinoPharm (Shanghai, China). The chemicals were all analytical grade. Hemoglobin (HEM) from bovine blood, cytochrome *c* (Cyt-*c*) from horse heart, lysozyme (LYS) from chicken egg, ovalbumin (OVA) from chicken egg, tosylphenylalanine trypsin from bovine pancreas, trifluoroacetic acid (TFA), and α-cyano-4-hydroxycinnamic acid (CHCA) were supplied by Sigma (St. Louis, MO). CTS (75–85% deacetylation) was obtained from Aldrich (Milwaukee, WI, USA). All aqueous solutions were made up in doubly distilled water. The stock solutions of HEM, Cyt-*c*, LYS, and OVA (1 mg mL^{-1} each) were prepared in 20 mM NH_4HCO_3 buffer solution (pH 8.1) and were denatured in a 95 °C water bath for 15 min. Normal human serum was kindly donated by the Clinical Laboratory of Zhongshan Hospital (Shanghai, China).

2.2. Fabrication of trypsin-immobilized miniature incandescent bulb

In this work, a piece of miniature incandescent bulb (rated voltage, 6.3 V; rated current, 0.12 A; rated power, 0.76 W; electric resistance, ~52.5 Ω; Shanghai Zhaomin Lighting Electric Factory, Shanghai, China) was employed to immobilize trypsin for IR-assisted proteolysis (Fig. S1 (Supplementary Information)). The length and diameter of the glass bulb were approximately 8.0 and 4.5 mm, respectively. The suction nozzles of 1-mL and 0.2-mL polypropylene pipette tips were cut with a knife to reduce their lengths to 47 and 37 mm, respectively. Two pieces of copper wires coated with polyvinyl chloride (PVC) insulation layers were connected to the electrical contacts of the miniature incandescent bulb. The wire-bearing bulb was inserted into the shortened 1-mL pipette tip until its glass bulb protruded outside the bulb holder. Subsequently, ethyl α-cyanoacrylate instantaneous adhesive (Beijing Chemical Plant, Beijing, China) was employed to glue the bulb in place. Finally, the attachment end of the shortened 0.2-mL polypropylene pipette tip was pressed into the polypropylene bulb holder with the two electric wires inserted inside (Fig. 1B).

Fig. 1A illustrates the immobilization process of trypsin on the surface of a miniature incandescent bulb. Prior to immobilization, the glass bulb was rinsed with absolute ethanol and doubly distally water successively. After it was immersed into CTS solution (1 g CTS dissolved in 100 mL of 10 mg mL^{-1} acetic acid aqueous solution) for 1 min, the surface of the bulb was allowed to dry at room temperature for 15 min. The CTS-coated glass bulb was then immersed in 0.1 M NaOH aqueous solution for 10 min to neutralize the acetic acid in the CTS coating so that it became undissolvable. After the bulb was dipped in glutaraldehyde solution (5 mg mL^{-1} in 20 mM NH_4HCO_3 buffer solution (pH 8.1)) at 4 °C for 20 min, it was treated with 5 mg mL^{-1} trypsin in 50 mM phosphate buffer (pH 8.0) containing 20 mM $CaCl_2$ at 4 °C for 30 min to immobilize trypsin covalently. Finally, the trypsin-immobilized miniature incandescent bulb was flushed with 20 mM NH_4HCO_3 buffer solution (pH 8.1) and was kept in a 4 °C refrigerator.

2.3. IR-assisted on-bulb proteolysis of proteins

The IR-assisted on-bulb protein digestion system consists of a trypsin-immobilized miniature incandescent bulb, a disposable 96-hole hemagglutination plate, and a APS3005S-3D DC power supply (output voltage, 0–30 V; output current, 0–5 A; Shenzhen Antai Technology Co., Ltd., Shenzhen, China). Before digestion, the stock solutions of HEM, Cyt-*c*, LYS, and OVA were diluted to the desired concentration (200 or 20 ng μL^{-1}) with 20 mM NH_4HCO_3 buffer solution (pH 8.1). Each diluted protein solution (20 μL) was dropped in a V-shaped well (8 mm diameter and 7.5 mm depth) on a hemagglutination plate (Fig. 1C). After the trypsin-immobilized bulb was immersed in the sample solution, a desired DC voltage (1.5–6.0 V) was applied to light it up (Fig. 1D and E). The protein samples were allowed to digest under IR radiation for 5 min except mentioned otherwise. For comparison, HEM and Cyt-*c* (200 ng μL^{-1} each) in 20 mM NH_4HCO_3 buffer (pH 8.1) were also digested by using conventional in-solution proteolysis in a 37 °C water bath for 12 h. The weight ratio between trypsin and protein substrate was 1:40. The obtained digests were further identified by MALDI-TOF-MS.