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Trypsin immobilization on three monolithic disks for on-line protein digestion

R. Nicoli [a], N. Gaud [a], C. Stella [b], S. Rudaz [a], J.-L. Veuthey [a,*]

[a] *Laboratory of Analytical Pharmaceutical Chemistry, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 20 Bd d'Yvoy, 1211 Geneva 4, Switzerland*
[b] *Laboratory of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest-Ansermet, 1211 Geneva 4, Switzerland*

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Abstract

The preparation and characterization of three trypsin-based monolithic immobilized enzyme reactors (IMERs) developed to perform rapid on-line protein digestion and peptide mass fingerprinting (PMF) are described. Trypsin (EC 3.4.21.4) was covalently immobilized on epoxy, carbonyldiimidazole (CDI) and ethylenediamine (EDA) Convective Interaction Media® (CIM) monolithic disks. The amount of immobilized enzyme, determined by spectrophotometric measurements at 280 nm, was comprised between 0.9 and 1.5 mg per disk. Apparent kinetic parameters K_m^* and V_{max}^*, as well as apparent immobilized trypsin BAEE-units, were estimated in flow-through conditions using *N*-α-benzoyl-L-arginine ethyl ester (BAEE) as a low molecular mass substrate. The on-line digestion of five proteins (cytochrome *c*, myoglobin, α_1-acid glycoprotein, ovalbumin and albumin) was evaluated by inserting the IMERs into a liquid chromatography system coupled to an electrospray ionization ion-trap mass spectrometer (LC-ESI–MS/MS) through a switching valve. Results were compared to the in-solution digestion in terms of obtained scores, number of matched queries and sequence coverages. The most efficient IMER was obtained by immobilizing trypsin on a CIM® EDA disk previously derivatized with glutaraldehyde, as a spacer moiety. The proteins were recognized by the database with satisfactory sequence coverage using a digestion time of only 5 min. The repeatability of the digestion (R.S.D. of 5.4% on consecutive injections of myoglobin 12 μM) and the long-term stability of this IMER were satisfactory since no loss of activity was observed after 250 injections.

Keywords: Immobilized enzyme reactors; Immobilized trypsin; Peptide mass fingerprinting; Convective interaction media® disks; LC–MS/MS

1. Introduction

Today, the use of liquid chromatography (LC) hyphenated with mass spectrometry (MS) has become the tool of choice for protein identification and characterization by peptide mass fingerprinting (PMF) [1,2]. Current protocols include proteolytic digestion of the sample followed by the separation of the resulting peptides using one-dimensional (1D) or two-dimensional (2D) LC [3]. Peptide identification is commonly performed using MS equipped with either matrix assisted laser desorption ionization (MALDI) [4,5] or electrospray ionization (ESI) sources [6,7]. However, ESI which can be coupled to all kinds of analyzers (single and triple quadrupole, ion trap or time-of-flight), is recognized as the source of choice. As a result, proteins can be identified by measuring the resulting peptide masses or by comparison of the obtained MS/MS fragmentation pattern with the theoretical proteolytic fragments from proteomic databases [8,9].

Trypsin is the most used proteolytic enzyme for protein digestion, which is usually performed in-solution with incubation protocols of 4–24 h [7,10]. The small amount of enzyme (trypsin to protein ratio of about 1:20 to 1:100 (w/w)) necessary to limit autoprotolysis induces long incubation times with important variability, since the generation of peptides from trypsin autodigestion may cause MS ionization suppression, making identification of the studied protein difficult. An interesting strategy to reduce autoprotolysis is achieved by immobilizing trypsin on solid supports to perform protein digestion in a continuous flow system [11].

* Corresponding author. Tel.: +41 22 379 63 36; fax: +41 22 379 68 08.
E-mail address: jean-luc.veuthey@pharm.unige.ch (J.-L. Veuthey).

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Immobilized enzyme reactors (IMERs) offer several advantages. First, the enzyme stability is improved and its catalytic activity is maintained for a longer period of time. Second, they are cost-effective and compatible with high-throughput analytical methods; they can be very easily inserted in a LC–MS system and used for several analyses [12,13]. Therefore, IMERs have received great attention in recent years and several enzymes have been already immobilized for proteomic [11,14–16] and metabolic studies [17–19], enantioselective analysis and synthesis [20,21], as well as for the identification of new potential enzyme inhibitors [22,23]. The choice of the ideal immobilization support depends both on the nature of the studied enzyme and characteristics of the selected sorbent in terms of surface area, mass transfer properties, thermal and chemical stability and costs [24]. In particular, trypsin was already immobilized on different kinds of supports, such as membranes [25], capillary columns and chips [26,27], porous polymeric or silica beads (e.g. Poroszyme® immobilized trypsin cartridge) [28,29], immobilized artificial membranes (IAM) [30], and silica [14–16,31] or polymeric [32–39] monolithic material. In bioreactors packed with porous beads, the substrate has to diffuse into the pores to interact with the active sites of the immobilized enzyme [32]. Thus, the low mass transfer observed with porous materials represents the rate limiting step. Because the generated backpressure in such bioreactors is high, the IMERs activity is often reduced with important digestion times. Recent development of silica or polymeric monolithic supports can provide useful alternative as they present good mass transfer properties, a large surface area and a low-pressure drop due to their macroporous and mesoporous structures [40,41]. In monoliths, the molecule reaches the interconnected pores by convection and the diffusion path is extremely short. Convective interaction media® (CIM) are methacrylate based (poly-glycidylmethacrylate-ethyleneglycol dimethacrylate) monolithic disks placed in dedicated housing [42] that have been already used in continuous flow systems as IMERs [39,41,43–45]. Different chemistries are commercially available and suitable for enzyme immobilization such as epoxy, carbonyldiimidazole (CDI) and ethylenediamine (EDA) derivatives.

To limit protein surface modifications and obtain stable linkage with the support, the immobilization technique must be carefully chosen. A variety of methods are now available, such as adsorption, entrapment, cross-linking and covalent binding [12]. For the latter, immobilized protein leakage is avoided and a multiple choice of supports is available. Moreover, to prevent loss or activity modifications of the immobilized enzyme, it is mandatory that the functional groups involved in linkage with the support are not situated in the enzyme active site.

Several strategies can be used to covalently immobilize trypsin on a CIM® disk. The coupling between native epoxy groups of CIM® disk and nucleophilic residues of trypsin under basic conditions was the first used technique. Recently, the CIM® CDI disk was made commercially available. The advantage of the latter is that the kinetic reaction between imidazole groups and nucleophilic residues of the enzyme occurs rapidly, which decreases the immobilization time from days to hours [39]. Both immobilizations are easily achieved, but, in some cases, the active sites of the immobilized enzyme are not accessible to the substrate resulting in the reduction of bioreactor efficiency. An improvement can be obtained by using a spacer, which enhances enzyme mobility and allows for higher enzymatic activity. As an example, the immobilization through Schiff base formation on a CIM® EDA disk, previously derivatized with glutaraldehyde, was successfully used by Bartolini et al. [22–24] for studies on immobilized acetylcholinesterase.

This paper describes the preparation and characterization of three trypsin-based monolithic bioreactors for PMF studies. Once the operating parameters assessed, the optimum conditions were retained for rapid (5 min) on-line digestion of five proteins by coupling the IMERs to an LC-ESI–MS/MS instrument through a switching valve. On-line results obtained with the disks were compared to in-solution digestions of 20 h in terms of obtained scores, number of matched queries and sequence coverages.

2. Experimental

2.1. Chemicals

CIM® epoxy, CDI and EDA disks (3 mm × 12 mm I.D.) were purchased from BIA Separations (Ljubjana, Slovenia). Trypsin from bovine pancreas, *N*-α-benzoyl-L-arginine ethyl ester (BAEE), bovine serum albumin (BSA; ∼66000 Da), myoglobin from equine heart (MYO; ∼16950 Da), albumin from chicken egg white (OVA; ∼42750 Da), α_1-acid glycoprotein from bovine plasma (AGP; ∼21560 Da), cytochrome *c* from equine heart (CYTC; ∼11700 Da), formic and phosphoric acids, potassium dihydrogen phosphate, ammonium bicarbonate (AMBIC), Tris(hydroxymethyl)aminomethane hydrochloride (TRIS–HCl), calcium chloride, urea, iodoacetamide, dithiothreitol, glutaraldehyde solution 25% in water and sodium cyanoborohydride were purchased from Sigma–Aldrich (St-Louis, USA). Acetonitrile was of HPLC grade from Panreac Quimica (Barcelona, Spain), water was obtained from a Milli-Q Waters Purification System (Millipore, Bedford, MA, USA) and was used to prepare buffers and standard solutions.

2.2. Protein pre-treatment and in-solution incubation protocols

A classical in-solution protocol was used to digest the five selected proteins (CYTC, MYO, AGP, OVA, BSA): 200 μg of the target protein was dissolved in 200 μl of 100 mM AMBIC buffer pH 8.0 containing 6 M urea and 10 mM dithiothreitol. The mixture was heated at 60 °C for 60 min in the dark using a thermostatic mixer under agitation (800 rpm) to denature the protein. Reduced cysteines were carbamidomethylated by adding 50 μl of 100 mM iodoacetamide in 100 mM AMBIC to the denatured protein-solution. The mixture was mixed and allowed to stay for 30 min at room temperature in the dark. A Microcon® YM-10 kDa centrifugal filter unit (Millipore, USA) was used to desalt and concentrate the protein sample up to 100 μl prior to in-solution and on-line digestions.