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Investigation of bi-enzymatic reactor based on hybrid monolith with nanoparticles embedded and its proteolytic characteristics

Lulu Shangguan[a,1], Lingyi Zhang[a,*,1], Zhichao Xiong[a], Jun Ren[a], Runsheng Zhang[b], Fangyuan Gao[a], Weibing Zhang[a]

[a] Shanghai Key Laboratory of Functional Materials Chemistry, East China University of Science and Technology, Shanghai 200237, P.R. China
[b] Shanghai Key Laboratory of Crime Scene Evidence, Shanghai Research Institute of Criminal Science and Technology, Shanghai Public Security Bureau, Shanghai, P.R. China

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ABSTRACT

The bottom-up strategy of proteomic profiling study based on mass spectrometer (MS) has drawn high attention. However, conventional solution-based digestion could not satisfy the demands of highly efficient and complete high throughput proteolysis of complex samples. We proposed a novel bi-enzymatic reactor by immobilizing two different enzymes (trypsin/chymotrypsin) onto a mixed support of hybrid organic–inorganic monolith with SBA-15 nanoparticles embedded. Typsin and chymotrypsin were crossly immobilized onto the mixed support by covalent bonding onto the monolith with glutaraldehyde as bridge reagent and chelation via copper ion onto the nanoparticles, respectively. Compared with single enzymatic reactors, the bi-enzymatic reactor improved the overall functional analysis of membrane proteins of rat liver by doubling the number of identified peptides (from 1184/1010 with trypsin/chymotrypsin enzymatic reactors to 2891 with bi-enzymatic reactor), which led to more proteins identified with deep coverage (from 452/336 to 620); the efficiency of the bi-enzymatic reactor is also better than that of solution-based tandem digestion, greatly shorting the digestion time from 24 h to 50 s. Moreover, more transmembrane proteins were identified by bi-enzymatic reactor (106) compared with solution-based tandem digestion (95) with the same two enzymes and enzymatic reactors with single enzyme immobilized (75 with trypsin and 66 with chymotrypsin). The proteolytic characteristics of the bi-enzymatic reactors were evaluated by applying them to digestion of rat liver proteins. The reactors showed good digestion capability for proteins with different hydrophobicity and molecular weight.

1. Introduction

The bottom-up strategy of proteomic profiling study based on mass spectrometer (MS) has drawn high attention, due to the high accuracy, reliability, and reproducibility in protein identification [1,2]. In this strategy, protein samples are firstly digested to peptides by a protease (typically trypsin), followed by LC-MS analysis for peptide and protein identification. Thus, how to realize rapid and efficient generation of peptides becomes one of the most challenging steps for such MS-based proteomics analysis. The challenges are particularly stringent for membrane proteins as they demonstrate poor solubility, low abundance and relative paucity of tryptic cleavage sites [3], which make them notoriously difficult to study and consistently underrepresented in proteomic analyses [4].

It has been shown that the use of multiple proteases can greatly increase the sequence coverage of identified proteins by effectively maximizing number of generated peptides for MS identification in large-scale proteomic analysis [5,6]. This versatile approach has made some success in solution-based tandem digestion. Glatter et al. [7] accomplished large-scale quantitative assessment of proteins by using different in-solution digestion protocols; the results revealed that superior cleavage efficiency of tandem Lys-C/trypsin proteolysis was over trypsin digestion. Wisniewski et al. [8] also performed consecutive proteolytic digestion with Lys C and trypsin by an enzyme reactor. The tandem use of endoproteinases enabled identification of up to 40% more proteins and phosphorylation sites in comparison to the commonly used one-step tryptic digestion. However, solution-based tandem digestion strategy not only has the conventional solution digestion drawbacks as autolysis, poor enzyme to substrate ratio, but also extends digestion time up to 24 h

* Corresponding author. Tel.: +86 021 64253977; fax: +86 021 64233161.
E-mail address: zhanglingyi@ecust.edu.cn (L. Zhang).
[1] These two authors contributed equally to this work.

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or more which greatly limits the sample processing throughput. Therefore, the application of enzymatic reactor with multiple proteases immobilized may combine the advantages of immobilized protease and multiple protease digestion to achieve fast, highly efficient and complete digestion for complex protein samples.

Due to the advantages of low enzyme autolysis, high enzyme-to-substrate ratio, time-saving and reusability, immobilized enzyme reactor (IMER) has drawn much attention recently [9–11]. For IMER, enzymes can be immobilized by several mechanisms: sol–gel trapping [10], layer-by-layer assembly [12], cross-linking [13], covalently bonding or physical adsorption [14,15]. And variety of materials have been used to support enzymes, including polymer membranes [16], microchips [17], capillary columns [18], micro/nanoparticles [19] and monolith materials [20]. Among various matrices, monolithic materials are used more widely owing to its obvious advantages such as easy fabrication and modification, good biological compatibility [21]. Combining the advantages of organic and inorganic monoliths, the organic–inorganic hybrid monoliths attract much attention due to the characteristics of better pH stability and less shrinkage [22]. In addition, with large surface area and some special surface properties [23], nanoparticle also shows great potential as excellent enzyme support [24,25]. In our recent work, we designed a novel IMER by immobilizing trypsin on a composite support of hybrid organic–inorganic monolith with SBA-15 nanoparticles embedded in silica capillary. Owing to higher trypsin immobilization amount, the IMER was successfully applied to digestion of standard proteins and rat liver proteins with high sequence coverages, while the digestion time was reduced to only 19 s in dynamic mode [26].

In general, only one protease was immobilized (typically trypsin) in IMER [17,27,28], which limits digestion efficiency and tends to generate missed cleaved peptides, especially for proteins lacking of lysine (R) and arginine (K) residues. Besides trypsin, other endoproteases such as chymotrypsin [16,29], Glu-C [30,31], Lys-C [32] can also be used for MS-based protein analysis to cover the complete sequence of proteins. The specific enzymolysis sites of chymotrypsin are phenylalanine (F), tryptophane (W), tyrosine (Y), and leucine (L), methionine (M) are less specific enzymolysis sites for it [33–35]. Considering the completely orthogonal specificities of these two proteases, the combination of trypsin and chymotrypsin may lead to better digestion. Fischer et al. [33] had used mixed enzymes of trypsin and chmotrypsin for hydrolysis of membrane proteins in an organic solvent; the combination significantly improved the identification of hydrophobic peptides with distinctly higher sequence coverage of transmembrane regions. Zhou et al. [16] proposed a novel enzymatic reactor by simultaneously immobilizing trypsin and chymotrypsin on biocompatible PVDF membranes, and this bi-enzymatic reactor could produce enhanced rapid digestion of standardized prototypic proteins, hydrophilic proteins and hydrophobic transmenbrane proteins. Temporini et al. [29] also proposed a multi-enzymatic approach by synchronously bonding trypsin and chymotrypsin to an monolithic silica column in a one-step reaction via epoxy-groups, which showed good digestion efficiency and increased the confidence degree in proteomic analysis.

In this paper, a bi-enzymatic reactor based on monolith with nanoparticles embedded was prepared to achieve complementary digestion of complex protein samples. The monolith and nanoparticles were modified with trypsin and chymotrypsin by using covalent bonding with glutaraldehyde as bridging reagent and chelation with copper ions, respectively. The preparation and operation conditions including nanoparticles percentage, the inner diameter of silica capillary and the length of the bioreactor were optimized. Carbonic anhydrase was used to evaluate the performance of the bi-enzymatic reactor. The advantages and proteolysis characteristics of the bi-enzymatic reactor were further demonstrated by its application in membrane proteins from rat liver and proteins of rat liver extract by comparing with two single protease reactors and the solution-based tandem digestion using free proteases.

2. Materials and methods

2.1. Instruments

A syringe pump (Baoding Longer Precision Pump Co., Ltd., China) was used to push the sample solution through capillary reactor. Sol–gel solution and samples were mixed by a vortex vibrator (QL-901, Kylin–Bell Lab Instruments Co., Ltd., China). The nanoparticles were homogeneously dispersed in solution by an ultrasonic cleaner (SZ-80, Hangzhou Sagee Instrument Co., Ltd., China). The temperature of digestion was controlled by a column oven (Dalian Elite Analytical Instruments Co., Ltd., China).

2.2. Reagents and materials

Carbonic anhydrase (bovine erythrocytes), BSA (bovine serum), trypsin (bovine pancreas), α-chymotrypsin (bovine pancreas), dithiothreitol (DTT) and iodine acetamide (IAA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Tetraethoxysilane (TEOS, 98%), 3-amino-propyltriethoxysilane (APTES, 99%) and tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, 99%) were purchased from ACROS (Shanghai, China). Cetyltrimethyl ammonium bromide (CTAB) was purchased from Crystal Pure Reagent (Shanghai, China). 3-Glycidoxypropyltrimethoxysilane (GLYMO), iminodiacetic acid (IDA), benzamidine hydrochloride, sodium cyanoborohydride ($NaBH_3CN$) and urea were purchased from Aladdin (Shanghai, China). Formic acid (FA) was from Fluka (Buches, Germany). HPLC-grade acetonitrile (ACN) was from Merck (Darmstadt, Germany). Fused silica capillary with diameters of 75 and 100 μm i.d. was obtained from Sina Sumtech Co., Ltd. (Hebei, China). C_{18} AQ beads (3 and 5 μm, 120 Å) were purchased from Daiso (Osaka, Japan).

2.3. Preparation and modification of nanoparticles

The SBA-15 nanoparticles were prepared as previous report [36]. The modification procedure of SBA-15 nanoparticles with carboxylic group is shown in Fig. 1A. Firstly, the iminodiacetic acid conjugated glycidoxypropyltrimethoxysilane (GLYMO-IDA-silane) was synthesized according to reference [37]. The SBA-15 nanoparticles (0.01 mg) were dispersed homogeneously in 25 mL of ethanol by vortexing and sonicating, and 5 mL of GLYMO-IDA-silane (pH 6.0) was added into the suspension. Then the mixture was incubated in a water bath at 40 °C for 24 h. Finally, the SBA-15 nanoparticles were cleaned with ethanol and then put into oven at 60 °C overnight to get IDA modified nanoparticles (SBA-15-COOH).

2.4. Preparation of hybrid organic–inorganic monolith with SBA-15 nanoparticles embedded

The hybrid organic–inorganic monolithic column was prepared as reported in our previous work [38]. TEOS (112 μL), APTES (118 μL), ethanol (215 μL), water (32 μL) and CTAB (8 mg) were mixed and sonicated to obtain a homogeneous solution (donated as MPS, monolith preparation solution). Then, 100 μL of MPS was mixed with 2.0 mg SBA-15 nanoparticles, followed by vortex shaking and sonication in turn 1 min for each to form a homogeneous solution at 0 °C, which was filled into the pretreated capillary with appropriate length by a syringe. After both ends being sealed, the capillary was put into a water bath at 40 °C for 24 h. Subsequently, the obtained hybrid monolithic capillary column was flushed with