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

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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

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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.

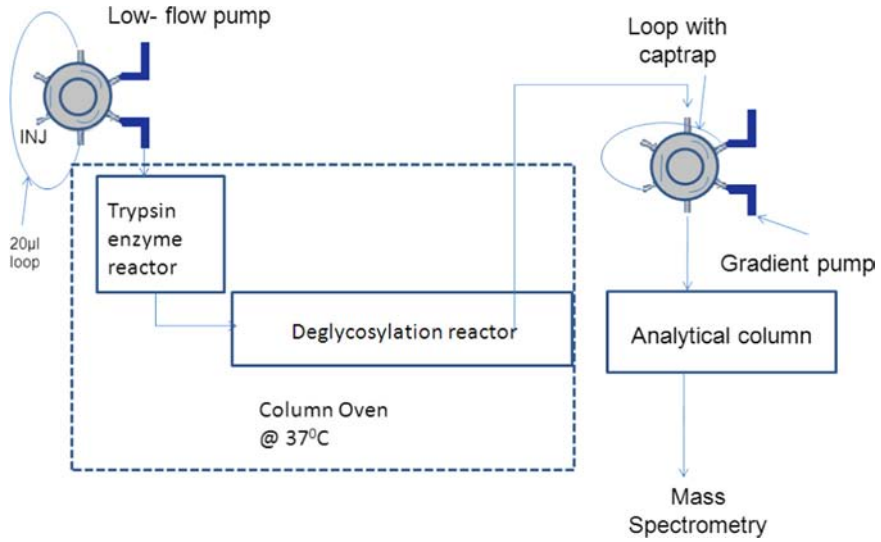

Fig. 1. On-line experimental set-up of enzyme reactors coupled to LC–MSMS.

2.2.2. MS conditions

The positive ion source voltage was set at 4500 V, GS 1 at 15, GS 2 at 10, curtain gas, CUR at 30 and TEMP at 350 °C.

2.3. Preparation of dextran-coated fused silica capillaries

Firstly, the 160 cm × 50 μm fused silica capillaries were cleaned at a flow rate of 5 μl/min with 2 M NaOH for 30 min, 0.1 M HCl for 30 min, water for 5 min and finally with ethanol for 5 min. The dextran-coated capillaries were prepared as follows: the clean capillaries were incubated for 60 min with a 10% APTES solution in ethanol. After this step, the capillaries were flushed at a flow rate of 5 μl/min with ethanol for 5 min and then dried overnight at 50 °C. To modify the surface with dextran, the capillaries were incubated for 30 min with a solution of 5% CMD in water also containing 200 mM EDC and 50 mM NHS. After 30 min they were flushed for 5 min at 5 μl/min with water, then filled with a solution of 10% AMD in water and incubated once more overnight. The resulting dextran-modified capillaries were washed at a flow rate of 5 μl/min with both water and methanol for 5 min, after which 100 mM CDI in acetone was injected for 30 min. After this activation step, they were flushed with ethanol and water for 5 min. A solution containing 2.5 mg/ml trypsin in 50 mM ammonium bicarbonate was injected at a flow rate of 2 μl/min and the enzyme coupling was allowed to proceed for 8 h. The reaction was stopped by injection of 1 M glycin pH 3. Finally the resulting trypsin micro-reactors were flushed with water and stored in a closed box at 4 °C or used immediately. All reactions were conducted at room temperature except for the drying step following APTES incubation. The chemistry of the surface modification and enzyme immobilisation in fused-silica capillaries has previously been described in Ref. [4].

2.4. Preparation of the co-polymerised monolith column

27 cm × 250 μm i.d. and 665 μm o.d. fused silica capillaries were pre-treated with 1 M NaOH for 30 min, then flushed with 3 ml of 0.1 M HCl, and finally, rinsed with 3 ml water. Thereafter, a 50% (v/v) Bind-Silane (bifunctional reagent) in acetone was introduced and left inside the column for 40 min at room temp. Finally, the capillary was rinsed with water and acetone, and then dried with N_2 gas. The monolith was prepared by dissolving 20 mg of acrylamide, 30 mg of N, N'-methylenebisacrylamide, and 30 mg of PEG per ml of 0.2 M sodium bicarbonate/0.5 M sodium chloride (pH∼8) buffer. The mixture was vortexed for a few seconds and heated at 55–60 °C for 15 min to completely dissolve the monomers. To 0.5 ml of this solution, 2 μl of 20% (v/v) TEMED were added. The mixture was then degassed for 15 min using N_2 gas delivered through fused silica tubing. Next 5 μl of N-acryloxysuccinimide (NAS) (140 mg/ml, 828 mM) dissolved in DMSO was added. Since DMSO is of a higher density than the buffer, the NAS was added on top of the solution, while degassing was used to ensure a thorough mixing of the NAS with the solution buffer. After 1 min, 2 μl of 20% (w/v) ammonium persulfate (APS) was added in order to initiate polymerisation. The APS was dispensed into the middle part of the solution while degassing, to ensure an efficient and rapid mixing. After 30 s, a 19 μl aliquot of this solution was removed and quickly mixed (so as to avoid contact with oxygen) with 1 μl of freshly prepared trypsin (20 mg/ml in a buffer containing 0.5 M benzamidine). This mixing step was performed without N_2 degassing. An activated (Bind-Silane) capillary was then inserted into the trypsin-monomer vial, whereby the solution filled the tubing by capillary action. Immobilisation/polymerisation were allowed to proceed for 30 min at room temperature with the capillary ends covered with parafilm [7].

2.5. Preparation of PNGase F immobilisation and polymerisation

Preparation of the co-polymerisation monolith column is described in Section 2.4. After 30 s, a 2 μl aliquot of the polymerisation solution was removed and mixed with 1 μl of PNGase F. The activated Bind-Silane fused silica capillary (6 cm in length) was immediately filled with the mixtures and immobilisation/polymerisation was allowed to proceed for 30 min at 37 °C with the capillary ends covered with parafilm [33].

2.6. Preparation of samples

10 μl of 84 ng/μl Eprex® was spiked into 100 μl of 50 mM ammonium bicarbonate buffer in an auto sampler vial. This vial was placed in the auto sampler for direct injection, 20 μl of the sample was injected into the on-line trypsin reactor and deglycosylation reactor, coupled to the analytical column and to the TIS source for detection.

3. Results and discussion

We elected to use silica capillaries derivatised with a dextran hydrogel to passivate the silica surface as these should provide less

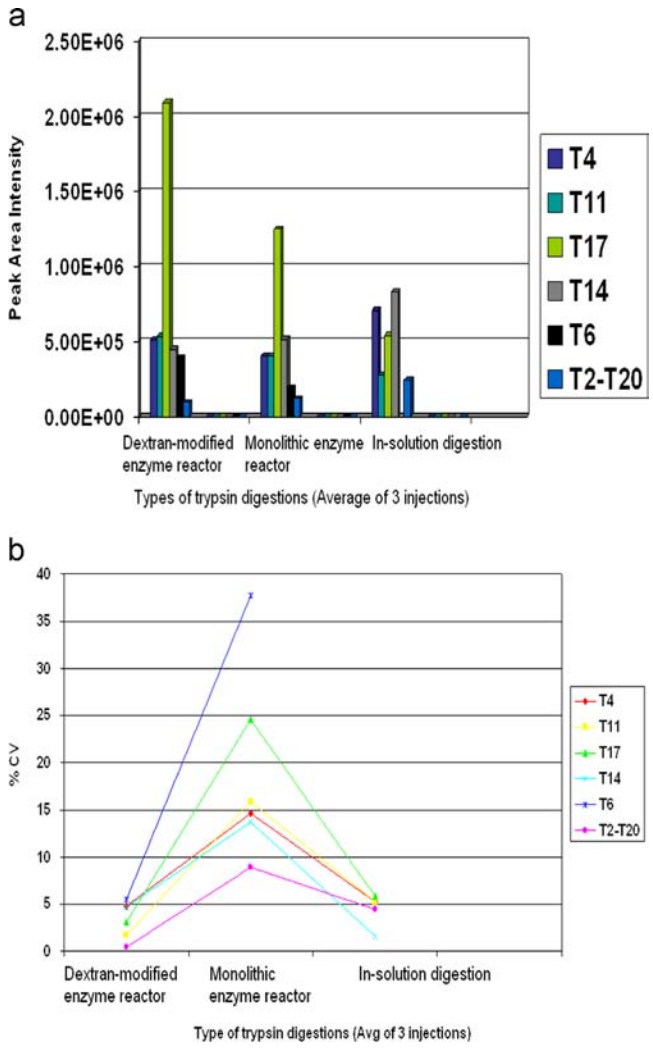

Fig. 2. (a) Graph summarising the peak area intensity of the two different types of enzyme reactors and in-solution enzyme digestion. (b) Graph summarising the reproducibility of the two different types of enzyme reactors and in-solution enzyme digestion.

non-specific adsorption of proteins and also offer a means to immobilise a larger amount of enzyme when compared to an unmodified surface [15,32]. As an alternative strategy, we coupled the enzyme to a monolithic polymer that had been fabricated inside a fused silica capillary. The digestion efficiencies of the two trypsin reactors fabricated in this manner were compared with the results from an in-solution digestion. This data is summarised in Fig. 2a, which shows that three of the peptides produced a higher response when using the dextran-modified fused silica reactor and that the other three peptides were more abundant when we utilised an in-solution digestion. The EPO peptide T17 (VYSNFLR) has shown a response that was approximately 4 times higher than that produced from the in-solution digest and almost twice as high as that obtained when the monolithic reactor was used. It was also interesting to note that the EPO peptide T6 (VNFYAWK), which was below our detection limit in the in-solution digestion, was readily identified from both of the enzyme reactors but could not be detected in our in-solution digests. Some of the other peptides in these digests showed a similar result for both of the immobilised enzyme reactors and also for the in-solution digestion. Triplicate injections onto the dextran-modified enzyme reactor were shown (Fig. 2b) to have a maximum coefficient variation (CV) of approximately 5% for all digested peptides and therefore this reactor also offers better reproducibility when compared to using trypsin bound to the monolith, where the variation between injections was found to be much higher, as in one case it was as high as 37%. From the six peptides injected, three peptides, most notably EPO peptide T17, showed an improved response when using an enzyme reactor. The extracted ion chromatogram was 4 × higher in the open tubular enzyme reactor and 2 × higher in the monolithic enzyme reactor when compared to the results from an in-solution digestion. The repeatability for the open tubular enzyme reactor was similar to the in-solution digestion, with both giving a CV of approximately 5%. Thus, in general, both of the on-line enzyme digestion reactors can be considered to offer reasonably robust performance and offered better sensitivity when screening for EPO peptides T17, T11 and T6. The use of the online reactor also reduced the digestion time when compared to the off-line in-solution enzyme digestion method. Another advantage is that these reactors were able to be used by us for more than 50 injections without an obvious loss of activity and this is a cost benefit in comparison to limitation of a one-time use of the enzyme during in-solution digestion.

In order to detect the tryptic glycopeptides, a deglycosylation step using the enzyme PNGase F to cleave the N-glycan is necessary to make these protein fragments compatible with LC–MS/MS detection. However, the PNGase F enzyme is offered by vendors in relatively small quantities at a comparatively high cost and so the high-wastage procedure for coating the dextran modified capillary was too costly to attempt. Thus the monolithic enzyme reactor was the only type prepared for this study and this provided good performance in comparison to the results that were achieved when we did an in-solution deglycosylation, as the peak abundances for deglycosylated peptides (EPO T5DAM and EPO T9DAM) obtained in-solution were lower than for the on-line deglycosylation reactors (Fig. 3a).

The trypsin and deglycosylation reactors (held inside an oven at 37 °C) were coupled to the autosampler and a trap column as shown in Fig. 1. The first factor that we evaluated was whether positioning the trypsin reactor either before or after the deglycosylation reactor in the flow path, produced any noticeable effect on the detection of the target peptides. Thereafter we tried sandwiching the trypsin reactor between two PNGase F reactors, but rather unexpectedly this orientation produced a 50% drop in the intensities for both T5 DAM and T9 DAM when compared to the TRYPSIN→PNGase F orientation. Lastly we doubled the length of the deglycosylation reactor that was connected after the trypsin reactor and this brought about a favourable increase in the XIC intensity for T5 DAM and T9 DAM. The comparison data for the four configurations used to carry out the digestions is summarised in Fig. 3b. Overall these results showed that there was a major difference in the abundance of the glycosylated peptides when using different on-line configurations. For example, when the PNGase F reactor was placed first followed by the trypsin reactor, it shows T9 DAM 10 times lower abundance than trypsin reactor comes before PNGase F reactor (Fig. 3b).

These results were promising, as even though it takes an hour (at 1 μl/min flow rate) to pass the sample through the reactors, this is considerably shorter than the two days that it normally takes us to complete the off-line sample preparation and we were also able to get an improvement in the sensitivity for both the non-glycosylated and glycosylated tryptic peptides in the Eprex™ digest.

4. Conclusions

Our goal to replace an off-line (in solution) enzyme digestion with a fast and efficient on-line method that could be applied to detect rhEPO in equine plasma extracts has been achieved. We have demonstrated that immobilising trypsin on a dextran-coated capillary offers better sensitivity in addition to efficiency and repeatability performance enhancements when compared to the use of a

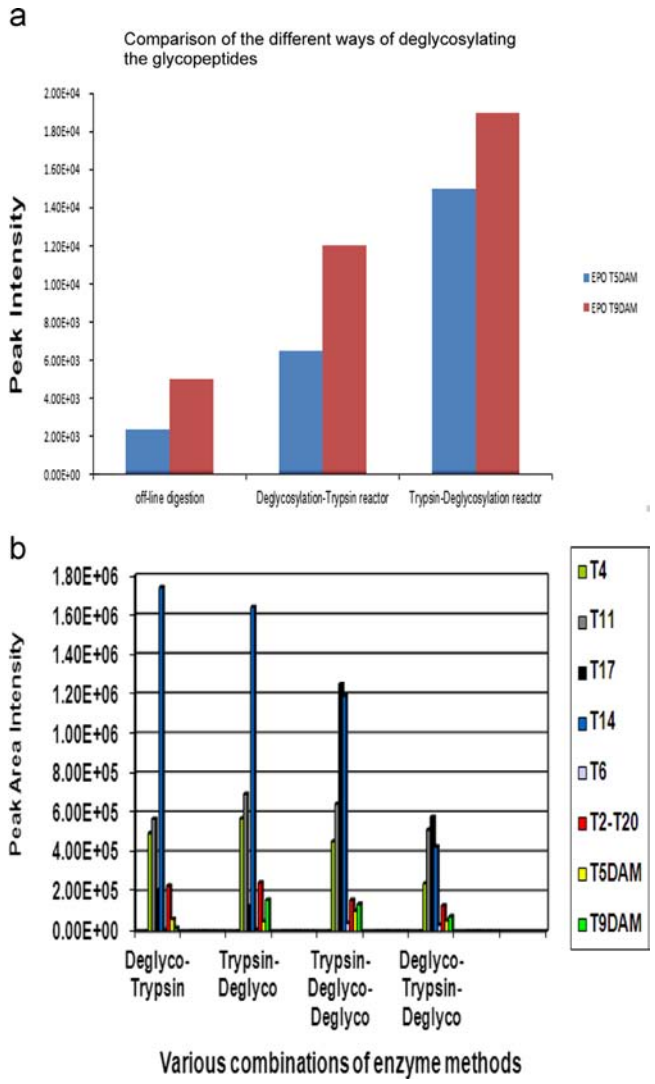

Fig. 3. (a) Comparison of the different ways of deglycosylating the glycopeptides. (b) Graph summarising the peak area intensity for the various orientations of the enzyme reactors.

monolithic capillary for this purpose. We have also demonstrated that the PNGase F enzyme can be successfully coupled to a monolithic column to fabricate a deglycosylation reactor and, in combination with the trypsin reactor, was used to detect both the digested non-glycosylated and glycosylated peptides in an on-line digestion/LC–MS/MS configuration with good sensitivity and acceptable repeatability. This time saving technique minimises the extent of manual sample handling, which reduces the potential sources of contamination. Future work will look at optimising the loading flow rate during the digestion/deglycosylation/trapping stage in order to reduce the cycle time between injections and/or multiplexing the analysis to improve the overall throughput of the method.

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