Chemical Engineering Journal 287 (2016) 148–154

Contents lists available at ScienceDirect

Chemical Engineering Journal

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

Low back-pressure hierarchically structured multichannel microfluidic bioreactors for rapid protein digestion – Proof of concept

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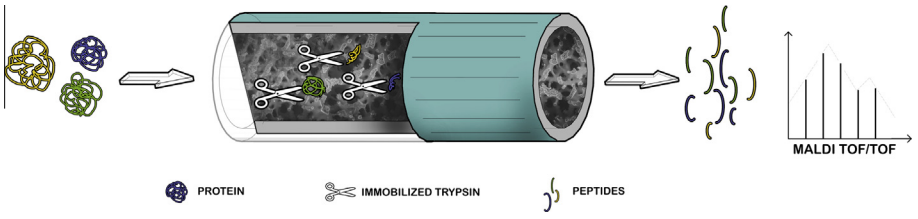
HIGHLIGHTS

- Low back pressure hierarchically structured microfluidic reactors.
- Intensive mass transport induced by torturous and meandering pore structure.
- Trypsin immobilized microreactors for efficient protein digestion.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 June 2015
Received in revised form 5 October 2015
Accepted 26 October 2015
Available online 14 November 2015

Keywords:
Continuous protein digestion
Immobilized enzyme reactor
Microfluidic reactor

ABSTRACT

A novel, easy-to-fabricate monolithic enzymatic microreactor with a hierarchical, torturous structure of flow-through channels of micrometric sizes and large mesopores was shown to enable rapid and very efficient digestion of proteins at high yields and exceptionally low back-pressures. Four silica monoliths with bi-modal 3D pore structure in micrometer and nanometer size scales were synthesized and characterized for structural and flow properties. The monolith with the highest total pore volume (4 cm^3/g) and flow-through channels 20–30 μm in size, was further functionalized with trypsin to obtain multichannel immobilized enzyme (proteolytic) reactor (IMER). The value of permeability coefficient K evaluated for water ($\sim 2.0 \cdot 10^{-11}$) was found to be two orders of magnitude higher in the novel reactor than reported before for high-performance IMERs, enabling the flow rates of 750 mL/cm^2 min at pressure gradients of 64 kPa/cm. Very high practical potentials of the novel microbioreactor were demonstrated in the proteolysis of cytochrome c (Cyt-c) and myoglobin (Myo), without any earlier pretreatment. MALDI-TOF/TOF mass spectrometry analysis of sequence coverage was high: 70% (Cyt-c) and 90% (Myo) for 24 min digestion, and 39% (Cyt-c) and 53% (Myo) when the proteolysis time was reduced to 2.4 min. The proposed microreactors make full use of all advantages of microfuidic devices and mesoporous biocatalysts, and offer exceptional possibilities for biochemical/proteolytic applications in both large (production) and small (analytical) scales.

1. Introduction

Protein digestion is an effective and inexpensive method to convert a protein into free amino acids and short chain peptides [1–4]. Therefore, it is indispensable for efficient protein identification, and hence crucial for the advancement of proteome studies [4–6], and also for the manufacture of bioactive peptides [3], which can play an important role in health promotion and risk reduction. But currently, the bioactive peptides are manufactured by rather expensive methods: transgenic, recombinant and complex synthetic procedures, that restrict their preparation and commercialization on a larger scale [3].

Conventionally, the proteolytical digestion of protein is performed in solution for several hours (12–24 h), with low concen-

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http://dx.doi.org/10.1016/j.cej.2015.10.120
1385-8947/

trations of enzyme to avoid the autodigestion of trypsin, which might produce excessive amounts of undesired tryptic fragments and complicate the unambiguous assignment of the studied protein [7,8]. The immobilization of trypsin on to various carriers has been proposed to speed up the process, while avoiding autodigestion [2,7,9–11]. This paved the way to the on-column digestion systems, which in the last decade evolved into continuous-flow immobilized enzyme microreactors (IMER) [7–10,12–14]. Microreactor technology offers several key advantages including: drastically reduced reaction time due to the large surface-to-volume ratio and very intensive mass transport typically observed in micrometric channels [15]. Unlike in-solution digestion, typically performed in batch reactors, a continuous protein digestion in IMERs offers important advantages: enzyme denaturation is reduced, improving efficacy, operational stability and reproducibility, and importantly, IMERs can easily be coupled with different mass spectrometry devices to obtain very efficient on-line systems for rapid protein digestion, identification and mapping [10].

For protein identification the most important factor is the sequence coverage. Therefore, small IMERs or chips have been used to efficiently produce the amount of peptides sufficient to be identified by MS [3,4,6]. For bioactive peptide manufacturing, the capacity and high productivity of IMERs are of major significance, enabling a simple, effective, inexpensive and rapid cleavage of peptides [3].

Irrespective of the applied enzyme support: polymer [16], silica or hybrid [17,18], the porous structure and surface character of IMERs are important factors. Applying hydrophilic supports decreases the nonspecific adsorption of proteins or peptides on to IMERs [1,2,19]. The porous structure of monolithic IMERs affects not only flow permeability (back pressure vs. flow rate dependence) but also digestion efficiency, which depends on the apparent rate of protein digestion controlled by the rate of diffusion into the pores and the size of activated surface area. In this respect, IMERs filled with structured packing or monolithic supports synthesized using templating approach or porogenes proved to be superior [8,20–22]. However, the back-pressure applied to reach the required flow rates was of the order of a few megapascal [9,12,23], and it was reduced to ca. 1.5–2 MPa in monolithic IMERs specifically designed for low-backpressure operation [8,21]. Clearly, they necessitate the use of high pressure metering pumps, and it is neither convenient nor cost-effective.

Significant progress in IMER-based protein digestion can be achieved by application of hydrophilic silica monoliths with a hierarchical bi-modal pore structure synthesized using the Nakanishi method [24]. Although initially devised for chromatographic applications, they have been more recently applied as microreactors for the efficient synthesis of fine chemicals [18,25,28]. Due to the abundant presence of large textural flow-through pores (micrometer in size), the backpressure could be reduced to about one megapascal, even for major flow rates [1,6]. Moreover, the application of a double templating approach, proposed by Smått et al. [26] and modified by Pudło et al. [27], gave the monoliths with even larger flow-through pores (30–40 μm), as applied in advanced microfluidic devices, and also larger surface areas, arising from the presence of mesopores of about 20 nm in diameter, thus similar to the most effective biocatalysts [20,22]. Herein, we propose the preparation of microfluidic IMERs making use of the latter concept and demonstrate their huge practical potentials in proteolytic digestion.

2. Materials and methods

2.1. Chemicals

Tetraethoxysilane (TEOS), polyethylene glycol 35,000 (PEG), cethyltrimethylammonium bromide (CTAB), myoglobin, cytochrome c, N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), 3-aminopropyltrimethoxysilane (APTS) trifluoroacetic acid (TFA) were from Sigma–Aldrich. 2-Cyanoethyltriethoxysilane (CNTS) was from Lancaster. Cyano-4-hydroxycinnamic acid (HCCA) was from Bruker. Glutaraldehyde (GLA) and other chemicals were purchased from Avantor.

2.2. Synthesis of silica monoliths (MH)

Four silica monoliths were prepared by means of the Nakanishi method with minor modifications [28–30], to obtain samples with bi- and even tri-modal hierarchical pore structure. The applied method is based on a meticulous control of concomitant phase separation and the sol–gel process. By changing the reagent' composition (Table 1) and the post synthesis treatment (vide infra) four monoliths (MH1–MH4) of different pore structures were obtained. The general procedure was as follows: PEG was dissolved in aqueous HNO_3, after which TEOS was added slowly to the PEG solution in an ice bath followed by the addition of CTAB. The solution was mixed, then left to gel in polypropylene tubes at 40 °C and aged for 10 days (MH1) or 3 days (MH2–MH4) at the same temperature. Next, the alcogels obtained were impregnated with ammonia solutions: 1 M for 9 h at 90 °C (MH1), or 0.1 M for 20 h at 40 °C (MH2 and MH4) or water (3 days at room temperature) followed by 1 M ammonia solution for 24 h at 80 °C (MH3). Before drying, the samples were washed with water and then calcined at 550 °C for 8 h (ramp of 1 °C min^{-1}) to obtain silica rods 40 mm in length and 4 or 6 mm diameter. The monoliths were functionalized and clad with polymer resin (L285MGS-H285MGS type) to obtain single-rod multichannel microfluidic microreactors.

2.3. Modification of monoliths and immobilization of trypsin

A single silica rod was immersed in either APTS or CNTS solution (0.15 mmol/mL) in toluene and held for 24 h at 80 °C under intensive stirring, and then extensively washed with ethanol and dried.

Prior to trypsin adsorption, the CNTS functionalized monolith of MH1 type was washed with ethanol and distilled water for 45 min (flow rate of 1 mL/min) and then with 0.05 M borate buffer (pH 7.5) containing 10 mM $CaCl_2$. Next, a solution of trypsin (5 mL) in borate buffer was pumped (1 mL/min, 2.5 h) through the reactor under recycling conditions to immobilize the protein. Excess protein was removed by washing the columns with a 0.05 M borate buffer (pH 7.5) with 10 mM $CaCl_2$ for 0.5 h.

Before the attachment of trypsin the APTS functionalized silica monolith (MH1) was washed with ethanol and distilled water for 45 min (1 mL/min), followed by 0.1 M phosphate buffer (pH 7.0). To attach aldehyde groups on to the monolith's surface a 2.5 vol. % GLA solution in 0.1 M phosphate buffer (pH 7.0) was cycled (1 mL/min) through the reactor for 45 min, whereupon it was washed with water and 0.05 M borate buffer (pH 7.5) containing 10 mM $CaCl_2$. Finally, 5 mL of trypsin solution in borate buffer was passed through the reactor as described above. Excess protein was removed by washing as was described in [20]. Prior to activity

Table 1
Molar ratio of reagents and parameters of monoliths pore structure determined from nitrogen adsorption and mercury porosimetry.

Sample	Molar ratio of reagents TEOS:PEG:H_2O:HNO_3:CTAB	S_{BET} [m^2/g]	V_{pN2} [cm^3/g]	d_{pN2} [nm]	V_{tHg} [cm^3/g]
MH1	1:0.52:14.25:0.26:0.027	287	1.02	2.5/22	4.0
MH2	1:0.59:14.17:0.27:0	650	1.06	7.8	2.5
MH3	1:0.63:14.92:0.25:0	413	1.17	11.3	3.2
MH4	1:0.63:15.58:0.41:0	595	1.16	7.8	3.0