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Development of a bioreactor based on trypsin immobilized on monolithic support for the on-line digestion and identification of proteins

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Abstract

The preparation and characterization of a new trypsin-based bioreactor is here described for on-line protein digestion and peptide analysis. Trypsin was immobilized on an epoxy-modified silica monolithic support with a single reaction step and the amount of immobilized enzyme was found to be 66.07 mg (±11.75 S.D.)/column ($n = 6$). The bioreactor was coupled through a switching valve to an analytical column for the on-line digestion, peptide separation and identification of test proteins by ESI-MS–MS. The influence of various parameters (flow rate, temperature, buffer pH and molarity, etc.) on enzymatic activity was investigated by an experimental design and the mostly significant factor was found to be the flow rate. The efficacy of the reported on-line bioreactor for tryptic mapping is reported for somatostatin and myoglobin, selected as model compounds. Tryptic peptide maps obtained by on-line digestion of myoglobin were compared to those obtained by traditional off-line digestion. Sequence coverage obtained with the on-line protocol (21 peptides, 75.16% coverage of myoglobin sequence) was found to be comparable to the one obtained with the off-line protocol (18 peptides, 76.47% coverage). Sensitivity for myoglobin digestion and identification was 0.1 mg/ml. The reproducibily of the peptide maps in terms of retention time was from 1.53 to 4.31%, R.S.D.

Keywords: Immobilized enzyme reactors; Immobilized trypsin; Monolithic columns; Peptides; Proteins

1. Introduction

The rapidly growing interest in the area of proteomics induces intensive efforts to find robust, automated and sensitive high-throughput analytical tools. The same types of analytical methods used in proteomics can be dedicated to the characterization of isolated or recombinant proteins [1,2]. One of the key advancements in rapid protein identification is peptide mapping, with the proteolytic digestion of the protein under study followed by separation and sequencing of the resulting peptide fragments. A powerful technique used for the separation and identification of peptides is high-performance liquid chromatography coupled with electrospray ionisation tandem mass spectrometry (LC–ESI-MS–MS). The acquired peptide fragments are compared with the theoretical proteolytic fragments predicted from proteomic database [3–5].

Protein identification via peptide mapping can be employed for a range of purposes including detection of pathological changes of proteins [6,7], screening of potential targets for drug interaction [8], detection of post-translational modification, identification of genetic variants and quality control of protein produced by recombinant DNA technology [9].

Trypsin is a widely used proteolytic enzyme and proteolysis can be performed in two ways: in solution (homogeneous) or by solid-phase digestion (heterogeneous). The established protocol for in solution tryptic digestion requires 4–24 h with extensive manual sample preparation and often it is the limitation step [10]. In view of the strong interest

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in high throughput analysis, the concept of solid-phase digestion (ex trypsin immobilization on a solid support) has received great attention in the last years because it offers several advantages [11–13]. Firstly, the rate of enzyme denaturation or inactivation is reduced allowing to maintain its catalytic activity for a longer time and providing good reproducibility. Secondly, immobilized trypsin is re-usable and finally the generation of peptide fragments caused by trypsin autoprotolysis, which may complicate the unambiguous assignment of the studied protein, is avoided. Therefore, the use of immobilized trypsin provides good reproducibility, better suitability for trace-level samples, is cost-effective and amenable to high-throughput automation and provide overall cost-effectiveness.

Trypsin can be immobilized on packing material, which can be either used for "batch wise" experiments, or directly immobilized into columns and used in continuous flow systems as immobilized enzyme reactors (IMERs) for the on-line proteolysis [14]. Several researchers have reported the use of immobilized trypsin columns for on-line digestion of proteins. In particular, elegant methods have been developed for the rapid digestion of proteins using Poroszyme immobilized trypsin cartriges [15–18]. These cartridges contain the enzyme attached to a hydrophilic cross-linked poly(styrene–divinylbenzene) support with a patented bimodal pore size distribution. Five percent methanol or acetonitrile is often recommended in order to reduce binding of hydrophobic peptides to the cartridges and enhance peptide recovery. In the reported applications mass spectrometry analysis of tryptic peptides is carried out after digest collection (i.e. in off-line method). On the other hand few papers report automated methods for the tryptic digestion, transfer of the peptides to a trapping column for concentration and desalting, automatic elution from the pre-column to the analytical column and analysis by ESI-MS–MS [19–21].

In bioreactors packed with enzyme-modified porous beads, the substrate has to diffuse into the pores of the support to interact with the active sites of the immobilized enzyme. The low mass transfer observed with conventional packed material is rate limiting and therefore, the flow rate cannot be increased. As a result, the apparent activity of the immobilized enzyme decreases when increasing the flow rate.

Recent development of monolithic supports, specific in terms of very low back-pressure and better accessibility of substrate to the active site, can provide useful alternative and can circumvent limitations of traditional materials [22]. In this case, the presence of large mesopores facilitates the fast transfer of macromolecules through the monolith. Hence, a great advantage of monoliths is the high liquid flow rate applied maintaining good efficiency. Based on the nature of the material from which they are made, monolithic columns can be classified as organic polymer- [23–25] or silica-based supports [26–28].

Different reactive monolithic polymers directly polymerized in microfluidic devices as supports for trypsin immobilization have been described for protein digestion followed by MS determination of the peptides [13,29–31]. Very high proteolytic activity of immobilized trypsin permitted digestion of model proteins with satisfactory sequence coverage. However, in only one paper the active microreactor was coupled on-line with ESI-MS detection. Furthermore no MS–MS analysis for complete sequence determination is reported [31].

Following the previous successful development of bioreactors based on monolithic silica support [32,33] this material was tested for tryptic digestion of proteins.

This paper describes the preparation and characterization of a silica monolithic column based on immobilized trypsin for the development of an automated bioreactor. Trypsin was covalently immobilized on epoxy-modified silica monolithic support with a single reaction step. Kinetic characteristics of the trypsin bioreactor were determined using a chromogenic low-molecular-mass substrate: *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA). The effects on enzymatic activity of different parameters such as flow rate, contact time, bioreactor temperature, buffer type and pH, were carefully studied. Once evaluated the operating parameters, the optimum conditions were retained for the on-line digestion of some proteins on the monolithic bioreactor, coupled through a switching valve to a HPLC–UV–ESI-MS–MS system for the on-line peptide separation and identification.

The applicability of the integrated chromatographic approach for protein identification and characterization has been studied with a peptide (somatostatin) and demonstrated on a recombinant protein (myoglobin).

2. Experimental

2.1. Reagents and materials

Trypsin from bovine pancreas (EC 3.4.21.4), *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), *p*-nitroaniline (*p*-NA) and somatostatin were purchased from Sigma (St. Louis, MO, USA). Potassium dihydrogenphosphate, dipotassium hydrogenphosphate and organic solvents were purchased from Merck (Darmstadt, Germany). Horse heart myoglobin (Mb) was kindly provided by Professor L. Casella (University of Pavia, Italy). Water was deionized by passing through a Direct-Q (Millipore) system (Millipore, Bedford, MA, USA).

Epoxy-modified silica Chromolith Flash (25 mm × 4.6 mm I.D.) supports were prepared as research samples at Merck (Darmstadt, Germany) following a previously reported procedure [34].

2.2. Equipment

Trypsin immobilization and column characterization were performed with an Agilent HP-1100 series (Palo Alto, CA, USA) modular system. The instrument is equipped with an

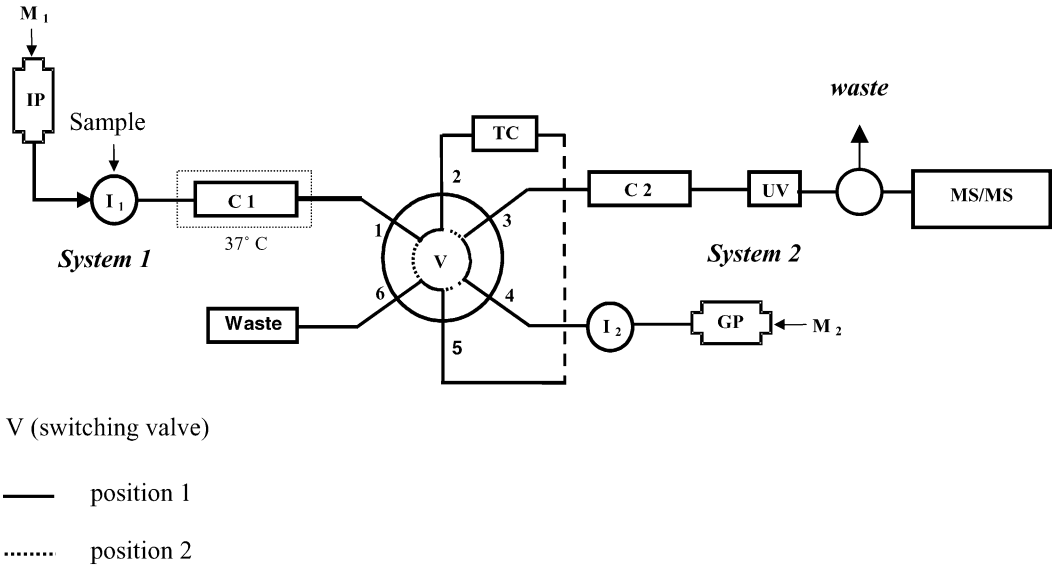

Fig. 1. Scheme of the chromatographic set-up for performing the on-column digestion and ESI-MS–MS analysis of peptide mixtures from the trypsin bioreactor. IP: isocratic pump, I_1: thermostated column oven autosampler set at 37.0 ± 0.1 °C, M_1: 100 mM phosphate buffer (pH 7.0) delivered at 1.0 ml/min, C1: trypsin bioreactor, TP: C_{18} trapping column, GP: quaternary gradient pump, M_2: gradient mobile phase delivered at a flow rate of 0.3 ml/min (see Table 1 for gradient conditions), C2: analytical column, UV: diode array detector, MS–MS: ion trap mass spectrometer with electrospray ionization ion source and V: automatically controlled six-port Rheodyne sample valve.

autosampler, a diode-array detector and a thermostated column oven (40.0 ± 0.5 °C). The system was connected to an HPLC ChemStation.

On-column digestions and peptide analysis were performed with the column-switching set-up reported in Fig. 1. Chromatographic experiments were carried out with two HPLC modular systems. System 1 consisted of an isocratic pump (IP) HP 1050, a thermostated column oven Surveyor autosampler (Thermo Finnigan, San Jose, CA, USA) set at 37.0 ± 0.1 °C, a trypsin bioreactor (25 mm × 4.6 mm I.D.) (C1) and a C_{18} trapping column (TC) (C_{18} Kromasil 100, 10 mm × 4.6 mm I.D). System 2 consisted of a quaternary gradient pump (GP) Surveyor LC system (Thermo Finnigan, San Jose, CA, USA) equipped with a diode array detector and a LCQ DECA ion trap mass spectrometer with ESI ion source controlled by Xcalibur software 1.3 (Thermo Finnigan, San Jose, CA, USA). The analytical column was a Symmetry 300 (Waters, Milford, MA, USA) (C2) (100 mm × 2.1 mm I.D, 3.5 μm).

Experiments were carried out in positive ion mode under constant instrumental conditions: source voltage 4.5 kV, capillary voltage −20 V, sheet gas flow 70 (arbitrary units), auxiliary gas flow 20 (arbitrary units), capillary temperature 200 °C, tube lens voltage −5 V. MS–MS spectra were obtained by CID (collision-induced dissociation). Studies in the ion trap were performed with an isolation width of 3 Th (*m*/*z*), the activation amplitude was around 35% of ejection radio frequency (RF) amplitude that corresponds to 1.85 V.

Acquired MS–MS spectra were automatically searched against protein database for equine proteins using the Bioworks 3.0 software package (Thermo Finningan) [35–37].

2.3. Chromatographic conditions

Systems 1 and 2 could be used independently or the eluent from system 1 could be directed onto system 2 through a six-port Rheodyne sample valve automatically controlled with a Kontron valve interface 492 (Kontron Instruments) (V) as shown in Fig. 1.

2.3.1. Step 1 (valve in position 1)

The sample was loaded on the enzymatic column (C1); 100 mM phosphate buffer (pH 7.0) (M_1) was used as eluent delivered by IP at 1.0 ml/min. A C_{18} column trap (TC) was inserted for concentration and desalting of the tryptic digest.

2.3.2. Step 2 (valve in position 2)

Peptides were back flushed from the column trap by GP. In order to elute peptides from the trapping column to the analytical column, the GP started a gradient with solvents A [water with 0.05 or 0.1% trifluoroacetic acid (TFA)] and B (acetonitrile with 0.05 or 0.1% TFA) at a flow rate of 0.3 ml/min. Before coupling the mass spectrometer, the effluent from the analytical column was diverted to waste for the first 3 or 10 min in somatostatin and myoglobin analysis, respectively.

2.3.3. Step 3 (valve in position 1)

The valve was switched back to the original position for trapping column conditioning.

Chromatographic conditions are summarized in Table 1.

Table 1
Chromatographic conditions for the on-line system

Somatostatin			Myoglobin		
Time (min)	Valve position[a]	B (%) (CH_3CN + 0.05% TFA)[b]	Time (min)	Valve position[a]	B (%) (CH_3CN + 0.1% TFA)[b]
0	1	0	0	1	0
2	1	0	10	1	0
2.01	2	0	10.01	2	0
14	2	50	75	2	55
19	2	95	78	2	100
23	2	95	80	2	100
38	2	0	85	2	0
38.01	1	0	85.01	1	0
40	1	0	90	1	0
45	1	0	95	1	0

[a] Six-port switching valve (V) programming (see text and Fig. 1).
[b] B% of mobile phase delivered by GP.

2.4. Trypsin immobilization

The epoxy-modified silica monolithic columns used for the immobilization of trypsin were prepared following a previously described procedure based on the in situ modification of silica-based monolithic supports [34].

The immobilization of trypsin was carried out with slight modification of previously described procedures [38,39]. Specifically, 250 mg of trypsin were dissolved in 25 ml of grafting solution (0.05 M phosphate buffer 1.9 M in ammonium sulphate, pH 8.0) and filtered with 0.45 μm filters. Before immobilization, the column was equilibrated with 50 ml of grafting solution. The ligand solution was then pumped through the support at 0.5 ml/min, flushing and back-flushing every 15 min. The reaction was stopped after 4 h in order to prevent the undesirable auto-digestion of the enzyme in solution.

After immobilization, the column was washed with 70 ml of 0.01 M phosphate buffer pH 6.0 at 0.2 ml/min, then with 100 ml of a 1 M glycine solution at 0.5 ml/min to cover unreacted epoxyde groups. Finally, the obtained bioreactor was equilibrated with pH 7.0 0.1 M phosphate buffer mobile phase. The amount of immobilized trypsin was determined by the UV absorbance decrease of the enzyme solution at 280 nm before and after the immobilization procedure.

2.5. Assay for trypsin activity

In order to measure the enzymatic activity of the IMER, two different procedures were applied, which provided both rapid information about the activity state of the column (hydrolytic activity test) and detailed determination of the Michealis–Menten kinetic parameters (V_{max} and K_m) of the reactor (trypsin column activity determination). The kinetic parameters of free trypsin were measured with a UV–vis spectrophotometer using a chromogenic substrate, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide, which gives the yellow colored *p*-nitroaniline (*p*-NA) upon hydrolysis monitored at 420 nm. The experiments were carried out as described in ref. [40]. The measured K_m and V_{max} were 4.325 mM and 0.0215 mM/min, respectively. The BAPNA activity of the soluble trypsin was 6.45 U/mg.

2.5.1. Hydrolytic activity test

The enzymatic activity of immobilized trypsin was checked by measuring the hydrolysis yield of a 0.1 mM standard solution of a chromogenic substrate, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide, which gives the yellow colored *p*-nitroaniline (*p*-NA) upon hydrolysis. The mobile phase consisted of 0.1 M phosphate buffer pH 7.0 and the flow rate was set at 1.5 ml/min. Experiments were performed by keeping column temperature at 37 °C. The area of the hydrolysis product *p*-NA was measured at 410 nm due to the lack of absorbance of BAPNA at this detection wavelength. The elution profile of the unreacted substrate was followed at 310 nm. The percentage of hydrolysis was calculated by comparing the area of *p*-NA product to the area of a *p*-NA standard solution of the same molar concentration and in the same chromatographic conditions according to following equation:

$$H(\%) = \frac{A_{p\text{-NApr}}}{A_{p\text{-NAst}}} \times 100 \quad (1)$$

where H (%) is the hydrolysis yield, $A_{p\text{-NApr}}$ and $A_{p\text{-NAst}}$ are the peak areas of produced *p*-NA and standard *p*-NA, respectively.

2.5.2. Trypsin column activity determination

The activity of immobilized enzyme was determined in terms of active units (U_{BAPNA}) following a previous described on-column procedure [32]. One unit of enzymatic activity is defined as the amount of enzyme catalysing the production of 1 μmol of *p*-NA/min, i.e. the number of micromoles of *p*-NA produced by hydrolysis of BAPNA. Aliquots of 20 μl V_{inj} BAPNA aqueous solutions at increasing concentration were injected in the system 1, at a flow

rate of 1.5 ml/min and *p*-NA directly monitored at 410 nm. The Michaelis–Menten trend was found in the concentration range between 0.1 and 30 mM by plotting the rate of enzymatic reaction (V) against the substrate concentration [S].

The rate of the enzymatic reaction (V) expressed as (Δarea *p*-NA/min) was calculated by:

$$V(\Delta\text{area } p\text{-NA/min}) = \frac{\text{area}(p\text{-NA})}{\text{time(min)}} \quad (2)$$

The values are the means of two independent measurements. Time indicates the reaction time defined by the time for complete elution of the product in the bioreactor, which is dependent upon flow rate in the column system.

Kinetic parameters V_{max} and K_m were obtained thanks to the Lineweaver and Burk plot, which is a linear transformation of the Michaelis–Menten plot. From the V_{max} value, the immobilized active units were determined by applying the following equation:

$$U(\mu\text{mol/min}) = \left[\frac{(\Delta\text{area/min})\text{max}}{\varepsilon p\text{-NA}}\right] \cdot \text{column void volume (ml)} \quad (3)$$

The *p*-NA extinction coefficient for concentration assessment corresponds to the area of a 1 mM solution.

The number of active units of the chromatographic bioreactor used in this study was 0.1996 U (±0.001).

3. Results and discussion

Monolithic silica support was used to prepare a new trypsin-based bioreactor.

The repeatability of the immobilization procedure was assessed by preparing six bioreactors. Under the described conditions the amount of immobilized enzyme was found to be 66.07 mg (±11.75 S.D.)/column with a satisfactory stability (approximately 250 digestions/column).

3.1. Study of experimental factors and their influence on enzymatic activity

Preliminary experiments were carried out to identify factors acting on the hydrolysis rate of trypsin-based bioreactor. The substrate used to test trypsin activity was *n*-benzoyl-DL-arginine-*p*-nitroanilide.

In this study, six factors mainly related to trypsin activity were examined by applying a Plackett–Burman (PB) design. As the latter saturated design assumes that interaction effects are negligible, only the main effects of factors can be evaluated. The six parameters, as well as their nominal values, were selected on the basis of theoretical considerations and/or preliminary experiments (Table 2).

Table 2
Experimental factors and selected levels of the Plackett–Burman design

Factors	Level	Value
(1) Flow rate (ml/min)	−1	0.5
	+1	3.0
(2) Injected m (mg/ml)	−1	0.5
	+1	1.5
(3) Molarity (mM)	−1	20
	+1	100
(4) Sample treatment	−1	Yes
	+1	No
(5) S.D.S. (%)	−1	No
	+1	Yes
(6) Buffer type	−1	Phosphate
	+1	Ammonium bicarbonate

3.1.1. Flow rate (X_1)

If on-line digestion is considered, flow rate determines the contact time between enzyme and substrate. Two extreme flow rate values (0.5 and 3.0 ml/min) were chosen representing a compromise between reaction time and chromatographic system constraints (i.e. back-pressure).

3.1.2. Analyte injected mass (X_2)

Two different sample concentrations (0.5 and 1.5 mg/ml) below the bioreactor saturation level were considered. The aim was to investigate the effect of the injected mass on the enzymatic activity.

3.1.3. Buffer concentration (X_3)

As reported in the literature [41] when hydrophobic interactions occur in enzyme substrate binding, high ionic strength can improve enzymatic activity by lowering the Michaelis–Menten constant (K_m). Thus, experiments were carried out with 20 and 100 mM buffers.

3.1.4. Sample treatment: alkylant and reducing agents (X_4)

A preliminary treatment is often necessary when performing tryptic digestion of proteins. This consists of a reduction—alkylation of disulfide bridges and denaturation of protein with high concentrations of urea [42], to enhance the accessibility of the substrate to the catalytic site. Treated and not treated samples were analyzed to verify a possible influence of the additives on trypsin activity.

3.1.5. Sample treatment: SDS (X_5)

In order to solubilise protein mixtures it is recommended to add sodium dodecyl sulphate (SDS) in the sample solution. The influence of this additive was also studied.

3.1.6. Kind of buffer (X_6)

Different buffers can be used to perform tryptic digestion as reported in the literature. The candidates evaluated in this study were phosphate, well adapted for chromatographic

Table 3
The six factors experimental design used to screen the relevant parameters

Number of experiments	Flow rate (ml/min)	Sample concentration (mg/ml)	Buffer concentration (mM)	Treatment	S.D.S. (%)	Buffer type
1	3.0	1.5	20	No	No	Phosphate
2	0.5	1.5	100	Yes	0.05	Phosphate
3	0.5	1.5	100	No	No	AmBic
4	3.0	0.5	100	No	0.05	Phosphate
5	0.5	0.5	20	No	0.05	AmBic
6	3.0	1.5	20	Yes	0.05	AmBic
7	3.0	0.5	100	Yes	No	AmBic
8	0.5	0.5	20	Yes	No	Phosphate
NP[a]	1.5	1.0	100	Yes	No	Phosphate

[a] Nominal conditions.

purposes, and ammonium bicarbonate, an MS compatible buffer.

The effect of each factor was examined by means of the percentage of hydrolysis, calculated as described in Section 2.5.1.

Experiments (Table 3) were performed randomly and response values were recorded for statistical evaluation. In addition to the eight experiments, three supplementary trials were conducted at conditions corresponding to the nominal conditions (NP) at the beginning, during, and at the end of the experiments, to check method performance as well as to estimate the experimental error.

The coefficients for the regression models were evaluated using the NEMROD (LPRAI; Marseille, France) software package.

Multiple regression enables the mathematical relationship between the response and the independent variables. The experimental design provides sufficient data to fit the mathematical model described below:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6$$

where Y corresponds to the experimental response (% of hydrolysis), X_i are the independent evaluated factors, b_0 is the intercept, and b_i are the parametric coefficients of the model obtained by multiple regression. The statistical evaluation of the model revealed that R^2 and R^2_{adj} were of 0.996 and 0.974 and thus confirmed the good fitting.

In Fig. 2, the effects and significance of studied factors on response are represented. For coefficient evaluation, a confidence interval was calculated as $(t \cdot \text{S.D.}_{exp})/\sqrt{N}$ where N is the number of experiments of the full factorial design (eight), S.D._{exp} the experimental standard deviation calculated with the three nominal points and t the Student variable at 95% confidence level.

From these results, only the sample concentration (X_2) did not influence the analytical response. All the others coefficients were statistically significant.

Phosphate was the buffer of choice to perform on-line digestion (X_6). Furthermore, independent experiments showed that digestion repeatability was lower with ammonium bicarbonate buffer.

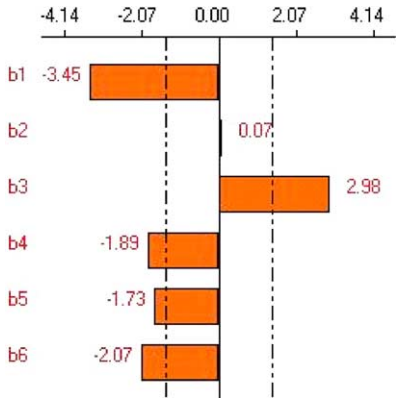

Fig. 2. Effects and significance of studied factors on response. b_i are the parametric coefficients for the independent evaluated factors (X_i) in the model obtained by multiple regression. The screening provided sufficient data to fit the mathematical model: $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6$.

The presence of SDS in the sample (X_5) negatively affected the hydrolysis rate, according to data reported in literature [43]. Such detergent noticeably decreases the rate of tryptic digest carried out in presence of 2 M urea. For this reason it should be adequately removed prior to digestion.

As proteins require a pre-treatment (e.g. reduction and alkylation of disulfide bridges, denaturation of the protein) in order to facilitate the tryptic digestion, this parameter (X_4) was studied and results showed that the absence of pre-treatment decreased the selected analytical response.

Buffer concentration (X_3) positively influenced the hydrolysis rate, probably due to the fact that a high salt concentration enhances substrate affinity for the catalytic site of the enzyme [41].

The mostly significant factor was the flow rate (X_1). By increasing the flow rate, lower hydrolysis yields were obtained due to a reduce contact time between substrate and enzyme. According to the analyte–enzyme interaction (thermodynamic and kinetic) this parameter was further studied, by measuring the hydrolysis rate at 25 and 37 °C. As reported in Fig. 3, trypsin activity is higher at 37 °C

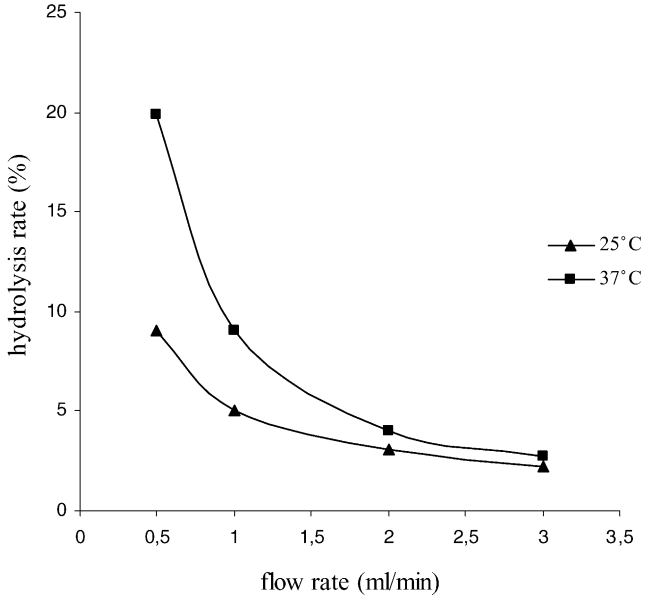

Fig. 3. Influence of temperature on hydrolysis rate evaluated at different flow rates. The hydrolysis rate was determined at 25 °C (▲) and 37 °C (■). Mobile phase of the trypsin column was 100 mM phosphate buffer, pH 7.0.

and more influenced by the flow rate. In all cases, the maximum conversion yields were obtained at low flow rate showing the importance of kinetics in the enzymatic process.

To investigate how the hydrodynamic features of the monolithic support influences the characteristics of the immobilized enzyme, the kinetic parameters (V_{max} and K_m) of trypsin bioreactor were determined at different flow rates (from 0.5 to 3 ml/min) and listed in Table 4. It can be seen that V_{max} values decreased when increasing the flow rate, this in agreement with the reaction time dependence on flow rate. Interestingly the effect of the latter on the K_m is very small. This is in accordance with the idea that the mass transfer of substrate in the immobilized monolithic reactor is mainly controlled by the convective flow, but not by diffusion rate as in traditionally porous particle packed columns [44]. The obtained results are in agreement with already described hydrodynamic features of monolithic supports regarding their application as separation units, where flow unaffected dynamic binding capacity and separation quality [28,34].

Table 4
Kinetic constants of immobilized trypsin at different flow rates, thus different residence times of substrate in the bioreactor

Flow rate (ml/min)	Residence time (min)	K_m (mM)	V_{max} (mM/min)
0.5	8.0	12.67	0.47
1.5	5.6	11.23	0.46
2.0	3.0	12.90	0.43
3.0	1.8	11.25	0.35

3.2. On-column digestion and tandem MS studies

The developed trypsin bioreactor was integrated in an analytical chromatographic system (Fig. 1). On the basis of the experimental design results, the buffer selected for digestion was 100 mM phosphate buffer and the temperature was set at 37 °C. The pH was maintained at 7.0 as a compromise between trypsin activity and stability of the silica–monolith support. These experimental conditions were applied in the on-line digestion and identification by tandem mass spectrometry peptide sequencing.

A peptide (somatostatin) and a recombinant protein (myoglobin) were chosen as test substrates to assess the performance of the on-line system.

A C_{18} trapping column was inserted on-line after the trypsin reactor to retain peptides in a high salt concentration environment and to compensate the pH shift between the two chromatographic systems.

The overall on-line system performance was verified using a 14-amino-acids peptide of pharmaceutical interest, somatostatin (M_r 1638) expected to yield two peptides (peptide 1 of M_r 933 and peptide 2 of M_r 741) upon cleavage by trypsin.

Before on-line experiments, a switching time was determined by injecting on system 1 a somatostatin sample (20 μl of a 0.5 mg/ml solution) at 1.5 ml/min and following the UV trace at 220 and 280 nm. Experimental conditions are shown in Table 1. In the case of somatostatin, the switching time was set at 2 min. In the first 2 min analysis the elution of products and unhydrolyzed somatostatin was considered as complete. In the reported chromatographic conditions three peaks appeared in the chromatogram that were recognised as peptide 1, partially hydrolysed somatostatin and peptide 2.

The system was tested in terms of peptide recovery at different sample concentrations (0.25, 0.50, 0.75 and 1.0 mg/ml) and reproducibility of obtained results. Peptides recovery was investigated by comparing MS traces of tryptic digest of somatostatin obtained: (a) with the standard on-line procedure (previously described); (b) with the injection on system 2 (valve position 2) of digested eluted solutions from the bioreactor and collected in 10 ml volumetric flask. These solutions were evaporated and reconstituted in 0.5 ml of 100 mM, pH 7.0 phosphate buffer.

No loss of peptides was observed up to 0.75 mg/ml samples, as confirmed by the comparable area percentage of the two peptides.

The repeatability of the on-line automated system was determined on the areas (1.2 and 3.7% R.S.D., $n = 3$ for peptide 1 and peptide 2, respectively) and retention times (0.3 and 1.0% R.S.D., $n = 3$ for peptide 1 and peptide 2, respectively) obtained by the complete digestion of a somatostatin sample (0.5 mg/ml). The R.S.D. values were considered acceptable in terms of digestion and analytical reproducibility.

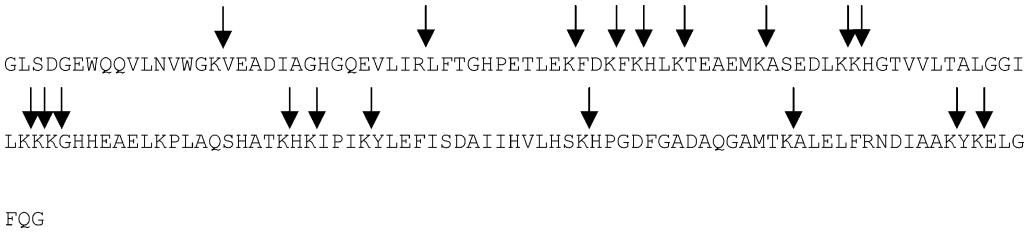

Fig. 4. Primary structure of myoglobin. Arrows indicate the expected (theoretical) tryptic cleavage sites.

3.2.1. Myoglobin

In order to test the efficacy of the on-line system, a recombinant protein (myoglobin) was on-line digested and mapped. Myoglobin was used mainly because its tryptic map has been well documented. Each myoglobin molecule contains one heme prosthetic group inserted into a hydrophobic cleft in the protein. The apo form of Mb was prepared by standard acid–2-butanone method [45]. The apomyoglobin solution was in 10 mM phosphate buffer pH 7.5.

The same protein was off-line digested following the manual protocol here reported. Approximately 100 μl of 1 mg/ml trypsin solution in 1.2 mM HCl was added to 5 ml of a 1.5 mg/ml apomyoglobin solution and the sample was incubated for 2.30 h at 37 °C. Digestion was stopped by the addition of concentrated HCl to pH 1.0 and storing the solution at 0 °C.

Myoglobin primary structure and theoretical tryptic cleavage sites are shown in Fig. 4. Molecular masses of peptides obtained by on-line and off-line digestion are listed in Table 5. Fig. 5A and B shows the peptide profiles of products obtained from off-line and on-line digestion, respectively, of 1.5 mg/ml Mb. The 10 peaks labelled with the same numbers indicate identical mass spectra, thus identical peptides. A good correspondence between the two analytical methods was observed in terms of their retention times as indicated by Δt values reported in Table 5.

Sequence coverage obtained with the on-line protocol (21 peptides, 75.16% coverage of myoglobin sequence) was found to be comparable to the one obtained with the off-line protocol (18 peptides, 76.47% coverage). More in particular, in the on-line trace, most hydrophilic peptides (low retention time) are missing, while peptides with miscleavages are present, in comparison to the off-line trace. These results can be ascribed to the different degree of digestion, partly due to the diverse reaction time of the on-line and off-line procedures. As an example, peptide 48–56 with one miscleavage is only present in the on-line trace while complete digestion occurs with a longer time as confirmed by the presence of peptides 48–50 and 51–56 in the off-line profile.

3.2.2. Effect of flow rate on protein identification

Enzymatic activity of the trypsin bioreactor can be directly controlled by the contact time between the enzyme and substrate, and thus by the flow rate, as previously

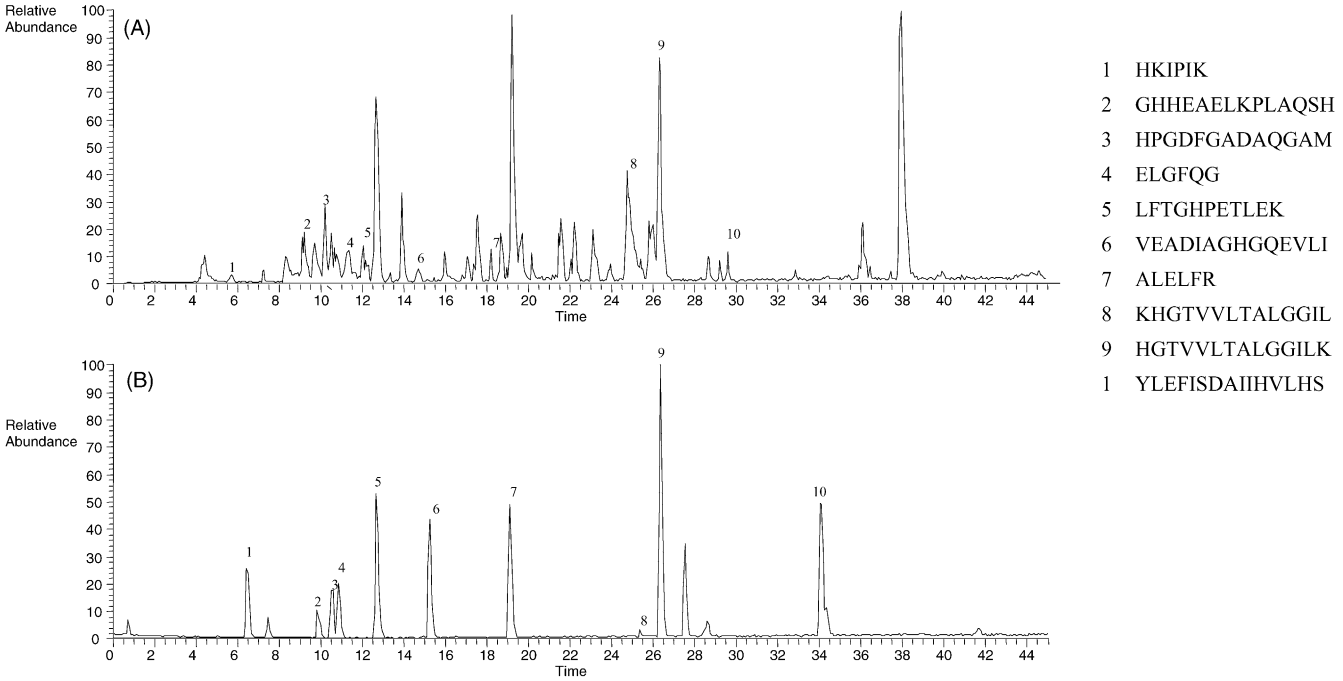

Fig. 5. Chromatograms of a tryptic digest of myoglobin 1.5 mg/ml using (A) digestion in an on-line trypsin reactor and (B) digestion off-line in solution.

Table 5
Peptide masses, sequences and chromatographic retention times (t_r) of the fragments obtained by on-line and off-line digestion of a 1.5 mg/ml myoglobin sample

Peptide mass $[M + H]^+$	Position	Sequence	Missed cleavages	On-line t_r (min)	Off-line t_r (min)	Δt_r (min)[a]
397	48–50	HLK	0	–	7.07	–
708	51–56	TEAEMK	0	–	10.00	–
790	57–63	ASEDLKK	1	–	10.74	–
1086	48–56	HLKTEAEMK	1	14.35	–	–
735	97–102	HKIPIK	1	14.41	16.44	2.03
470	99–102	IPIK	0	–	15,02	–
1982	79–96	KGHHEAELKPLAQSHATK	1	18.31	–	–
1854	80–96	GHHEAELKPLAQSHATK	0	19.70	19.84	0.14
1503	119–133	HPGDFGADAQGAMTK	0	20.46	20.52	0.06
650	148–153	ELGFQG	0	21.35	20.88	−0.47
1272	32–42	LFTGHPETLEK	0	22.00	22.67	0.67
941	146–153	YKELGFQG	1	22.70	–	–
1607	17–31	VEADIAGHGQEVLIR	0	24.71	25.23	0.52
1662	32–45	LFTGHPETLEKFDK	1	26.41	–	–
1554	140–153	NDIAAKYKELGFQG	2	27.12	–	–
748	134–139	ALELFR	0	28.69	29.10	0.41
1937	32–47	LFTGHPETLEKFDKFK	2	29.18	–	–
1361	134–145	ALELFRNDIAAK	1	29.23	–	–
1652	134–147	ALELFRNDIAAKYK	2	30.18	–	–
1635	64–79	HGTVVLTALGGILKKK	2	34.49	–	–
1507	63–77	KHGTVVLTALGGILK	1	34.76	35.37	0.61
922	140–147	NDIAAKYK	1	36.37		
1379	64–77	HGTVVLTALGGILK	0	36.67	36.35	−0.32
1816	1–16	GLSDGEWQQVLNVWGK	0	–	37.54	–
2859	17–42	VEADIAGHGQEVLIRLFTGH PETLEK	1	–	38.60	–
1885	103–118	YLEFISDAIIHVLHSK	0	39.22	44.03	4.81
3404	01–31	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR	1	47.90	–	–
Peptide number	–	–	–	21	18	–
Percentage AA	–	–	–	75.16	76.47	–
Score	–	–	–	148.30	98.40	–

Trypsin column chromatographic conditions: mobile phase 0.1 M KH_2PO_4; pH 7.0 and flow rate 1.0 ml/min; temperature 37 °C. Analytical column chromatographic conditions: see Table 1.

[a] Δt_r = (off-line t_r − on-line t_r).

discussed (Section 3.1). For this reason, myoglobin was introduced in the trypsin bioreactor at various flow rates (0.5, 1.0 and 1.5 ml/min). In Table 6 are reported the number of recognized peptides, scores and peptide sequence coverage achieved at different flow rates. As reported in Table 6, an increasing number of peptides was obtained by decreasing the flow rate and 17 common peptides were found within the three different peptide maps. The additional peptides obtained at lower flow rate are the result of complete digestion of peptides containing uncleaved fragments, this in agreement with the higher reaction time.

By decreasing the flow rate from 1.5 to 1.0 ml/min an improvement in both score and peptide sequence coverage was observed; but no further enhancement was obtained reducing the flow from 1.0 to 0.5 ml/min.

Table 6
Influence of flow rate on peptide map obtained by on-line digestion of a 1.5 mg/ml myoglobin sample

Flow rate (ml/min)	Number of peptides[a]	Score[a]	Peptide recovery (%)[a]
0.5[b]	27	148.3	74.5
1.0[b]	21	148.3	75.2
1.5[b]	18	122.3	64.7

[a] Results from LC–MS. The analytical column chromatographic conditions are given in Table 1.

[b] Trypsin column chromatographic conditions: mobile phase 0.1 M KH_2PO_4; pH 7.0 and temperature 37 °C.

3.2.3. Sensitivity

The dependence of peptide coverage on injected protein amount was studied by injecting different concentration myoglobin samples (from 0.1 up to 1.5 mg/ml). On-line digestion of myoglobin sample at the lowest concentration (0.1 mg/ml) did not give a significant peptide map (data not shown). On the other hand, by increasing the concentration from 0.5 to 1.5 mg/ml, the obtained sequence coverage increased from 33.33 to 75.16% with score values of 40.4 and 148.3, respectively. Therefore, this method is suitable for the analysis of recombinant proteins.

Table 7
Cromatographic retention times repeatability in peptide mapping for a 1.5 mg/ml myoglobin sample after digestion on trypsin column

Peptide mass $[M + H]^+$	Day 1			R.S.D. (%)
	Analysis 1[a], t_r (min)	Analysis 2[a], t_r (min)	Analysis 3[a], t_r (min)	
470	26.84	–	–	–
650	20.43	21.35	20.86	2.2
735	14.82	14.41	13.61	4.3
748	26.97	28.69	27.79	3.1
922	34.27	36.37	–	4.2
941	21.28	22.70	21.99	3.2
1086	14.69	14.35	13.67	3.6
1272	20.5	22	21.21	3.5
1361	27.39	29.23	28.15	3.3
1379	34.27	36.67	34.95	3.5
1503	19.03	20.46	19.49	3.7
1507	32.35	34.76	33.27	3.6
1554	25.45	27.12	26.29	3.2
1607	23.32	24.71	24.06	2.9
1635	31.86	34.49	32.79	4.0
1652	28.18	30.18	28.97	3.5
1662	24.46	26.41	25.40	3.8
1854	18.21	19.7	18.63	4.1
1885	38.11	39.22	38.32	1.5
1937	26.9	29.18	27.85	4.1
1982	16.94	18.31	17.2	4.2
3404	46.85	47.9	46.49	1.6

Chromatographic conditions: mobile phase 0.1 M KH_2PO_4; pH 7.0; temperature 37 °C and flow rate 1.0 ml/min.
[a] Analytical column chromatographic conditions are given in Table 1.

3.2.4. Reproducibility of peptide maps

In view of routine application of the proposed system, the repeatability of peptide maps obtained with the on-line system was measured as relative standard deviation of retention time of identified common peptides as reported in Table 7. Three analyses of the same sample (1.5 mg/ml) were performed in sequence. The maps were fairly reproducible in terms of retention time (from 1.53 to 4.31%, R.S.D., $n = 3$).

Same results in terms of peptide number, retention times and R.S.D. values were observed in different days (data not shown).

4. Conclusion

The preparation of a bioreactor based on trypsin immobilized on epoxy-modified silica monolithic support has been described. The monolithic material is suitable for enzyme immobilization in terms of repeatability of the grafting reaction, long-term stability of the bioreactor and catalytic efficacy. The experimental design was used to study the influence of different parameters on enzymatic activity. The flow rate is the most important factor to control the enzymatic activity of the trypsin bioreactor as an increasing number of peptide was obtained by decreasing the flow rate.

On-line coupling of the monolithic trypsin bioreactor with an analytical column is demonstrated as an integrated analytical tool enabling protein digestion, high resolution peptide separation and protein identification using ESI-MS–MS.

Sequence coverage obtained with the proposed on-line procedure was comparable to the one given by the off-line standard method, however the total analysis time decreases compared to traditional protein analysis. The sensitivity demonstrated for the peptide map was as good as expected for a 4.6 mm I.D. monolithic column using a flow rate of 1 ml/min and suitable for the analysis of recombinant proteins. Sensitivity of the system will be improved by using similar columns with smaller I.D.'s as previously demonstrated for other polymeric-based microreactors.

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