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RESEARCH PAPER

Preparation and Application of Novel Thermo-sensitive Matrix-based Immobilized Enzyme for Fast and Highly Efficient Proteome Research

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Abstract: By massively analyzing proteins extracted from cells, tissues and organisms using mass spectrometry, proteomics is capable of providing information about the change in proteins expression, modification, composition and quantification. However, most immobilized enzymes used in mass spectrometry based “shotgun” proteomic strategy are prepared using solid materials as the immobilization matrix and digest the substrate proteins in heterogeneous system. The inherent mass transfer resistance in the solid-liquid interface and steric hindrance of the solid matrix limits the digestion efficiency and sample processing throughput. Here, we prepared a novel immobilized enzyme using soluble thermo-sensitive polymer as the matrix material by exploiting the thermo-responsive ability of the polymer to environmental temperature change. The thermo-sensitive immobilized trypsin had the feature of “homogeneous digestion at high temperature and heterogeneous separation at low temperature” and the advantages of significantly shortened digestion time and recover & reuse of the enzyme. When BSA was digested 1 min by immobilized enzyme, the amino acid sequence coverage was up to 94%, higher than 74% amino acid sequence coverage in traditional enzyme solution. Finally, the immobilized-trypsin was successfully applied in fast and highly efficient digestion of complex proteome extracted from HeLa cell. The efficiency of immobilized enzyme digestion in 1 min was similar to that of solution digestion in 12 h, which sufficiently demonstrated the application potential of this thermo-sensitive immobilized trypsin in proteomics research.

Key Words: Immobilized enzyme; Thermo-sensitive polymer; Mass spectrometry; Proteomics; Fast digestion

1 Introduction

The proteome is the entire set of proteins, produced or modified by extracting from the cell, tissue or organism. Generally, proteomics refers to the large-scale experimental analysis and identification of proteins, and is specifically used for protein purification and mass spectrometric analysis[1,2]. Currently, shotgun proteomics is the most preferred method for proteome profiling. In the "shotgun" strategy, protein samples were digested by protease, and the hydrolysates were identified by liquid chromatography-mass spectrometry (LC-MS). Finally, the detailed information of proteins was obtained from mass spectral data[3,4]. Hydrolysis of protein is the crucial step for qualitative and quantitative analysis of protein in this strategy. Previously, it took too long time (12–24 h) for hydrolysis of protein to use enzymatic solution in the "shotgun" experiment, severely limiting the speed and throughput of sample preparation. Otherwise, compounding the problem was that the enzymatic solution could be used only one time without recycling, resulting in higher cost. Immobilized enzyme can avoid hydrolysis of protease itself, which significantly improves the efficiency of hydrolysis and

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feed ratio of enzyme and substrate, shorting reaction time and reducing cost in recovery[5–7].

At present, different immobilization methods and immobilization carrier materials, such as mesoporous materials[8,9], quartz capillary column[10,11], chip material[12,13], silica material[14,15], magnetic nanoparticles[16,17], etc. have been developed, and solid phase material were usually used as the immobilization carrier . However, as immobilized protease substrate protein performs enzymatic reaction in a heterogeneous phase system, the mass transfer resistance of solid-liquid and steric hindrance of solid phase material might limit the efficiency of hydrolysis and probability of collision between immobilized enzyme and substrate. To solve these technical problems, we developed a thermo-sensitive polymer immobilized-enzyme, which utilized the thermo-sensitive features achieving "homogeneous digestion at high temperature and heterogeneous separation at low temperature" by controlling its environment temperature in the mutual transformation between the dissolved and insoluble state. In general, thermo-sensitive polymer solution had the characteristics of phase-transfer temperature, the upper critical solution temperature (UCST) was the main type. When the environment temperature was higher than the phase-transfer temperature, the polymer was completely dissolved in solution; nevertheless, when the phase-transfer temperature was up to or below phase-transfer temperature, polymer began to gather, precipitating out sediment[18,19].

Based on *N*-acryloyl gump ammonia amide (NAGA) as the temperature response groups, UCST thermo-sensitive *N*-acryloyl gump 11 olefine aldehyde ammonia amide copolymer [poly real (NAGA-co-UnAl)] was synthesized as carrier materials. After a series of further preparation, a novel thermo-sensitive matrix-immobilized trypsin was obtained. Commonly, most of the immobilized enzymes were prepared with solid phase carrier materials, enzymatic reaction was carried out in a heterogeneous phase system and digestion in heterogeneous system. However, the immobilized enzyme reagent which we prepared, was dissolved by ultrasound at 37 ºC, and it could undertake homogeneous reaction with protein substrates, realize heterogeneous separation, high efficiency of enzyme hydrolysis and high recovery rate of hydrolysate.

2 Experimental

2.1 Instruments and reagents

Matrix assisted laser desorption ionization mass spectrometer (New ultrafleXtreme MALDI-TOF/TOF-MS), EQUINOX55 Fourier Infrared Spectrometer (FT-IR) were from BRUKER (Germany). Quadruple-electrostatic field orbit trap combination high resolution tandem mass spectrometer (Q-Exactive Plus Orbitrap MS), liquid chromatography (Easy-nLC 1000) were from Thermo Fisher Scientific(USA).

Bovine serum albumin (BSA, 98%), trypsin (90%), glycinamide hydrochloride (98%), acryloyl chloride (98%), 1-undecen-10-al (UnAl, 95%), sodium cyanoborohydride (99%) and azodiisobutyronitrile (AIBN, 99%) were purchased from Sigma-Aldrich (USA). All other reagents were of analytical reagent grade and used without further purification. Aqueous solutions were prepared with Milli-Q doubly-distilled water.

2.2 Experimental methods

2.2.1 Synthesis of *N*-Acryloyl ammonia amide (NAGA)

The thermo-sensitive morphon of NAGA was synthetized following the protocol described in literature [18].

2.2.2 Synthesis of UCST thermo-sensitive polymer (NAGA-co-UnAl)

NAGA-co-UnAl polymers were synthesized by free-radical polymerization of organic monomers, and the mole ratio of NAGA and UnAl was 4:1. The synthetic steps referred to the protocol were as described in reference [18].

2.2.3 Fixation of trypsin to poly(NAGA-co-UnAl)

Approximately 10 mg poly(NAGA-co-UnAl) was dissolved in 500 μL phosphate buffer solution (PBS, pH 7.4), and then 4 mg trypsin and 5 mg sodium cyanoborohydride were added and the mixture solution was stirred at 24 ºC for 3 h. Then, the mixture solution was incubated at aqua astricta for 2 min until turbidness appeared. The mixture was centrifuged at 10000 rpm for 10 min at 4 ºC. The supernatant was collected. The insoluble were dissolved in 1.0 mL sodium bicarbonate solution (50 mM), and the solution was again placed in an ice water bath, and then centrifuged for separation. Finally, this procedure was repeated three times to wash away non-immobilized trypsin and the final product poly(NAGA-co-UnAl)-trypsin immobilized enzyme was yielded. The supernatants were collected during the process of cleaning, which was used to determine the amount of trypsin immobilized on carrier material.

2.2.4 Hydrolysis of protein standards by Poly(NAGA-co-UnAl)-trypsin immobilized enzyme

BSA was dissolved in 50 mM ammonium bicarbonate solution, and 1 μg μL^{-1}, dithiothreitol (DTT) was added to a final concentration of 10 mM, then the solution was incubated at 95 °C for 10 min. After cooling down, indoacetamide (IAA) was added to the solution of final concentration of 50 mM, and then the solution was placed under dark for 1 h.

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