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

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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].

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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.

For the in-solution tryptic digestions, a freshly prepared 0.8 mg/ml trypsin-solution in 100 mM AMBIC was added to the protein-solution to obtain a trypsin/protein ratio of 1:50 (w/w). The incubation was performed for 20 h at 37 °C under stirring on a thermostatic mixer (800 rpm). The reaction was stopped in ice adding 10 μl of formic acid 50% (v/v) (pH around 3). The obtained supernatant was injected (5 μl) into LC–MS/MS system for peptides separation and proteins identification (Section 2.5).

2.3. Trypsin immobilization on CIM® disks

Three monolithic disks (CIM® epoxy, CDI and EDA) were used as supports for trypsin immobilization (Fig. 1).

2.3.1. Immobilization on CIM® epoxy disk

At first, trypsin was covalently immobilized on a CIM® epoxy disk at pH 8.0 for 24 h at 25 °C under gentle shaking. A 2.5 mg/ml solution of trypsin was freshly prepared in 0.5 M phosphate buffer pH 8.0 and, after equilibrating the CIM® epoxy disk with this buffer for 1 h, 2.0 ml of the trypsin-solution (containing 50 mM benzamidine as autodigestion inhibitor) was percolated through the disk inserted into its housing to completely fill the monolithic pores, as described by Vodopivec et al. [43]. The monolithic disk was removed and placed in a glass beaker with the trypsin-solution and incubated for 24 h at 25 °C. After, the support was washed with 2.0 ml of 0.5 M phosphate buffer pH 8.0 for 1 h. The residual enzyme and washing solutions were recovered and the immobilized enzyme determined by UV-spectroscopy at 280 nm. The disk was then stirred with 2 ml of 1 M monoethanolamine in 0.5 M phosphate buffer pH 7.5 for 3 h at 25 °C for the end-capping of the remaining epoxy groups. The disk was rinsed with 2 ml of 0.5 M phosphate buffer pH 8.0 for 30 min, inserted into its housing, connected to the HPLC system and equilibrated with the mobile phase consisting of 20 mM AMBIC pH 8.0 for 1 h at a flow rate of 1.0 ml/min. If not used immediately, the immobilized trypsin CIM® epoxy disk was stored in 0.5 M phosphate buffer pH 3.0 at 4 °C.

2.3.2. Immobilization on a CIM® CDI disk

The disk was inserted into its housing and washed by percolating 2 ml of water and 2 ml of 0.5 M phosphate buffer pH 8.0 to remove ethanol 96% (shipping solution) and stabilize the disk. A syringe was filled with 2.0 ml of freshly prepared trypsin-solution (2.5 mg/ml in 0.5 M phosphate buffer pH 8.0) and was percolated through the CIM® CDI disk at a flow rate of 0.4 ml/min. An empty syringe was connected to the other side of the housing to collect the flow through. This procedure was repeated for 2 h at 25 °C at regular time intervals of 15 min. Afterwards, the immobilized disk was washed with 2 ml of 0.5 M phosphate buffer pH 8.0 and 2 ml of water. The residual enzyme and washing solutions were recovered and the immobilized enzyme determined by UV-spectroscopy at 280 nm. After immobilization, the residual groups spontaneously self-deactivate in aqueous solution avoiding the use of monoethanolamine. The disk was connected to the HPLC system and equilibrated with the mobile phase consisting of 20 mM AMBIC pH 8.0 for 1 h at a flow rate of 1.0 ml/min. If not used immediately, the immobilized trypsin CIM® CDI disk was stored in 0.5 M phosphate buffer pH 3.0 at 4 °C.

2.3.3. Immobilization on CIM® EDA disk

The CIM® EDA disk was first equilibrated with 50 mM phosphate buffer pH 7.5 in a glass beaker for 1 h and then covered with 10 ml of a 10% glutaraldehyde solution (v/v) in 50 mM phosphate buffer pH 7.5 and reacted overnight in the dark at 25 °C. Then, the derivatization solution was removed and the disk was rinsed with 0.5 M phosphate buffer pH 7.5 for 30 min. After equilibrating the disk with 0.5 M phosphate buffer pH 3.0 for 1 h, 2.0 ml of the trypsin-solution (2.5 mg/ml

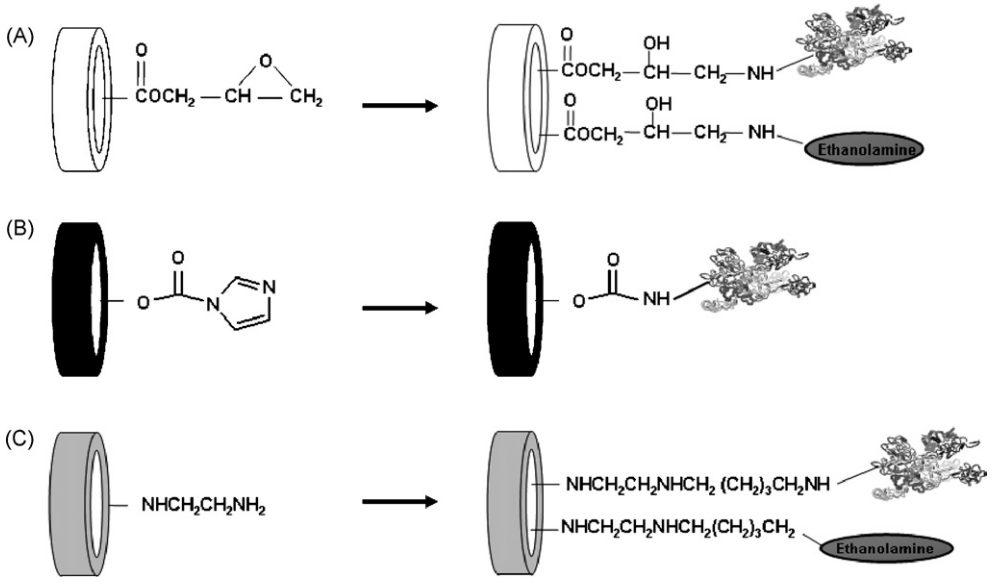

Fig. 1. Trypsin immobilization on a: (A) CIM® epoxy disk; (B) CIM® CDI disk; (C) CIM® EDA disk. For detailed protocols see: Section 2.3.

in 0.5 M phosphate buffer pH 3.0) was percolated through the disk inserted into its housing to fill the monolithic pores. Then, the monolith was removed and placed in a glass beaker with the trypsin-solution for 24 h at 25 °C. The disk was washed with 2.0 ml of 0.5 M phosphate buffer pH 3.0 for 1 h. The residual enzyme and washing solutions were recovered and the immobilized enzyme was determined by UV-spectroscopy at 280 nm. The Schiff bases were reduced by stirring the immobilized trypsin disk with 10 ml of 0.1 M cyanoborohydride solution in 0.5 M phosphate buffer pH 3.0 for 2 h at 25 °C in the dark. The disk was then washed with 0.5 M phosphate buffer pH 3.0 for 30 min to remove the reductive agent and covered with 2 ml of 1 M monoethanolamine in 0.5 M phosphate buffer pH 7.5 for 3 h at 25 °C for the end-capping of the remaining free groups. The disk was then washed with 2 ml of 50 mM phosphate buffer pH 7.5 for 30 min, inserted into its housing, connected to the HPLC system and equilibrated with the mobile phase consisting of 20 mM AMBIC pH 8.0 for 1 h at a flow rate of 1.0 ml/min. If not used immediately, the immobilized trypsin CIM® EDA disk was stored in 0.5 M phosphate buffer pH 3.0 at 4 °C.

2.4. Assays for determining IMERs activity

2.4.1. Amount of immobilized trypsin

The amount of immobilized trypsin was evaluated by measuring the difference in the UV absorbance at 280 nm of trypsin-solutions before and after the immobilization procedures using a PerkinElmer double-beam Lambda 20 UV/Vis spectrophotometer (Waltham, MA, USA).

2.4.2. Michaelis–Menten studies

Apparent kinetic parameters, K_m^* and V_{max}^*, were estimated by inserting the bioreactors in a continuous flow system composed of a HP1100 isocratic pump (Agilent, Waldbronn, Germany) set at 1.0 ml/min and an autosampler. Increasing concentrations of the substrate BAEE (5–200 mM) were injected (V_{inj} = 5 μl) through the selected IMER (maintained at 25 °C) with 50 mM TRIS pH 8.0 containing 10 mM $CaCl_2$ as the mobile phase. Fractions were collected in volumetric flasks for 3 min and the absorbance of the obtained product benzoyl-L-arginine (BA) was measured by UV-spectroscopy at 255 nm, as described by Schwert and Takenaka [46]; the contribution of the substrate BAEE was subtracted for each concentration. The initial reaction velocity (expressed in ΔA/min) was plotted as a function of the injected BAEE concentration and the apparent K_m^* and V_{max}^* were estimated by fitting the experimental points with the Michaelis–Menten equation using Prism 4.0 (GraphPad Software, San Diego, CA, USA).

2.4.3. Estimation of the apparent immobilized trypsin BAEE-units

The apparent international enzyme units (U^*), defined as the amount of immobilized enzyme that converts 1 μmol of BAEE per minute at pH 8.0 and 25 °C can be obtained from the apparent V_{max}^* by using Eq. (1):

$$U^*\,(\mu\text{mol/min}) = \frac{(V_{max}^*) \times V \times 10^6}{808} \quad (1)$$

where V corresponds to the collected volume (3 ml) and 808 corresponds to the differential molar absorbance of BAEE against BA at 255 nm, as determined by Kedzy et al.[47]. These units can be easily converted to the most used apparent immobilized trypsin BAEE-units, defined as the amount of enzyme that catalyzes a change in absorbance of 0.001 per minute at 253 nm, 25 °C, pH 7.6 in a reaction volume of 3.2 ml [48], using the conversion factor determined by Bergmeyer et al. [49] (i.e. 270 BAEE-units correspond to 1 international enzyme unit at 25 °C).

In-solution, the determination of BAEE-units was performed using a standard protocol to certify that no trypsin degradation occurred after long-term storage in the freezer (−20 °C).

2.5. IMERs-LC-ESI–MS/MS system and on-line protein digestion

Once the kinetic characteristics were determined, IMERs were compared for the on-line digestion of proteins using an HPLC HP1100 series modular system (Agilent, Waldbronn, Germany) coupled through the column-switching set-up reported in Fig. 2 to an ion trap MS Esquire 3000+ (Bruker Daltonics, Billerica, MA, USA). The system was controlled by Agilent Chemstation (v. 10.02) and Esquire Control (v. 5.2.) softwares. The first dimension of the set-up consisted of an isocratic pump, an autosampler, an oven set at 37 °C containing the bioreactor, a C_{18} trapping column (C_{18} Nucleosil, 5 μm, 8 mm × 4 mm I.D.) and an UV–vis detector. The C_{18} trapping column was inserted to retain, desalt and concentrate the digested peptides. The protein sample (50 μl) was loaded onto the enzymatic column, with a solution of 20 mM AMBIC pH 8.0 used as mobile phase at 0.4 ml/min. The second dimension consisted of a binary gradient pump coupled to a diode-array detector (DAD) and the ion trap MS. The analytical column was a C_{18} X-Bridge 3.5 μm, 100 mm × 2.1 mm I.D. (Waters, Milford, MA, USA). Retained compounds were back flushed from the trapping column in the gradient mode (solvent A: water + 0.1% formic acid, solvent B: acetonitrile + 0.1% formic acid; 0–30 min 4–27% B, 30–30.1 min 27–80% B, 30.1–40.0 min 80% B, 40.1–60 min 4% B) at 0.2 ml/min and the first 0.4 ml was diverted to the waste. MS experiments were carried out in positive mode under constant electrospray conditions: high voltage (HV) capillary 4000 V, nebulizer gas 60 psi, drying gas 10.0 l/min and dry temperature 350 °C. To detect peptides, the instrument was set in Auto (MS^2) mode with an ion charge control (ICC) target of 40000. Five full MS scans between 100 and 3000 Th were acquired followed by 15 average MS/MS spectra with one precursor ion selected for MS/MS experiments. Mascot® software and Swissprot® database were used to identify the studied proteins from uninterpreted MS/MS spectra of the obtained peptides. The MS/MS ion search parameters were set as follow: 2 missed cleavages were allowed with variable peptide modifications (carbamidomethyl (C) and oxidation (M)), peptide mass tolerance of ±2 Da and MS/MS tolerance of ±1.5 Da.

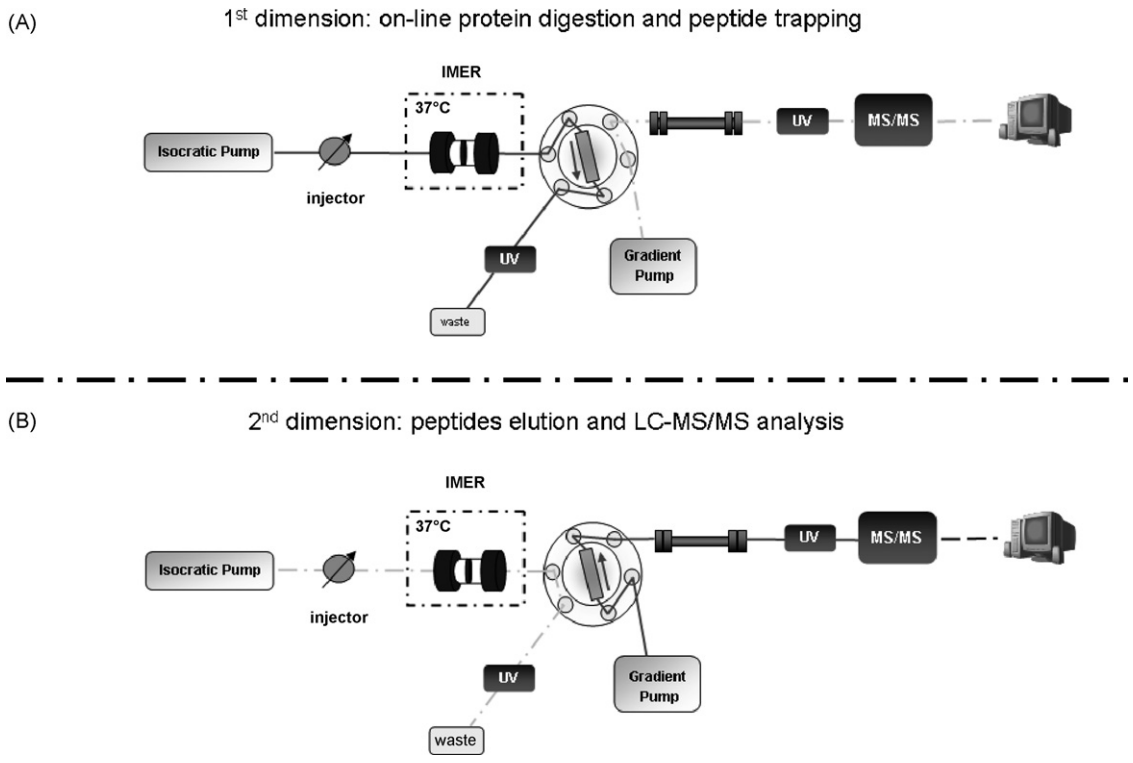

Fig. 2. Chromatographic set-up to perform on-line protein digestion (see Section 2.5).

3. Results and discussion

3.1. Assays for determining IMERs activity

3.1.1. Amount of immobilized trypsin

The amount of immobilized trypsin was evaluated by measuring the decrease in UV absorbance (at 280 nm) of the trypsin-solution before and after immobilization procedures. A calibration curve using several trypsin-solutions (12.5–500 μg/ml) was used for quantification ($y = 0.0012x + 0.011$; $r^2 = 0.9994$). For the three bioreactors, the amount of immobilized enzyme was comprised between 0.9 and 1.5 mg (Table 1), in agreement with the literature [43,50]. However, the amount of immobilized enzyme only represents the binding capacity of the support without any information on some putative steric hindrances that may interfere and reduce IMERs activity. Therefore, the apparent kinetic parameters K^*_m and V^*_{max} as well as the apparent immobilized trypsin BAEE-units were obtained to compare enzymatic activity.

3.1.2. Michaelis–Menten studies

According to the theory, Michaelis–Menten kinetic parameters, K_m and V_{max}, can only be evaluated in the presence of a catalytic amount of enzyme. In case of IMERs, the direct estimation of such parameters is impossible due to the large amount of immobilized trypsin, making any comparison with in-solution parameters difficult. In fact, the active trypsin to protein ratio in such heterogeneous systems is about 5:1 (w/w), instead of 1:50 (w/w) for the in-solution digestions. Moreover, the mobility of the immobilized enzyme can be severely restricted, due to the coupling with the support, requiring several modifications on the conventional Michaelis–Menten kinetics model, based on diffusion-limited reactions.

In this study, the apparent kinetic parameters, K^*_m and V^*_{max}, were estimated for the comparison of IMERs efficiency in the conversion of BAEE and the calculation of the apparent immobilized BAEE-units (Section 3.1.3). The initial reaction velocity was obtained by dividing the BA absorbance at 255 nm (the contribution of BAEE was subtracted for each concentration) by the contact time (0.34 min), estimated as the ratio of CIM disks volume (0.34 ml) and flow rate (1 ml/min). The apparent K^*_m and V^*_{max} were extrapolated plotting the initial reaction velocity (expressed in ΔA/min) to the injected BAEE concentrations (Fig. 3) and fitting the experimental points with the Michaelis–Menten equation (Table 1). The CIM® EDA disk

Table 1
Amount of immobilized trypsin, apparent kinetic parameters and apparent IMERs activity

CIM® disks	Immobilized trypsin (mg)	K^*_m (mM)	V^*_{max} (ΔA/min)	IMER activity (BAEE-units/mg)
Epoxy	0.9	81.7	0.43	479
CDI	1.5	46.4	0.23	157
EDA	1.1	105.8	0.59	539

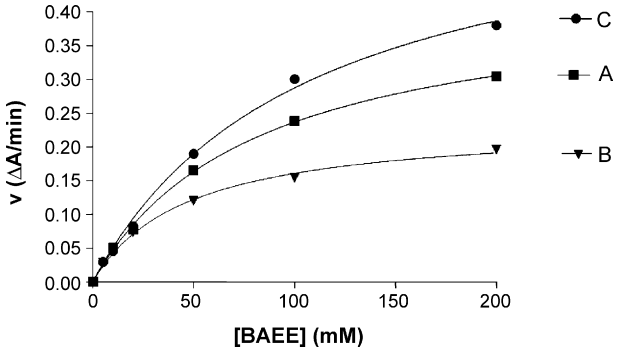

Fig. 3. Michaelis–Menten plot using immobilized trypsin on: (A) CIM® epoxy disk; (B) CIM® CDI disk; (C) CIM® EDA disk (see Section 2.4.2).

based bioreactor had the highest maximum reaction velocity ($V^*_{max} = 0.59\,\Delta A/\text{min}$) with an apparent affinity constant K^*_m of 105.8 mM. For the other IMERs, the obtained V^*_{max} values were lower (0.43 and 0.23 ΔA/min for CIM® epoxy disk and CIM® CDI disk-based bioreactors, respectively), suggesting minor activity in the conversion of BAEE.

3.1.3. Estimation of the apparent immobilized trypsin BAEE-units

First, the in-solution determination of BAEE-units was performed. The obtained value (13164 BAEE-units/mg of trypsin) was in agreement with the value determined by the manufacturer (12700 BAEE-units/mg of trypsin), indicating that no degradation occurred after long-term storage at −20 °C.

In a second step, the activity of the three IMERs was compared and the estimation of the apparent immobilized BAEE-units was performed using Eq. (1) and the conversion factor determined by Bergmeyer et al. (×270) [49]. The EDA bioreactor presented an activity of 539 apparent immobilized BAEE-units/mg (Table 1). Similar results were obtained for the CIM® epoxy disk bioreactor (479 apparent immobilized BAEE-units/mg), while the CIM® CDI disk based bioreactor presented 157 apparent immobilized BAEE-units/mg only. For the latter, the low activity could be explained by the choice of pH 8.0 and the absence of benzamidine during the immobilization process. To confirm these results, IMERs were compared for the on-line digestion of five proteins.

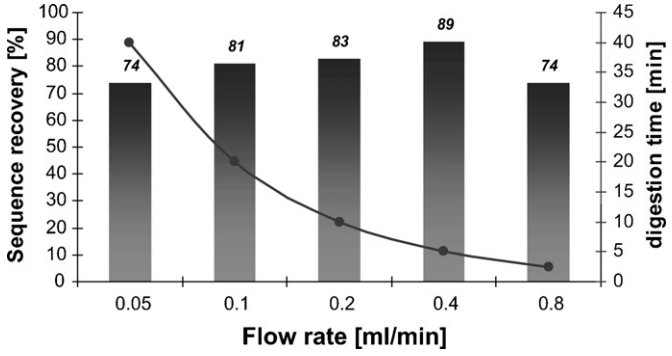

Fig. 4. Influence of the digestion flow rate on sequence recovery of MYO 12 μM standard samples. A flow rate of 0.4 ml/min was retained, corresponding to a digestion time of 5 min. For detailed analytical conditions, see: Section 2.5.

Table 2
Comparison between off-line (in-solution) and on-line digestion of tested proteins in terms of matched queries, score and sequence coverage

	CYTC			MYO			AGP			OVA			BSA		
	Matchs	Score	Seq. cov. (%)	Matchs	Score	Seq. cov. (%)	Matchs	Score	Seq. cov. (%)	Matchs	Score	Seq. cov. (%)	Matchs	Score	Seq. cov. (%)
In-Solution	4	190	54	34	829	88	13	709	50	15	763	46	31	1368	51
CIM® Epoxy	5	247	54	12	530	64	3	165	19	0	0	0	24	878	42
CIM® CDI	0	0	0	6	301	43	5	234	27	0	0	0	13	457	22
CIM® EDA	9	416	62	29	751	89	19	572	47	13	684	41	47	1977	62

3.2. IMERs-LC-ESI–MS/MS system and on-line protein digestion

After IMERs characterization and before performing on-line digestion studies, the appropriate digestion flow rate was determined by injecting a standard sample of MYO 12 μM. As reported in Fig. 4, the best sequence coverage was obtained using a flow rate of 0.4 ml/min, corresponding to a digestion time of 5 min. These results show that the use of high digestion flow rates (up to 0.4 ml/min in our study) increases the enzymatic activity. Analytes diffusion path in monolithic supports is extremely short and immobilized enzyme-protein interaction is based mainly on convection. This phenomenon is in agreement with results already observed by Josic et al. [41,51].

After, five carbamidomethylated proteins, namely CYTC (∼11700 Da), MYO (∼16950 Da), AGP (∼21560 Da), OVA (∼42750 Da) and BSA (∼66000 Da), were injected (12 μM, 50 μl) into the column switching set-up reported in Fig. 2, at a flow-rate of 0.4 ml/min. The system was fully automated and peptides were retained, desalted and concentrated on the C_{18} trapping column prior to their separation and MS identification. Obtained MS/MS spectra for each peptide were compared to the theoretical proteolytic fragments using Swissprot® database and Mascot® software. Bioreactors were characterized in terms of obtained scores, number of matched queries and sequence coverages with in-solution digestions (20 h) and results are reported in Table 2.

3.2.1. Immobilized trypsin CIM® epoxy disk

Trypsin immobilization on CIM epoxy disk was performed for 24 h at pH 8.0 in a single reaction step. First, the trypsin-solution was percolated through the disk to completely fill the monolithic pores as described by Vodopivec et al. [43]. This technique facilitates the access of trypsin to the pores enhancing the immobilization yield by reducing the diffusion process. The pH 8.0 was chosen as the optimal pH to perform the coupling reaction between the epoxy groups and the amino residues of trypsin, even if at this pH, the enzyme is potentially subjected to autodigestion. To prevent autoprotolysis, a solution of 50 mM benzamidine was used during the immobilization. The importance of the inhibitor was demonstrated by Deutscher et al. [52] and used by Bencina et al. [39] for trypsin immobilization. A solution of 1 M monoethanolamine was employed for the end-capping of the remaining free epoxy groups, preventing possible side reactions between immobilized trypsin and the disk and limiting non-specific interactions with the injected proteins.

This IMER presented, except for CYTC, lower sequence coverages compared with in-solution digestions. However, all injected proteins were recognized (except OVA), but with low and unsatisfactory scores (Table 2). The repeatability of the digestions was assessed on the sequence coverage percentages of a MYO digestion and a relative standard deviation of 7.1% ($n = 3$) was obtained. This kind of immobilization was easily achieved, but all active sites of the immobilized enzyme were probably not accessible to the protein due to steric hindrances. Moreover, the relatively low activity could be due to immobilization performed at pH 8.0; trypsin should be subjected to autodigestion and partially immobilized in its inactive form, reducing the bioreactor efficiency. The presence of an autodigestion inhibitor, benzamidine, was not sufficient to recover satisfactory activity.

3.2.2. Immobilized trypsin CIM® CDI disk

As a result of this particular surface chemistry, immobilization was achieved through the imidazole groups of the support and the nucleophilic residues of trypsin. This immobilization

Table 3
Comparison of peptide patterns of MYO 12 μM standard sample obtained using in-solution digestion (20 h) and immobilized trypsin CIM® EDA disk (5 min)

Peptide mass (Da)	# Missed cleavages	Peptide sequence	In-solution	CIM® EDA
2150.2543	2	ASEDLKKHGTVVLTALGGIL K		√
2110.1515	2	KKGHHEAELKPLAQSHATK	√	
1937.0167	2	LFTGHPETLEKFDKFK		√
1982.0566	1	KGHHEAELKPLAQSHATK	√	√
1661.8533	1	LFTGHPETLEKFDK	√	
1506.9366	1	HGTVVLTALGGILKK	√	√
1360.7583	1	ALELFRNDIAAK	√	√
1083.5612	1	HLKTEAEMK	√	
941.4727	1	YKELGFQG		√
735.4875	1	HKIPIK	√	
1885.0218	0	YLEFISDAIIHVLHSK	√	√
1853.9616	0	GHHEAELKPLAQSHATK	√	√
1815.9024	0	GLSDGEWQQVLNVWGK	√	√
1606.8547	0	VEADIAGHGQEVLIR	√	√
1502.6692	0	HPGDFGADAQGAMTK	√	√
1378.8416	0	HGTVVLTALGGILK	√	√
1271.6630	0	LFTGHPETLEK	√	√
Seq. cov. (%)		88		89
Score		829		751
Queries matched		34		29

approach is very rapid due to the favourable kinetic characteristics of the coupling and was performed in 2 h at pH 8.0 instead of 24 h for other described immobilization protocols. Moreover, free remaining imidazole groups of the supports rapidly self-deactivate after the immobilization process, avoiding the use of end-capping agents such as monoethanolamine.

However, the bioreactor activity was low and poorly satisfactory in terms of sequence coverage percentages obtained for the injected proteins (Table 2). MYO, AGP and BSA gave lower scores and sequence coverages compared to in-solution digestions. OVA and CYTC were not recognized by the database, supporting the idea that this IMER was not fully adapted to rapid on-line protein digestion. Concerning MYO, 43% of the sequence was covered with a R.S.D. of 35.6% on three consecutive injections. This high variability was difficult to explain and several hypotheses could be made such as the trypsin overloading. This phenomenon was already observed by Temporini et al. [15], who indicated that the amount of immobilized enzyme and accessibility of active sites for the substrate have to be simultaneously considered for optimal IMERs activity.

Finally, as reported for the CIM® epoxy based bioreactor, the immobilization was performed at pH 8.0, where trypsin autoprotolysis could occur, even if the immobilization time was reduced to 2 h instead of 24 h. Complementary experiments should be performed at different immobilization times and in the presence of benzamidine to avoid or reduce autodigestion.

3.2.3. Immobilized trypsin CIM® EDA disk

In this case, trypsin was covalently immobilized on a CIM® EDA disk, previously derivatized with glutaraldehyde as a spacer molecule. The trypsin linkage was performed for 24 h in 0.5 M phosphate buffer pH 3.0. Schiff bases were reduced with 0.1 M cyanoborohydride solution in the same buffer (2 h at 25 °C in the dark) and free remaining aldehydic groups were end-capped with 1 M monoethanolamine at pH 7.5 (3 h at 25 °C). This procedure was completed in about 72 h.

From a qualitative point of view, no significant differences between results obtained with the CIM® EDA disk based bioreactor and in-solution digestions were obtained (Table 2). However, on-line digestions were performed in 5 min instead of 20 h. The peptide patterns obtained for MYO digestion by the on-line and off-line approaches were comparable (Table 3), with respect to molecular weight and number of missed cleavages (0, 1 or 2 missed cleavages). A satisfactory R.S.D. of 4.7% was

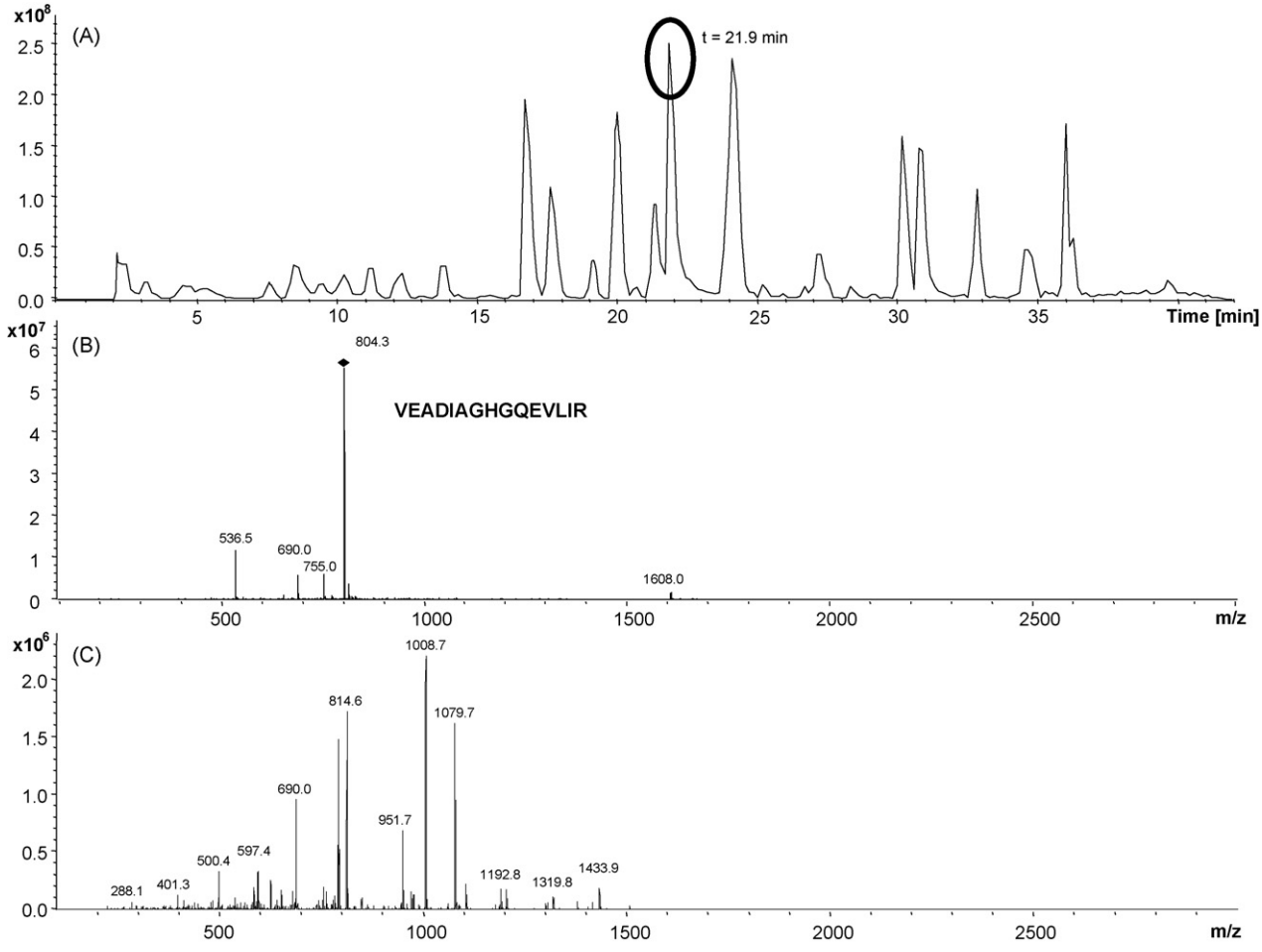

Fig. 5. On-line digestion of horse myoglobin 12 μM (50 μl) on immobilized trypsin CIM® EDA disk: (A) example of obtained LC–MS chromatogram (TIC); (B) example of full scan MS spectra (t = 21.9 min); (C) example of MS/MS spectra of the precursor ion detected at 804 Th.

obtained on three consecutive MYO injections. These results demonstrated that the presence of a spacer is necessary to maintain an excellent trypsin-based IMER activity. In fact, it is well known that the spacer increases the mobility of the immobilized enzyme on the surface of the monolithic structure facilitating the interaction between active sites and injected proteins. Another advantage of this IMER is the selected pH for performing the immobilization. The Schiff bases were formed for 24 h at pH 3.0 avoiding trypsin autodigestion and immobilization of the denaturated enzyme. LC–MS chromatogram, as well as MS and MS/MS spectra corresponding to the peptide detected at 804.3 Th of a MYO sample are reported in Fig. 5. Due to the excellent results obtained with this IMER, it was used daily in our laboratory. The stability was evaluated by performing on-line digestions of MYO 12 μM standard samples. Obtained sequence coverages were reproducible (±10%) through approximately 250 injections performed in a 6-month period (data not shown).

4. Conclusions

The preparation and characterization of three new trypsin based monolithic IMERs for rapid on-line protein digestion have been described. Trypsin was covalently immobilized on CIM® epoxy, CDI and EDA disks and resulting bioreactors used for on-line digestion, peptide separation and PMF studies. The apparent kinetic parameters, K_m^* and V_{max}^*, as well as the apparent immobilized trypsin BAEE-units, were estimated and compared for the three bioreactors with BAEE as a low molecular mass substrate. Efficiency was then assessed with the rapid (5 min) on-line digestion of different proteins, by coupling the IMERs to a LC-ESI–MS/MS through a column switching configuration. The activity was compared in terms of matching queries, obtained score and sequence coverage percentages with classical in-solution digestions performed in 20 h. The most efficient IMER was obtained by immobilizing trypsin for 24 h at pH 3.0 on a CIM® EDA, using glutaraldehyde as a spacer. The use of a spacer enhanced trypsin mobility, facilitated the interaction with injected samples and improved the enzymatic activity. In fact, this IMER, with a V_{max}^* of 0.59 ΔA/min, a K_m^* of 105.8 mM, and 539 apparent immobilized BAEE-units/mg, was the most active IMER in the conversion of the low-molecular mass substrate BAEE. Moreover, all the on-line digested proteins were identified with satisfactory sequence coverage percentages and repeatability (R.S.D. of 5.4% for injections of three samples of MYO 12 μM). In addition, no significant difference in cleavage patterns was obtained relative to the in-solution digested proteins.

The bioreactor obtained with the CIM® epoxy was characterized by reduced activity likely due to spacer absence and the choice of pH 8.0, probably indicating trypsin autodigestion during the immobilization process. The addition of benzamidine was not sufficient to recover complete activity. The CIM® CDI disk based bioreactor was not suitable, but other immobilization experiments have to be outperformed to confirm these results.

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