Biochemical and Biophysical Research Communications 473 (2016) 54–60

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

An easily regenerable enzyme reactor prepared from polymerized high internal phase emulsions

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ARTICLE INFO

Article history:
Received 1 March 2016
Accepted 11 March 2016
Available online 17 March 2016

Keywords:
Immobilized enzymatic reactor
Protein digestion
High internal phase emulsion
PolyHIPE monoliths

ABSTRACT

A large-scale high-efficient enzyme reactor based on polymerized high internal phase emulsion monolith (polyHIPE) was prepared. First, a porous cross-linked polyHIPE monolith was prepared by in-situ thermal polymerization of a high internal phase emulsion containing styrene, divinylbenzene and polyglutaraldehyde. The enzyme of TPCK-Trypsin was then immobilized on the monolithic polyHIPE. The performance of the resultant enzyme reactor was assessed according to the conversion ability of N_{α}-benzoyl-L-arginine ethyl ester to N_{α}-benzoyl-L-arginine, and the protein digestibility of bovine serum albumin (BSA) and cytochrome (Cyt-C). The results showed that the prepared enzyme reactor exhibited high enzyme immobilization efficiency and fast and easy-control protein digestibility. BSA and Cyt-C could be digested in 10 min with sequence coverage of 59% and 78%, respectively. The peptides and residual protein could be easily rinsed out from reactor and the reactor could be regenerated easily with 4 M HCl without any structure destruction. Properties of multiple interconnected chambers with good permeability, fast digestion facility and easily reproducibility indicated that the polyHIPE enzyme reactor was a good selector potentially applied in proteomics and catalysis areas.

1. Introduction

Recently, protein digestion has been studied in many research areas including proteomics, bioengineering and food industry, and the high-throughput and high proteolytic enzyme reactor is expected for efficient protein digestion. Comparing with the traditional protein digestion in solution suffered some drawbacks, such as enzyme autodigestion, low efficiency, extended incubation time and sample loss or contamination, [1], the immobilized enzymatic reactors (IMERs) had great advantages in the aspect of protein digestion [2–4]. Therefore, many IMERs have been studied by immobilizing enzymes on different materials such as polymeric membranes and nanofibers, [5,6], core–shell magnetic nanoparticle, [7,8], graphene oxide nanosheets [9] and organic-inorganic hybrid monolith [10]. These rapid and low carry-over IMERs were successfully applied to protein digestion and proteomic analysis [11].

Among these IMERs, the monoliths, composed of porous solid with small-sized skeletons and relatively large through-pores, could offer fast mass transfer and high enzyme binding capacity [12,13]. Generally, monolithic supports were prepared as silica-based monoliths [14,15] and organic polymers [16]. Silica-based monoliths had silica skeletons and through-pores via a sol–gel process or hybrid organic-inorganic process [17,18]. which made them high permeability, high mechanical strength, and good organic solvent tolerance [19]. But, the preparation of these monoliths was complicated, sometimes leading to poor reproducibility [20]. Another problem was that the nonspecific adsorption of the silica-based monolith was obvious due to the residual silanols, and the uncontrollable polymerization procedure lead it to an irregular pore size and complicated absorption/desorption behavior at the surface of the monolith. In comparison, with good porosity, mild biocompatibility and high-throughput conveying capacity, [21], [22] organic polymer materials had been widely applied in many research areas

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http://dx.doi.org/10.1016/j.bbrc.2016.03.049
0006-291X/

like scaffolds for in vitro 3D cell culture, [23], protein separation and phosphopeptides enrichment, [24], biomedical sensor support, [25], biocatalysts [26] and biomacromolecules trapping [27]. But the swelling of organic solvents might lead to the change of pore structure and the decrease of mechanical stability.

Notably, polymerized high internal phase emulsions (polyHIPEs) have been developed for extensive application in biocompatibility substrates [28,29]. After polymerization, polyHIPEs were formed and the droplets of dispersed phase were removed yielding a highly interconnected network pores with quite well defined diameter. Depending on dispersed phase in emulsion, polyHIPEs had adjustable meso- or macropore skeleton which avoided the problems of nanoparticle agglomerating, low permeability and surfactants demanding [30]. Moreover, the inherent meso- and macropore of polyHIPEs were benefit for specific in-situ absorbents of biological macromolecules when some functional group reagents were added in the polymerization procedure [31].

Here, we developed an easy-preparing and regenerable polyHIPE based IMER for trypsin immobilization and protein digestion. Our studies suggested that the developed IMERs not only have high trypsin immobilization and bioactivity in protein digestion but also have strong regeneration capacity and long-term durability. According to our knowledge, this is the first time report that using polyHIPEs as immobilized enzyme reactor support for protein digestion research.

2. Materials and methods

2.1. Materials and chemicals

Reagents including styrene (STY), divinylbenzene (DVB), sorbitan monooleate (Span80), TPCK-trypsin (Type I, >10 000 units/mg N_α-benzoyl-L-arginine ethyl ester hydrochloride, TPCK treated), N_α-benzoyl-L-arginine ethyl ester (BAEE, 98%), N_α-benzoyl-L-arginine (BA, 98.5%), Tris (Hydroxymethyl)aminomethane (Tris), iodoacetamide (IAA), dithiothreitol (DTT), Cytochrome C (Cyt-c) and bovine serum albumin (BSA) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Potassium persulfate ($K_2S_2O_8$, AR), Glutaraldehyde (GA, 25%, w/v aqueous solution) were obtained from Xilong Chemical Reagent (Guangzhou, China). Methanol and acetonitrile were of HPLC grade from Dikma Co., Ltd. (CA, America). All other reagents were of analytical grade, such as sodium hydroxide, hydrochloric acid and ammonium acetate. The water was deionized by an arium® 611 system (Sartorius, Germany) with resistance ≥18.2 MΩ/cm. All solutions and deionized water were filtered through 0.22 μm filter membrane before HPLC and LC-MS analysis.

Before using, STY and DVB were washed with 5% NaOH aqueous solution and then with water to remove the polymerization inhibitor. After washed to pH 7.0 with water, the obtained solution was dried with $MgSO_4$.

2.2. Preparation of polyHIPE monolith

Firstly, the dispersed phase of PGA and $K_2S_2O_8$ solution was prepared as following: polyglutaraldehyde (PGA) was obtained with GA solution reacted with 1.0 M NaOH at pH 10.5 for 30 min and then the solution was adjusted to pH 7.0 by 1 M HCl [32]. 6.531 mL 0.02% (g/mL) $K_2S_2O_8$ solution was mixed with 0.417 mL PGA solution as dispersed phase for the preparation of HIPE.

0.772 mL continuous phase containing STY, DVB and Span 80 was added into centrifugation tube and rotated in a vortex mixer (IKA MS3 basic, Germany) at 3000 rpm for 30 s, then 6.948 mL dispersed phase was added drop-wise to the continuous phase under condition of gentle stirring. The emulsion was finally stirred for 3 min to form a uniform emulsion. About 1.5 ± 0.2 mL emulsion was transferred into 2.5 mL sealed syringe and polymerized at 50 °C for 24 h. The synthesized polyHIPEs was washed with deionized water and ethanol-water solution (1:1 V/V). Finally, the monoliths were washed and soaked with ammonium acetate buffer (AAB, 0.1 mol/L, pH 8.0). It was measured that a dried polyHIPE monolith was about 0.7 ± 0.1 g.

The freshly prepared emulsions were placed for several hours to observe the stability and variation then the two phases was observed under the microscope in 400 and 1000 times. After polymerization, deionized water was used to investigate the mechanical strength and flow velocity of the monoliths at a pressure of 70 kPa. The morphology and cavity size distribution of monoliths were determined by SEM image analysis (S4800 field emission scanning electron microscope, Hitachi, Japan). Nitrogen adsorption/desorption measurements were valuated on a Micromeritics® ASAP 2020 adsorption apparatus using a BET model for surface area evaluation. To valuate the immobilization probability, the experiments of FTIR spectrum (Nicolet iS10, Thermo Fisher, USA) and elemental analysis (EA2400II, PerkinElmer, USA) were carried out.

2.3. Enzyme immobilization on polyHIPE monolith

For enzyme immobilization, the prepared monolith was connected to a constant flow pump (HL-2D, Shanghai Huxi Analysis Instrument Factory Co., Ltd) and kept at 37 °C. 2.0 mL TPCK-trypsin solution (0.2 mg/mL) was slowly injected and immobilized naturally onto the monolith for 10 min. The residue trypsin solution was pumped out and the reactor was washed with 2 mL AAB solution. After the procedure of enzyme immobilization, the prepared IMER was stored at 4 °C. For long-term storage, the IMERs were soaked in AAB and stored at −20 °C in fridge.

2.4. Investigation of monolithic polyHIPE based IMER

The TPCK-trypsin immobilization rate was calculated by conversation rate of BAEE to BA. Also, the conversion rate of BAEE to BA was used to optimize the synthesis conditions of monolith and assess the properties of IMER [33]. In brief, the prepared monolithic polyHIPE based IMER was heated in a cabinet drier at constant temperature of 37 °C, and then 0.650 mL solution of Tris−HCl and BAEE (0.3 mL of 0.1 mol/L Tris−HCl and 0.35 mL of 5.0 mg/mL BAEE) was pumped through the IMER and reacted for 10 min at a constant velocity. After that, 1.95 mL water was used to wash out the BAEE and BA and deionized water was used to clean the IMER. All eluents were respectively collected and the solution was filtered through 0.22 μm filter membrane before HPLC analysis.

To confirm the limitation of reusability of IMER, it was used many times. Once the enzyme activity of IMER was dramatically decreased, 15 mL AAB was injected to monolith to clean up and 20 mL HCl (4 mol/L) was used to regenerate the monolith for breaking the −C═N− bond between the TPCK-trypsin and PGA based on the Schiff base reaction. The remnant HCl was washed out by 6 mL deionized water and followed by 15 mL AAB. After that, TPCK-trypsin was immobilized on monolith once again and the regenerated IMER was reused.

For protein digestion, 1 mL of 10 mg/mL BSA and Cyt-c were respectively dissolved in 1 mL of 50 mM Tris−HCl (pH 8.1) containing 8 M urea and then the solution was reduced via 0.1 mL of 0.1 M DTT for 20 min at 50 °C. After cooling to room temperature, BSA and Cyt-c were alkylated in the dark with 0.1 mL 0.1 M IDA for 15 min at room temperature. The pretreated BSA and Cyt-C sample (0.2 mg/mL) was pump through the IMER by using the peristaltic pump at 37 °C in cabinet drier for 10 min. After digestion, the