Talanta 135 (2015) 18–22

Contents lists available at ScienceDirect

Talanta

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

Fabrication of an on-line enzyme micro-reactor coupled to liquid chromatography–tandem mass spectrometry for the digestion of recombinant human erythropoietin

Hsiao Ching Foo [a,b,*], Norman W. Smith [a], Shawn M.R. Stanley [b]

[a] Analytical & Environmental Science Division, School of Biomedical Sciences, King's College London, London SE1 9NH, UK
[b] Singapore Turf Club Laboratory, 1 Turf Club Ave, Singapore Racecourse, Singapore 738078, Singapore

ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form
19 December 2014
Accepted 20 December 2014
Available online 31 December 2014

Keywords:
Recombinant human erythropoietin
On-line digestion
Immobilised enzyme microreactors
Dextran-modified
Peptide-N-glycosidase F

ABSTRACT

Our aim was to develop a fast and efficient on-line method using micro-reactors for the digestion and deglycosylation of recombinant human erythropoietin extracted from equine plasma. The trypsin digestion micro reactors were fabricated using fused silica capillaries with either a dextran-modified coating or a porous monolith that was able to immobilise the enzyme. These were both found to be reasonably robust and durable, with the trypsin immobilised on dextran-modified fused silica capillaries offering better reproducibility than the micro-reactor based upon covalent attachment of this enzyme to the polymer. It is also evident that the enzyme attached micro reactors produced some tryptic peptides in a greater yield than in-solution digestion. A peptide-N-glycosidase F reactor was also fabricated and, when coupled with the trypsin reactor, the deaminated peptides T5 DAM and T9 DAM from recombinant human erythropoietin could also be detected by LC–ESI–MS/MS analysis. These results were better than those achieved using off-line digestion plus deglycosylation reactions and the analysis required far less time and effort to complete. The use of this on-line approach improved the sensitivity, efficiency and speed of our confirmation methodology that is based upon detecting the unique peptide segments of recombinant human erythropoietin that has been affinity extracted from positive equine plasma samples.

1. Introduction

Digestion of proteins with a highly specific enzyme that produce fragments of the amino acid sequence which are characteristic of the intact material, and are also compatible with detection/identification using liquid chromatography–mass spectrometry, is an important step in contemporary proteomics. In-solution based protein digestion is the traditional way of achieving this, but this procedure has several drawbacks. Firstly it is a time consuming step which requires up to 18 h of incubation in order to produce a reasonable amount of the peptides [1–3]. Secondly the by-products generated by the auto-digestion of the enzyme add chemical noise and, in some cases, the similarity between the autolysis fragments and the peptides from the protein can even complicate the interpretation of the results. These issues can be minimised by immobilisation of the protease onto a solid support [4], since the digestion time is dramatically reduced because the enzyme-to-substrate ratio is significantly enhanced. By doing this immobilisation the opportunity for enzyme autolysis by-products to be formed is significantly reduced. Furthermore, attaching an enzyme to a solid support provides increased stability towards any chemical denaturants and organic solvents that may be present as a consequence of the preparation steps conducted prior to the digestion [5]. When used in an on-line configuration, this method can also reduce the amount of sample handling required [4] and offers a more efficient way to process samples where the amount of material available is limited.

Enzymes can be entrapped in polyacrylamide gels [6,7] or covalently attached onto the surface of micro-beads [8,9], monolithic columns [10–14] and the inner walls of open capillaries or microchannels in microfluidics devices [15,16]. Out of all of these available options, the use of monolithic supports has attracted the most attention because they are easy to fabricate and the polymeric support generally provides fast mass transfer and low

Abbreviations: rhEPO, recombinant human erythropoietin; DPO, darbepoetin; IMERs, immobilised enzyme reactors; EPO, erythropoietin; PTM, posttranslational modification; PNGase F, peptide-N-glycosidase F; Asn, asparagine; TIS, turbo-ion spray; XIC, extracted ion chromatogram; LC/ESI/MS/MS, liquid chromatography/electrospray ionisation/mass spectrometry/mass spectrometry; DAM, deaminated

* Corresponding author at: Singapore Turf Club Laboratory, 1 Turf Club Ave, Singapore Racecourse, Singapore 738078, Singapore.
Tel.: +65 68791067; fax: +65 68791939.
E-mail address: foohsiaoching@turfclub.com.sg (H.C. Foo).

http://dx.doi.org/10.1016/j.talanta.2014.12.033
0039-9140/

backpressure [17,18]. Their large accessible surface area combined with a limited diffusion path length facilitates the rapid conversion of substrates [19–22] resulting in the limited generation of autolysis products due to the site isolation effect [23–25]. The fused silica capillary format for containing the immobilised enzyme reactors provides a convenient way to combine this step directly with hyphenated liquid chromatography–mass spectrometry [26–28].

An open tubular capillary can be modified using aminopropyltriethoxysilane and then subsequently derivatised with carboxyl-modified dextran combined with an amino-modified dextran hydrogel to passivate the silica surface. The resulting dextran hydrogel serves as an immobilisation surface for trypsin which is coupled via carbonyl diimidazole activation. The polymer produced in this manner provides the possibility to immobilise larger amounts of the enzyme and therefore should offer improved interaction capacity when compared to the limited area available when using the internal surface of the capillary alone [15,29].

An alternative approach is to fabricate an enzyme reactor with trypsin immobilised on a macroporous polymer monolith. This simple single step method, which involves the co-polymerisation of trypsin with acrylamide in capillary columns, requires a short fabrication time of only an hour [7]. These biocompatible porous monoliths produced in this manner are highly flow permeable and, provide high enzyme stability and fast mass transfer characteristics for the immobilised enzymes [7].

EPO is a regulator of erythropoiesis, the process that controls the production of red blood cells in mammals and, therefore it is likely to be performance enhancing when given to racehorses [30]. Although the intact rhEPO can be detected by mass spectrometry [30], the most common way of screening for its presence is to digest an affinity extracted sample with trypsin and then conduct a target analysis for the characteristic peptides using LC/MS/MS [31]. It is also possible to use this method to differentiate rhEPO from the synthetic epogen analogue Darbepoetin Alpha (DPO) since in addition to the presence of several peptides that are characteristic to both proteins, the peptides T5 (21–45) and T9 (77–97) have unique amino acid sequences that are specific to each protein. Unfortunately, this is not a straight forward process as the T5 and T9 product from both rhEPO and DPO are a population of glycopeptides with highly variable sialic acid containing glycan motifs that severely hinder detection under ESI/MS conditions. Consequently, an efficient process to remove the sugar moieties from the peptides is a key requirement for a successful analysis and PNGase F, which selectively releases N-linked glycans by hydrolysing the amide bond at the asparagine side chain [32], is frequently used for this purpose. This reaction is usually performed in solution using PNGase F mixed with the substrate in a small volume of buffer and, to keep cost under control, only a small quantity of the enzyme is typically used to catalyse the cleavage. As a result, the ratio of the glycolytic enzyme to substrate is usually lower than optimal. However, by efficiently immobilising the same amount of enzyme onto a solid support, this catalysis could be improved because only a small percentage of the sample is ever in contact with the enzyme at any time during the passage of the liquid through the reactor. Furthermore, only a small aliquot from the extract requires deglycosylation before each LC/MS/MS analysis, and this further enhances the overall enzyme to substrate ratio.

In this paper we report on the results obtained using serially connected trypsin and PNGase F reactors that were coupled in an on-line configuration with LC–ESI–MS/MS. Firstly an evaluation of the relative efficiency of the digestion technique was carried out by comparing the results obtained from the off-line (in-solution trypsin digestion) method versus the on-line approach with either trypsin immobilised inside an open tubular dextran-modified capillary or a monolithic column where the trypsin was covalently bound to the surface. Following on from this initial study, a monolithic PNGase F enzyme reactor was also made. Coupling of the on-line trypsin digestion reactor and PNGase F deglycosylation reactor in series considerably reduced the preparation time and by linking these directly to the mass spectrometer, the sample losses and/or overall potential for contamination with plasticisers and other chemical noise could be minimised. We demonstrate in this publication that both the non-glycosylated peptides and the (previously) glycosylated tryptic peptides could be detected within a single analytical run.

2. Materials and methods

2.1. Materials

Aminopropyl triethoxysilane (APTES) and N-(3-dimethylaminopropyl) – N'ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich Singapore. Carbonyl diimidazole (CDI) was purchased from Alfa Aesar Chemical Company (Singapore) while carboxyl-modified dextran 10 (CMD) was purchased from pK Chemicals A/S (Denmark). Amino-modified dextran (AMD) 3000 MW was from Invitrogen, Eugene, Oregon, USA. Sodium hydroxide, polyethylene glycol 10,000 (PEG), sodium bicarbonate, sodium chloride, dimethoxysulfoxide (DMSO), ammonium persulfate (APS), dimethyl sulfoxide (DMSO), ammonium persulfate (APS) were all from Merck (Singapore). Methacryloxypropyltrimethoxysilane (Bind-silane), acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′ tetramethylethylenediamine (TEMED), N-acryloxysuccinimide (NAS) and benzamidine were purchased from Acros Singapore. Trypsin lyophilised from Bovine pancreas was purchased from Thermo Scientific Singapore. Peptide-N-glycosidase (PNGase F) 500,000 U/ml was purchased from New England Biolabs and the fused silica capillary 50 μm and 100 μm i.d. were supplied by Polymicro USA. The water used throughout all experiments was Milli-Q Gradient A10 from Millipore (Singapore). Epoetin alfa, Eprex® the recombinant human erythropoietin, 10,000 IU/ml was purchased from Jassen-Cilag AG, (Schaffhausen, Switzerland). Darbepoetin alfa, Aranesp® 40 μg/0.4 ml were obtained from Amgen Manufacturing Limited a subsidiary of Amgen Inc. (Thousand Oaks, CA, USA).

2.2. Instrumentation

Experiments were carried out on a Tempo™ nano MDLC by Eksigent, with an Eksigent AS1 Autosampler coupled to the TIS source of an AB Sciex 4000 Qtrap LC/MS/MS operated by Analyst 1.5 software. The autosampler has a 6-port injection valve with a 20 μl injection loop. The trypsin enzyme reactor was coupled directly from the injection port to the deglycosylation reactor in the oven at 37 °C and the deglycosylation reactor was connected to the 6-port switching valve, passing through a CAPTRAP™, PEPTIDE (Michrom Bioresources, Inc.). When this switching valve is switched, the pump running an organic solvent gradient, delivers the sample from the CAPTRAP to the HALO™ C18 0.3 × 150 mm^2, 2.7 μm analytical column and then to the TIS source (Fig. 1).

2.2.1. LC conditions

The mobile phase used for both loading and gradient pumps were (A) 0.15% Formic acid in water and (B) ACN. The loading pump was set at 1 μl/min isocratic for 95% A and 5% B. The gradient pump was set at a flow rate 4 μl/min, at an initial condition of 95% A and 5% B followed by a liner gradient 10 min gradient to 5% A and 95% B, holding at 95% B for 5 min, and returning to initial conditions at 16 min and re-equilibrating in preparation for the next injection between 16 and 25 min.