To elute or not elute prior to digestion in immunocapture-based bottom-up LC-MS

Maren C. S. Levernæs[†], *Marianne N. Broughton*[‡], *Léon Reubsaet*[†] *and Trine G. Halvorsen*[‡*]

[†]Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, Norway

[‡]Department of Medical Biochemistry, Norwegian Radium Hospital, Oslo University Hospital, Norway

KEYWORDS

Immunocapture, antibody coated magnetic beads, tryptic digestion, bottom-up proteomics

ABSTRACT

Immunocapture-based bottom-up LC-MS is a promising technique for the quantification of low abundant proteins. The use of magnetic immunocapture beads provides efficient enrichment from complex samples through the highly specific interaction between the target protein and its antibody. In this article, we have performed the first thorough comparison between digestion of proteins while bound to antibody coated beads versus digestion of proteins after elution from antibody coated beads. Two previously validated immunocapture based MS-methods for the quantification of progastrin-releasing peptide (ProGRP) and human chorionic gonadotropin (hCG) were used as model systems. The tryptic peptide generation was shown to be protein dependent

and influenced by protein folding and accessibility towards trypsin both on-beads and in the eluate. The elution of proteins bound to the beads was also shown to be incomplete. I addition, the on-bead digestion suffered from unspecific binding of the trypsin generated peptides. Thus, as described for both model proteins, a combination of on-beads digestion and elution may be applied to improve both the quantitative (based on peak area of the signature peptides) and qualitative yield (defined as number of missed cleavages, total number of identified peptides, coverage, signal intensity and number of zero missed cleavage peptides) of the target proteins. The quantitative yield of signature peptides was shown to be reproducible in all procedures tested.

INTRODUCTION

In the recent years, the bottom-up LC-MS approach for quantification of peptide and protein biomarkers has gained much interest[1-4]. Due to their high molecular weight, proteins are most often determined through their proteotypic peptides, also known as signature peptides, which are generated after proteolysis and are specific for their parent proteins[4-6]. For quantitative purposes and to obtain good accuracy, precision and low detection limits, it is essential that this proteolytic step is reproducible[4,7,8].

Another important step to ensure reproducible results and low quantification limit, is the sample preparation. The increased sample complexity after proteolysis of serum proteins is a challenge especially when targeting low-abundant protein biomarkers. To increase the sensitivity, specific enrichment and removal of interfering proteins is needed. Antibody-based sample preparation through immunocapture or immunoaffinity enrichment prior to LC-MS [1,4,8,9] is the preferred approach in order to be able to determine very low abundant proteins in serum/plasma[10-12]. This is mainly due to the highly specific interaction between the target protein and its antibody, providing

efficient clean-up and potential enrichment. There are several validated methods for the detection of very low-abundant diagnostic proteins using magnetic immunocapture beads for sample clean-up and enrichment[12-16].

However, there seems to be no clear consensus on whether or not the target protein should be eluted from the antibody prior to digestion[12-16]. The main goal of either procedure is however to lay the foundation for a reproducible and efficient proteolytic step in order to reach the lowest possible quantification limit. The purpose of the current work was to perform a thorough comparison between digestion of the protein after elution from the antibody (figure 1a)[12,16] versus digestion of the protein while bound to the antibody (on-beads digest, figure 1b)[13-15]. Special emphasis was made on the qualitative yield (defined as number of missed cleavages, total number of identified peptides, coverage, signal intensity and number of zero missed cleavage peptides), the quantitative yield (based on peak area) of previously described signature peptides[13,14], and remains after elution (figure 1c) and digest (figure 1d). In addition, the importance of applying predigest treatment (reductive alkylation) for proteins without disulphide bonds were investigated.

MATERIALS AND METHODS

Chemicals

The pharmaceutical formulation Ovitrelle (recombinant hCG) was distributed by Merck Serono Europe Ltd. (Bari, Italy). Cloned ProGRP isoform 1, anit-ProGRP (monoclonal antibody E146) and anti-hCG (monoclonal antibody E27) were provided by the Department of Medical Biochemistry, Norwegian Radium Hospital (Oslo, Norway). Trypsin (TPCK treated, from bovine pancreas, sequencing grade), 1,1-dithiothretiol (DTT, BioUltra, for molecular biology, ≥ 99.5 %), iodacetic acid (IAA, > 99 %), trifluoroacetic acid (TFA, reagent grade, ≥ 98 %), and formic acid

(FA, for mass spectrometry, 98 %) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade. Human serum from healthy subjects was obtained from Oslo University Hospital, Ullevaal (Oslo, Norway).

Solutions

ProGRP isoform 1 (AA 1-125 + 8) was cloned from the Small Cell Lung Cancer cell line NCI-H128 (ATCC No. HTB-120), expressed in *Escherichia coli* (Promega) using pGEX-6P-3 constructs (GE Healthcare) and purified as described elsewhere[17]. The concentration of the ProGRP stock solution was determined by absorbance at 280 nm (A280). Working solutions were prepared by dilution with 50 mM ammonium bicarbonate buffer solution (ABC-buffer) and stored at -4 °C.

One syringe of Ovitrelle (250 μg/mL koriongonadotropin alfa) was transferred to a Protein LoBind Eppendorf tube from Eppendorft AG (Hamburg, Germany) and stored at 4 °C. Working solutions of hCG were made by diluting the stock solution with ABC-buffer.

Spiked serum samples were prepared by adding working solutions of ProGRP and hCG to human serum from healthy subjects. The standards were added to serum immediately before the experiments were performed.

Coating of monoclonal antibodies on magnetic beads

Coating of magnetic beads was performed as described elsewhere[18], using 20 mg of antibody to 1 g of magnetic beads (Dynabeads M280 tosylactivated, Life Technologies Corporation, Oslo, Norway). The anti-ProGRP was activated with p-toluene sulfonyl chloride (Life Technologies Corporation, Oslo, Norway) and yielded a concentration of 20 mg mab E146/g beads in a 1 mg/mL

bead solution. To improve the orientation of the hCG antibody, hydrocholorid acis (HCl) was added to pH 2.5 prior to incubation for 1 h at 0 °C, before coupling at pH 9.5. The solution was diluted to 10 mg mab E27/g beads in a 1 mg/mL bead solution using phosphate buffer saline pH 7.4 (PBS).

Immunoaffinity extraction

The antibody-coated magnetic beads were prewashed to remove any unbound antibodies as described elsewhere[14]: desired volume of beads was washed with 1 mL PBS containing 0.05 % Tween 20, and re-dissolved in PBS , yielding a solution with the initial bead concentration, ready for use.

The immunoaffinity extraction was performed as follows: Protein LoBind Eppendorf tubes (Eppendorf AG, Hamburg, Germany) containing 1 mL of spiked serum were added 20 µL of prewashed antibody-coated magnetic beads. Samples were rotated and shaken for 1 hour on a HulaMixer (Life Technologies Corporation, Oslo, Norway) to facilitate the antigen-antibody interaction. The beads were immobilized using a magnetic rack (DynaMag-2 from Life Technologies Corporation, Oslo, Norway) and the solution was removed before the beads were washed with 500 µL of PBS containing 0.05 % Tween 20, 500 µL of PBS, 300 µL of Tris-HCl (pH 7.4), and 300 µL of 50 mM ABC buffer.

Elution of bound proteins from the antibody-coated magnetic beads

To elute the bound proteins from the antibodies after immunoextraction, or to evaluate which peptides remained bound to the beads after on-beads digestion, a volume of 15 µL of either freshly

prepared 2 % FA or 0.1 % TFA was added to the washed beads. The samples were shaken (800 rpm) at room temperature for 5 min, placed in magnetic rack and the supernatant containing eluted proteins was either transferred (digestion without beads present) to new Protein LoBind Eppendorf tubes or kept together with the beads (digestion with beads present). This step was performed twice and for the samples to be digested without beads present the two supernatants were combined. The solution was neutralized to pH 7 by adding 24 μL 0.53 M NH_3 and 33.6 μL 0.013 M NH_3 to the FA and TFA eluates respectively, and freshly prepared ABC buffer to a final concentration of 50 mM to ensure optimal pH during tryptic digestion.

To investigate the impact of combining on-beads digestion and elution 2 µL of FA (100 %) or TFA (5 %) was added to the samples after ended digestion to give a final concentration of 2 % and 0.1 %, respectively. The samples were then shaken (800 rpm) at room temperature for 10 min.

Reduction, alkylation and tryptic digestion

Prior to digestion all samples were added freshly prepared 50 mM ABC buffer to a final volume of 80 μL (90 μL if no reduction and alkylation was performed). The samples that were reduced and alkylated were then added 5 µL of freshly prepared 100 mM DTT. The samples were placed in a thermomixer (Eppendorf) and subjected to vibration (800 rpm) at 60 °C for 15 min. When the samples had cooled to room temperature, 5 µL of freshly prepared 400 mM IAA was added, and the samples were placed in the dark for 15 min.

The digestion in the presence of beads was initiated by adding freshly prepared trypsin to a final enzyme-to-antibody ratio of 1:5 (w/w), while the digestion in the absence of beads was performed with an enzyme-to-protein ratio of 1:40 (w/w). Samples were incubated overnight at 800 rpm at 37 °C. The following morning the Eppendorf tubes were briefly centrifuged (Centrifuge model

5804, Eppendorf) and if beads were present placed in the magnetic rack. The tryptic peptide solution were transferred to new Protein LoBind Eppendorf tubes and centrifuged at 5000 rpm for 2 min to sediment remaining trace particles. Fifty µL of the supernatant was then transferred to vials for LC-MS/MS analysis.

Nano LC-MS/MS analysis

The Nano LC-MS/MS analysis was carried out using the Chromeleon Xpress controlled Dionex Ultimate 3000 chromatographic system (Thermo Fischer, Bremen, Germany) connected to an Xcalibur controlled LTQ Discovery Orbitrap MS equipped with a Nano-ESI ion source (Thermo Fischer, Bremen, Germany). Twenty µL of each sample was injected and trapped on a C18 Acclaim PepMap 100 enrichment column (300 μm i.d. x 5 mm, 5 µm; Thermo). The loading mobile phase, 20 mM FA and MeCN (97:3, v/v), was delivered at 10 µL/min for 4 min. The analytes were then transferred to a C18 Acclaim PepMap 100 analytical column (75 μm i.d. x 15 cm, 3 µm; Thermo) at 300 nL/min. Mobile phase A consisted of 20 mM FA and MeCN (95:5, v/v) and mobile phase B of 20 mM FA and MeCN (5:95, v/v). A linear gradient was run from 0 to 50 % B in 60 min, and then increased to 100 % for 4 min before switching back to 100 % A in order to regenerate the column. The total analysis time per run was 89 min. The nanospray ionization source was operated in the positive ionization mode and the spray voltage was set to 2.2 kV. The heated capillary was kept at 150 °C. The capillary voltage was set at 45 V, and the tube lens offset was 100 V. Data-dependent acquisition was performed in the orbitrap mass analyser at a resolution of 30 000 over a mass range between m/z 300-2000 Da with charge state disabled. Up to six of the most intense ions per scan were fragmented by collision induced dissociation (CID) at 35 % relative collision energy, activation time of 30 ms, and analysed in the linear ion trap. The

fragmented m/z values were dynamically excluded for 15 s to minimize the extent of repeat sequencing of peptides and to fragment lower intensity m/z values.

Data interpretation

The MS raw files were processed with Proteome Discoverer 1.4 (Thermo Fischer, Bremen, Germany), using the SEQUEST algorithm, searching against ProGRP, hCG, human proteome and mice proteome databases generated from sequences obtained from UniProt (January, 2015). Up to five missed cleavages were considered using trypsin as enzyme (only three for the human and mice proteome searches). Methionine oxidation and cysteine carboxylmethylation were chosen as variable modifications. The initial parent and fragment ion maximum mass deviation was set to 10 ppm and 0.8 Da, respectively.

The Thermo Scientific Xcalibur software version 2.1 (Thermo Fischer, Bremen, Germany) was used to manually extract ion chromatograms (XICs), signal intensities and peak area of selected tryptic peptides.

RESULTS AND DISCUSSION

Choice of model compounds

Human chorionic gonadotropin (hCG) and pro-gastrin releasing peptide (ProGRP) were chosen as model compounds because quantitative immunocapture based MS-methods previously have been developed and validated in house[13,14], and due to easy access to well characterized antibodies against both proteins. Both methods used as model systems are based on on-beads digestion, and the limits of quantification in human serum are 3 pM and 10 IU/L for ProGRP and hCG, respectively.

Choice of eluent

The most commonly used agents for elution of proteins and peptides from antibody coated magnetic beads are acetic acid, FA, TFA and glycine buffer. Elution with FA and TFA were chosen as these agents are directly compatible with LC-MS. The chosen elution conditions were based on literature studies[1,12,16,19,20].

Both elution with 2 % FA and 0.1 % TFA were found to be effective for releasing the bound antigens from its antibody. Some differences between the two eluents where however observed indicating that the elution should be optimized depending on the protein(s) and peptide(s) in question to increase the signal intensity in quantitative applications (figure S1). The detailed discussion of the results can be found in the supplementary information. As TFA may increase the peak retention time and suppress the ESI-MS signal[21], figures presenting the FA results are displayed throughout the article, while the TFA figures can be found in the supplementary information. Since both FA and TFA are widely used, both eluents were included in the study.

Comparison of digestion performed on-beads and after elution

Qualitative and quantitative yield of target proteins

A comparison between digestion after elution (figure 1a) and on-beads digestion (figure 1b) was performed for both proteins with emphasis on qualitative yield; number of missed cleavages, total number of identified peptides, coverage, signal intensity and number of zero missed cleavage peptides, and quantitative yield of signature peptides.

When comparing on-beads digest and digest after elution of ProGRP there were distinct differences between the two procedures (figure 2a); on-beads digestion generated less peptides,

more zero missed cleavages and higher signal intensity. In addition the quantitative yield of the signature peptides NLLGLIEAK and LSAPGSQR was considerably higher after on-beads digestion ($p < 0.01$). The sequence coverages, however, were similar with 97.6 % and 100 % for on-beads digestion and the TFA eluate (table 1), respectively (a bit less for the FA eluate (90.4 %)). These differences may be due to protein folding and accessibility towards trypsin in the eluate and while bound to the antibody coated beads[22].

For hCG, the differences between elution and on-beads digest were less distinct compared to ProGRP (figure 2b and 2c). Both the sequence coverage and the number of identified peptides of both subunits were similar or higher after on-beads digest (table 1). In addition, for the β-subunit the number and signal intensity of zero missed cleavages were similar or higher after elution, while for the α-subunit one less zero missed cleavage was observed after elution with FA (figure 2c). Although the coverages for the β-subunit after on-beads digestion and TFA elution are similar (table 1), different parts of the sequence was covered by the two procedures; β21-42 was only covered using on-beads digestion, while β132-144 was covered in the TFA eluate (figure S3b); β113-131 was not covered by any of the procedures. There were also differences in the quantitative yields of the signature peptides (table 2); VLQGVLPALPQVVCNYR (β-subunit) being significantly higher in the FA eluate ($p < 0.05$), but not in the TFA eluate ($p < 0.1$), compared to on-beads digestion, and AYPTPLR (α-subunit) being significantly lower in the TFA eluate ($p < 0.01$), but not in the FA eluate ($p < 0.1$), compared to on-beads digestion. These results indicate the importance of optimizing the procedure for the individual peptide(s) of interest.

Figure 2 demonstrates a difference between the two proteins; for hCG most zero missed cleavages was identified after elution, while on-beads digestion provides most zero missed cleavages of ProGRP. In addition, the overall signal intensity of hCG was similar after on-beads

digestion and elution, while on-beads digestion of ProGRP gave considerably higher signal intensity compared to elution. The quantitative yield of the signature peptides of ProGRP was significantly higher after on-beads digestion, whereas the yield of the hCG signature peptides was more peptide dependent: the yield of the β-subunit signature peptide was significantly higher in the FA eluate and the yield of the α-subunit signature peptide was higher after on-beads digestion. There are several differences between the two antigens that might affect the digestion step: The size of the antigen, elution efficiency, the location of trypsin cleavage sites relative to the epitope, epitope configuration (linear (E146) versus non-linear (E27)), and differences in the local conformation of the protein bound to beads and in the eluate. Several of these points can explain why digestion of hCG after elution gives less missed cleavages and higher signal intensity compared to on-bead digestion. For instance, the antibody E27 binds to a non-linear epitope on the hCG β-subunit and may reduce the digest efficiency on beads due to steric hindrance. Still, the differences between the digested eluate and on-beads digestion are small most likely due to the assumption that the 3D structure of hCG and its antibody will be destroyed by reductive alkylation prior to digestion, assumed to provide similar conformation of hCG during on-beads digestion and digestion of the eluate. On the other hand, it is not that easy to explain the behaviour of ProGRP: For instance, the localization of the signature peptide NLLGLIEAK close to the epitope, which ProGRP is bound to during on-beads digestion, favours digestion after elution. However, it might be the case that epitope binding alters the conformation of the protein to increase the accessibility compared to a folded protein after elution. In summary, the described results demonstrate that the choice of procedure may influence both the qualitative and quantitative yield of the protein and should therefore be optimized individually.

General clean-up efficiency

In addition to the targeted yield/comparison of the two procedures, some non-targeted aspects may be worth to consider; the amount of background, with emphasis on co-elution of other proteins and/or peptides and overall effect of sample clean-up, and the economic aspect with the possibility of reusing the antibodies after elution.

The basepeak chromatograms after on-beads digestion (red) and elution with FA (blue) of ProGRP (a) and hCG (b) are shown in figure 3. As one might expect, figure 3a shows that the observed background was considerably higher after on-beads digestion compared to digestion after elution. One might expect that this could be due to digestion of the anti-proGRP antibodies (E146) during on-beads digestion; however, as only 35 peptides from 18 proteins were identified from the E146 antibodies, the antibodies seems to be quite resistant to the applied amount of trypsin. The main contribution to the increased background after on-beads digestion is the 155 unique peptides identified from 32 human proteins present in serum. This is considerably fewer peptides than observed for these antibodies earlier [manuscript submitted]. The reason for this is most likely the presence of ProGRP in the current experiments which reduce the non-specific interactions of other proteins and peptides to the antibody. In comparison, only 29 unique peptides from 5 proteins were identified after elution and digestion without beads present. For hCG on the other hand (figure 3b), no difference in the background was observed; both the on-beads and elution chromatograms has more background compared to those from ProGRP. Again, the major contribution to the observed background is 189 unique peptides from 39 human proteins after on-beads digestion and 175 unique peptides from 43 proteins after elution and digestion without beads present; 29 unique peptides from 9 proteins were identified from the anti-hCG antibodies (E27). The observed differences in the amount of identified peptides from the ProGRP and hCG samples might be due

to digestion of proteins strongly bound to the beads not being co-eluted with the target protein (observed for ProGRP) or the digestion of proteins unspecific bound to the target protein (observed for hCG). These results indicate that the elution approach might provide the highest degree of sample clean-up; however, it will depend on the unspecific binding to the antibody and protein in question.

Being both time-consuming and expensive, the development of monoclonal antibodies is limited to the most well-characterized and desirable assays[23]. The possibility of reusing the antibodies would thus be beneficial. Affinity agents has already been reused in affinity columns[24,25] and recently Zhou et al demonstrated that antibody coated magnetic beads could be reused up to 10 times in solution-phase immuno-MRM assays without compromising the antibodies performance[23]. This implies that the sample cost would be considerably reduced using the elution approach and reusing the antibody-coated beads, thus increasing the number of samples analysed with the same batch of antibodies.

These results imply that elution prior to digestion may be the preferred option since the qualitative and quantitative yield of the target protein is similar to that of on-beads digestion, in addition to the possibility of giving cleaner chromatograms, which might prolong the lifetime of the analytical column, and the possibility of reusing the antibody coated magnetic beads, thus reducing the cost per sample. The additional steps in the sample preparation due to elution and neutralization (15 min) prior to digestion are thus of less importance compared to the gained benefits.

Evaluation of elution efficiency and on-beads digestion efficiency

To evaluate the efficiency of the elution step, an on-beads digestion of the washed eluted beads (figure 1c) was performed. Figure 4 shows that the elution the antigens with the current conditions of neither FA nor TFA are complete. Based on the number and intensity of peptides identified in the digested eluates in figure S1, the elution efficiencies of the two eluents were considered to be equal. The on-beads digestion of the eluted beads did however identify more peptides with higher signal intensity in the beads eluted with TFA, suggesting that TFA is a less efficient eluent than FA. Still, the signal intensity of the identified peptides from the two eluates was equal (figure S2).

When comparing the identified zero missed cleavage peptides of ProGRP in the eluate (figure 2a) and the on-beads digestion of the eluted beads (figure 4a), the signal intensities were in the same order of magnitude or higher in the on-beads digest of the eluted beads. This suggests that the elution efficiency from the anti-ProGRP coated beads was about 50 %, assuming equal digest conditions in solution and on-beads. The elution efficiency of hCG was considered to be slightly higher than for ProGRP as the signal intensity of the zero missed cleavage peptides were similar or higher in the eluates than remaining on beads (figure 2b vs figure 4b).

Similarly, an elution was performed to investigate which peptides were still bound to the beads after on-beads digest (figure 1d). The beads were washed thoroughly prior to elution to minimize unspecific binding of generated peptides. Figure 5a shows that only three peptides, all with missed cleavages, were identified in both the FA and TFA eluates of remaining peptides after on-beads digestion of ProGRP. The most abundant of these peptides were GSLKQQLREYIRWEEAARNLLGLIEAK which contains the linear epitope recognized by the monoclonal antibody E146, LREYI[17]. This demonstrates the selectivity of the antibody. The other two peptides partly overlap with the amino acids GNHWAVGHLMGK, and may bind non-

specific to either the antibody or the beads itself[26,27]. The overall sequence coverage of the remaining peptides eluted after on-beads digestion was 55.2 %.

For the hCG β-subunit, the combined coverage in the FA and TFA eluates of remaining peptides after on-beads digestion was 85.4 %. Different peptides were identified in the two eluates (figure 5b). This could either be due to elution differences between FA and TFA or more likely differences in the unspecific binding of peptides to the beads after digestion. Still, the first might be the case as only the TFA eluate identified peptides from the α-subunit (figure 5c), covering 52.7 % of the sequence. The epitope recognized by the hCG antibody, E27, is non-linear and positioned at the very top of the two adjacent peptide loops of the β-subunit, consisting of the β20-25 (KEGCPC) and β68-77 (LPGCPRGYNP) residues[28]. Five out of the ten eluted peptides contained some of these residues (figure 5b). The overall signal intensity of the identified peptides were in the same order of magnitude, implying no enhanced specificity towards any of them. Unspecific binding was also the cause of the identified α-subunit peptides in the TFA eluate, as the α-subunit is not recognized by this antibody.

These results show that several of the generated peptides after on-beads digestion remains bound to the antibody and that the elution of neither antigen from the antibodies is complete (current conditions). This suggests that the peptide yield for both digestion on-beads and after elution may be increased by either leaving the beads in the eluate during digestion or reducing the pH after on-beads digestion.

Impact of combining elution and on-beads digestion

With the intention to improve the peptide yield of both methods the impact of combining elution and on-beads digestion were investigated. 1) The eluted samples were digested in the presence of

beads (figure 1e) in order to investigate if this would increase the peptide yield by digesting both the eluted portion of the proteins and the proteins still bound to the antibody. 2) Similarly, the on-beads digested samples were added the eluting agents after ended digestion (figure 1f) to investigate whether or not this would reduce the unspecific binding and thus increase the peptide yield. The identified peptides from experiment 1) and 2) are shown in figure 6 and 7, respectively. The individual discussions of elution followed by digestion in the presence of beads and on-beads digestion followed by elution can be found in the supplementary information. Below is an overall comparison of the two combinations.

There was seen a difference between the two proteins. For ProGRP, digestion of the eluate in the presence of beads considerably reduced the number of identified peptides (figure 6a). In addition both the number and signal intensity of zero missed cleavage peptides increased. For hCG however, digestion of the eluate in the presence of beads had no impact on the number of identified peptides, nor the overall signal intensity of the generated peptides (figure 6b and 6c). On the other hand, elution following on-beads digest had no impact on overall signal intensity, nor the total number of identified peptides for ProGRP (figure 7a). For hCG, both the number and signal intensity of identified zero missed cleavages were improved, as well as sequence coverage (figure 7b and 7c). These results imply that any combination of elution and on-beads digestion may improve signal intensity and number of zero missed cleavages, as well as reduce unspecific binding. The combination of choice is however protein dependent and should be optimized in each application.

When comparing the two combinations, digestion of the eluate in the presence of beads (figure 1e), and on-beads followed by elution (figure 1f) there were no significant differences in the quantitative yield of any of the signature peptides for the two proteins ($p > 0.05$). In addition

approximately the same number and signal intensity of zero missed cleavages were identified. The major difference between the two options being that the on-beads digest followed by elution is less time-consuming as it is not necessary to readjust the pH of the eluate prior to digestion.

Impact of applying predigest treatment to a protein not containing disulphide bonds

Reductive alkylation is standard predigest treatment when a protein contains cysteine residues. As the use of DTT reduction followed by IAA alkylation involves the use of hazardous chemicals and denaturing temperature which are resource consuming and may contribute to increased sample complexity, these steps should be avoided when signature peptides and/or target protein does not contain cysteine residues or when the protein is known to not contain disulphide bridges. However, this predigest treatment might affect the peptide yield due to changes in steric conditions and/or liberation from beads during on-beads digestion. To investigate the impact of predigest treatment on a protein known to not contain disulphide bridges, reductive alkylation was applied to ProGRP prior to digestion both on-beads and after elution.

Figure 8 shows that the treated samples generated peptides with higher signal intensity compared to the untreated samples, both after elution and on-beads digest. In addition more zero missed cleavages were identified in the treated TFA eluate, while both the treated eluates generated less missed cleavages peptides compared to the untreated eluates. The quantitative yield of the signature peptides after on-beads digest were significantly higher in the treated samples ($p < 0.05$), while the quantitative yield of the digested eluates were unchanged ($p > 0.05$).

The predigest treatment were also tested on both combinations of elution and on-beads digest. However, no differences in signal intensity, missed cleavages or quantitative yield were observed.

Still, these results suggest that even proteins that do not contain disulphide bonds may benefit from applying reductive alkylation prior to digestion.

CONCLUDING REMARKS

To our knowledge, this is the first thorough comparison between on-beads digest and elution of proteins in immunocapture based bottom-up LC-MS methods. The presented research demonstrates that there is no clear answer to whether or not the protein should be eluted from the antibody prior to digestion, with the quantitative yield of the ProGRP signature peptides being significantly higher after on-beads digestion and those of hCG being higher after elution. Differences in the amount of protein folding and accessibility towards trypsin both in the eluate and while bound to the antibody may influence which is the preferred option for the protein(s) in question.

On-beads digestion is less time-consuming as elution has to be proceeded by neutralization of the eluate prior to digestion and hence more steps are introduced to the sample preparation procedure. However, these additional steps might give you less background in your chromatograms, prolong your columns lifetime, and reduce the cost per sample by providing the opportunity to reuse the antibody coated magnetic beads.

Generally, there was one major disadvantage with the immunocapture step in each approach; First of all elution of the captured proteins was found to be incomplete with the current elution conditions for both our model proteins. However, the quantitative yield of the signature peptides was found to be reproducible. Still, digestion of the eluate in the presence of beads may increase the signal intensity due to digestion of peptides bound to the antibody beads, as demonstrated for ProGRP. Secondly, unspecific binding of proteins and peptides to antibodies and magnetic beads

is a well-known phenomenon. Peptides generated from on-beads digestion may bind non-specific to the beads and thus reduce the signal intensity. By including an elution step following on-beads digestion the unspecific binding of such peptides may be reduced. This combination was shown to have an impact on hCG. These results indicate that any combination of elution and on-beads digestion might improve both the qualitative and quantitative yield of the target protein.

FIGURES

Figure 1. Overview of the experimental set-up. **A**: Elution and digestion without beads present, **B**: On-beads digestion, **C**: On-beads digestion of remaining proteins after elution, **D**: Elution of remaining peptides after on-beads digestion, **E**: Elution and digestion with beads present, and **F**: On-beads digestion followed by elution.

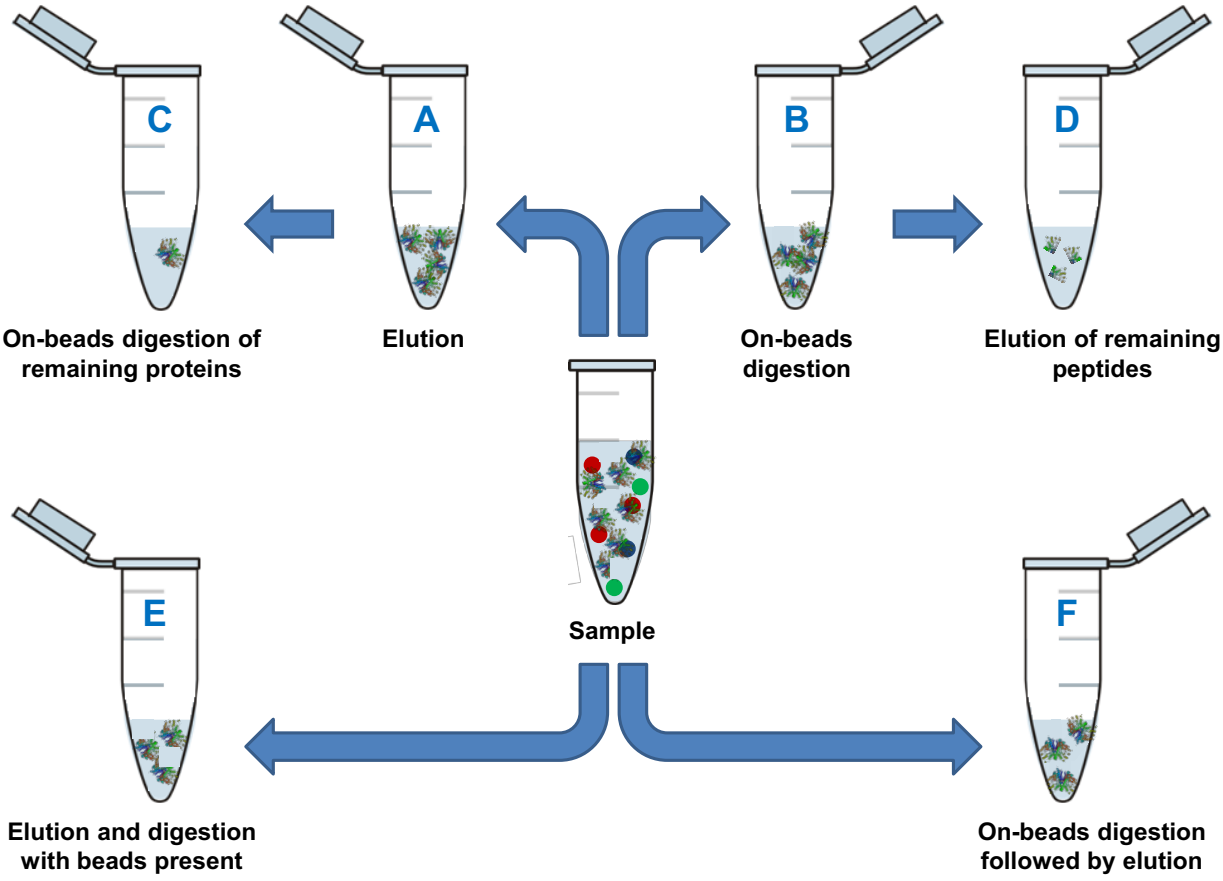


Figure 2. Comparison of on-beads digestion (above sequence) and digestion after elution with FA (below sequence) for **A**: ProGRP, **B**: hCG β-subunit and **C**: hCG α-subunit. Zero missed cleavage peptides are displayed closest to the sequence (grey box). Signal intensity: – low, – medium, – high and – very high. All the individual signal intensities are available in supplementary information.

Figure 3. Basepeak chromatograms (m/z = 55.0-2000.0) after on-beads digestion (red) and digestion after elution with FA (blue) of ProGRP (**A**) and hCG (**B**).

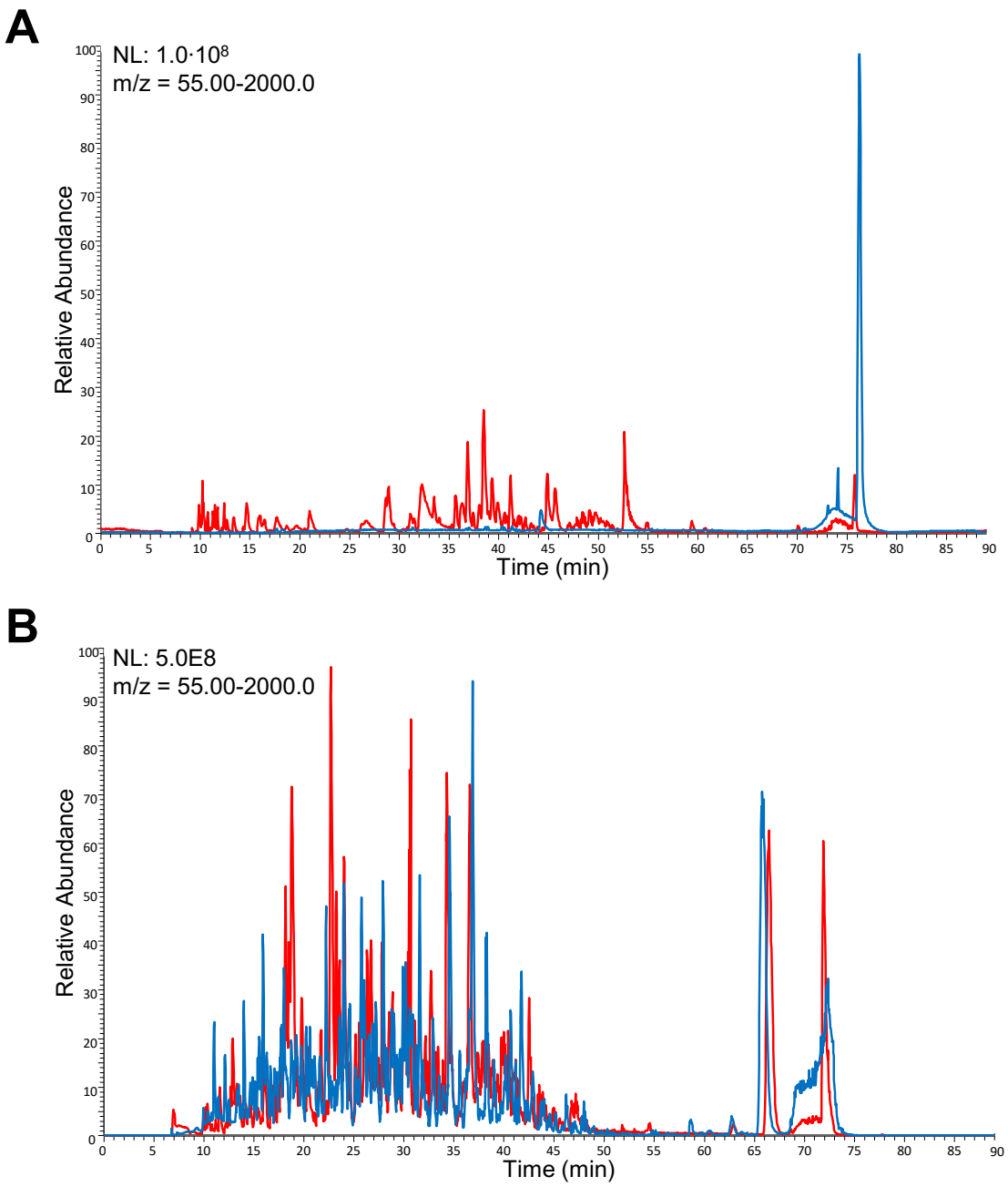


Figure 4. On-beads digestion of proteins remaining on the beads after elution with FA (above sequence) and TFA (below sequence) for **A**: ProGRP, **B**: hCG β-subunit and **C**: hCG α-subunit. Zero missed cleavage peptides are displayed closest to the sequence (grey box). Signal intensity:

– low, – medium, – high and – very high. All the individual signal intensities are available in supplementary information.

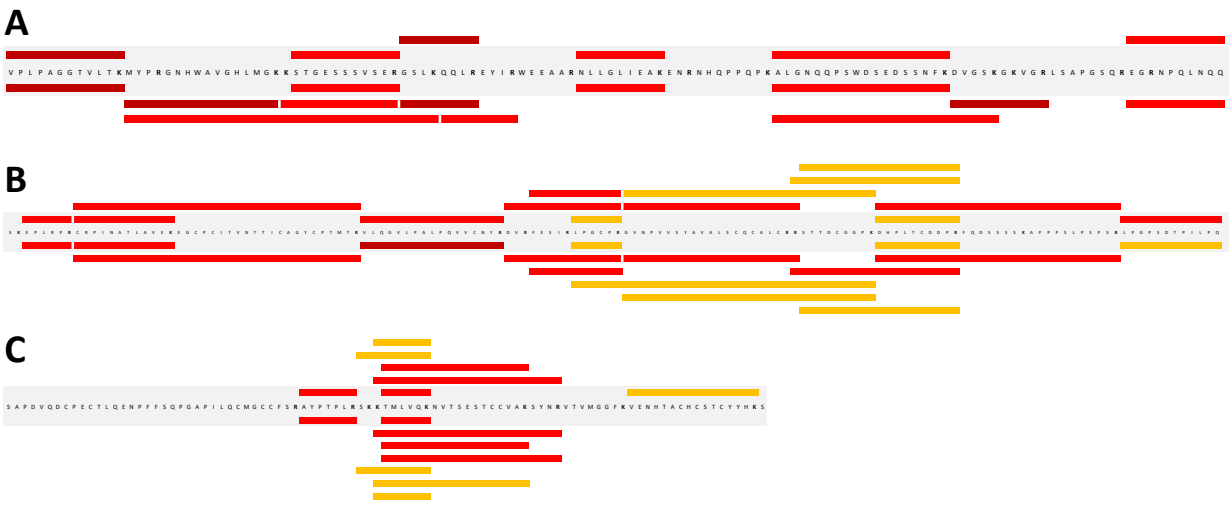


Figure 5. Elution of remaining peptides after on-beads digestion with FA (above sequence) and TFA (below sequence) for **A**: ProGRP, **B**: hCG β-subunit and **C**: hCG α-subunit. Zero missed cleavage peptides are displayed closest to the sequence (grey box). Signal intensity: – low, –

medium, – high and – very high. All the individual signal intensities are available in supplementary information.

Figure 6. Digestion of the FA eluate without (above sequence) and with (below sequence) the presence of beads for **A**: ProGRP, **B**: hCG β-subunit and **C**: hCG α-subunit. Zero missed cleavage peptides are displayed closest to the sequence (grey box). Signal intensity: – low, – medium, –

high and – very high. All the individual signal intensities are available in supplementary information.

Figure 7 – Identified peptides after on-beads digest (above sequence) and on-beads digest followed by FA elution (below sequence) for **A**: ProGRP, **B**: hCG β-subunit and **C**: hCG α-subunit. Zero missed cleavage peptides are displayed closest to the sequence (grey box). Signal

intensity: – low, – medium, – high and – very high. All the individual signal intensities are available in supplementary information.

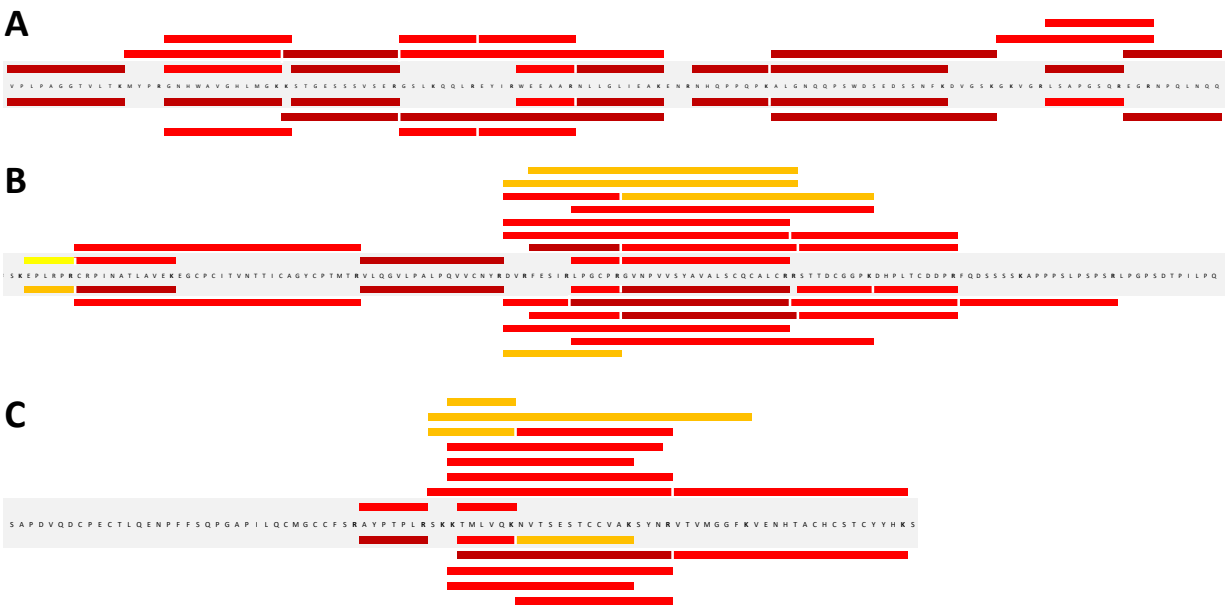
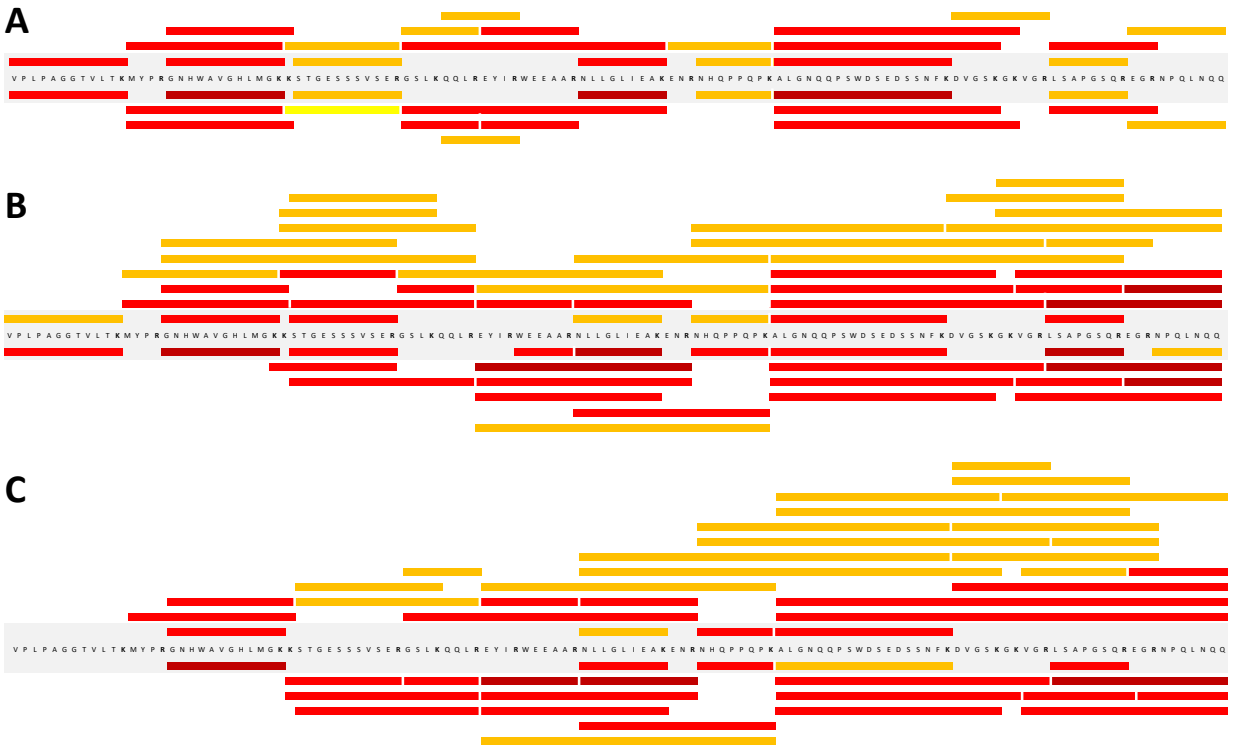


Figure 8 – Impact of applying predigest treatment to a protein not containing disulphide bridges for **A**: on-beads digestion, **B**: digestion after FA elution and **C**: digestion after TFA elution. Peptides generated from the untreated samples are displayed above the sequence and peptides

generated from the treated samples below the sequence. Zero missed cleavage peptides are displayed closest to the sequence.



TABLES

Table 1. Qualitative yield after digestion of the eluates and after on-beads digestion

Protein	FA eluate			FA eluate with beads present			TFA eluate			TFA eluate with beads present		
	# 0 missed cleavages	# Peptides	Coverage (%)	# 0 missed cleavages	# Peptides	Coverage (%)	# 0 missed cleavages	# Peptides	Coverage (%)	# 0 missed cleavages	# Peptides	Coverage (%)
ProGRP	4	32	90.4	9	14	83.2	7	38	100	8	17	95.2
βhCG	6	12	61.8	7	15	85.4	7	16	70.1	7	15	85.4
αhCG	1	8	62.4	2	9	41.9	2	6	41.9	2	8	59.1

Protein	On-beads digest			On-beads digest followed by FA elution			On-beads digest followed by TFA elution		
	# 0 missed cleavages	# Peptides	Coverage (%)	# 0 missed cleavages	# Peptides	Coverage (%)	# 0 missed cleavages	# Peptides	Coverage (%)
ProGRP	8	18	97.6	8	15	90.4	8	18	97.6
βhCG	5	18	77.1	7	18	90.3	8	15	85.4
αhCG	2	11	61.3	3	8	59.1	2	5	44.1

Table 2. Quantitative yield after digestion of the eluates and after on-beads digestion

Signature peptide	FA eluate			FA eluate with beads present			TFA eluate			TFA eluate with beads present		
	Intensity	Area	RSD (%)	Intensity	Area	RSD (%)	Intensity	Are	RSD (%)	Intensity	Area	RSD (%)
NLLGLIEAK	3.0·10⁷	4.6·10⁷	28.7	2.8·10⁶	4.9·10⁷	11.8	9.3·10⁶	1.5·10⁷	44.1	2.8·10⁷	2.9·10⁷	46.7
LSAPGSQR	-	-	-	6.2·10⁵	1.1·10⁷	20.3	1.7·10⁶	3.4·10⁶	74.1	4.1·10⁷	6.3·10⁷	3.4
VLQGVLPALPVVCNYR	5.6·10⁷	8,25·10⁷	8.4	4.7·10⁶	6.3·10⁷	19.4	3.7·10⁶	2.9·10⁶	30.5	4.0·10⁷	6.4·10⁷	10.7
AYPTPLR	7.5·10⁷	1.5·10⁸	9.0	1.2·10⁶	2.5·10⁷	5.7	5.1·10⁷	1.1·10⁸	12.9	8.5·10⁷	1.8·10⁸	6.5

Signature peptide	On-beads digestion			On-beads digestion followed by FA elution			On-beads digestion followed by TFA elution		
	Intensity	Area	RSD (%)	Intensity	Area	RSD (%)	Intensity	Are	RSD (%)
NLLGLIEAK	1.5·10⁷	2.8·10⁷	18.9	2.3·10⁷	3.4·10⁷	32.4	1.3·10⁷	2.1·10⁷	19.2
LSAPGSQR	1.2·10⁷	1.5·10⁷	9.6	9.8·10⁶	1.3·10⁷	33.6	6.0·10⁶	1.7·10⁷	31.7
VLQGVLPALPVVCNYR	4.1·10⁷	6.3·10⁷	13.2	4.9·10⁷	8.7·10⁷	9.8	4.0·10⁷	6.1·10⁷	13.1
AYPTPLR	9.6·10⁷	1.8·10⁸	12.8	1.3·10⁷	2.7·10⁷	17.1	8.2·10⁷	1.7·10⁸	10.5

ASSOCIATED CONTENT

Supporting information. The following files are available free of charge.

Supplementary information. Discussions regarding the choice of eluent, effect of eluent on tryptic digestion, elution followed by digestion in the presence of beads, and on-beads digestion followed by elution. Supplementary figure S1. Comparison of the generated peptides after FA and TFA elution. Supplementary figure S2. Comparison of in solution digest in ABC-buffer, neutralized FA and neutralized TFA of ProGRP. Supplementary figure S3. Comparison of on-beads digestion and digestion after elution with TFA. Supplementary figure S4. Digestion of the

TFA eluate without and with the presence of beads. Supplementary figure S5. Identified peptides after on-beads digest and on-beads digest followed by TFA elution. Supplementary figure S6. Impact of applying predigest treatment to a protein not containing disulphide bridges.

Supporting information signal intensities. Individual signal intensities for all peptides from all experiments. Peak area of signature peptides. T-tests.

AUTHOR INFORMATION

Corresponding Author

*Tel: +47 22855735. Fax: +47 22854402. E-mail: t.g.halvorsen@farmasi.uio.no.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

This work has been performed within DIATECH@UiO, a strategic research initiative at the Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway.

REFERENCES

(1) Becker, J. O.; Hoofnagle, A. N. *Bioanalysis* **2012**, *4*, 281.

(2) Calvo, E.; Camafeita, E.; Fernández-Gutiérrez, B.; López, J. A. *Expert review of proteomics* **2011**, *8*, 165.

(3) Picotti, P.; Aebersold, R. *Nature methods* **2012**, *9*, 555.

(4) Dittrich, J.; Becker, S.; Hecht, M.; Ceglarek, U. *PROTEOMICS-Clinical Applications* **2015**, *9*, 5.

(5) Anderson, L.; Hunter, C. L. *Molecular & Cellular Proteomics* **2006**, *5*, 573.

(6) Lange, V.; Picotti, P.; Domon, B.; Aebersold, R. *Molecular systems biology* **2008**, *4*, 222.

(7) Brownridge, P.; Beynon, R. J. *Methods* **2011**, *54*, 351.

(8) Fung, E. N.; Bryan, P.; Kozhich, A. *Bioanalysis* **2016**, *8*, 847.

(9) Ackermann, B. L.; Berna, M. J. **2007**, *4*, 175.

(10) Winther, B.; Nordlund, M.; Paus, E.; Reubsaet, L.; Halvorsen, T. G. *Journal of separation science* **2009**, *32*, 2937.

(11) Lund, H.; Torsetnes, S. B.; Paus, E.; Nustad, K.; Reubsaet, L.; Halvorsen, T. G. *Journal of proteome research* **2009**, *8*, 5241.

(12) Hoofnagle, A. N.; Becker, J. O.; Wener, M. H.; Heinecke, J. W. *Clinical chemistry* **2008**, *54*, 1796.

(13) Lund, H.; Løvsletten, K.; Paus, E.; Halvorsen, T. G.; Reubsaet, L. o. *Analytical chemistry* **2012**, *84*, 7926.

(14) Torsetnes, S. B.; Nordlund, M. S.; Paus, E.; Halvorsen, T. G.; Reubsaet, L. o. *Journal of proteome research* **2012**, *12*, 412.

(15) Torsetnes, S. B.; Løvbak, S. G.; Claus, C.; Lund, H.; Nordlund, M. S.; Paus, E.; Halvorsen, T. G.; Reubsaet, L. *Journal of Chromatography B* **2013**, *929*, 125.

(16) Zhao, C.; Trudeau, B.; Xie, H.; Prostko, J.; Fishpaugh, J.; Ramsay, C. *Proteomics* **2014**, *14*, 1311.

(17) Nordlund, M. S.; Fermer, C.; Nilsson, O.; Warren, D. J.; Paus, E. *Tumor Biology* **2007**, *28*, 100.

(18) Paus, E.; Nustad, K. *Clinical chemistry* **1989**, *35*, 2034.

(19) Stefanescu, R.; Born, R.; Moise, A.; Ernst, B.; Przybylski, M. *Journal of the American Society for Mass Spectrometry* **2011**, *22*, 148.

(20) Vogel, M.; Blobel, M.; Thomas, A.; Walpurgis, K.; Schänzer, W.; Reichel, C.; Thevis, M. *Analytical chemistry* **2014**, *86*, 12014.

(21) Chakraborty, A. B.; Berger, S. J. *Journal of biomolecular techniques: JBT* **2005**, *16*, 327.

(22) Chiva, C.; Ortega, M.; Sabidó, E. *Journal of proteome research* **2014**, *13*, 3979.

(23) Zhao, L.; Whiteaker, J. R.; Voytovich, U. J.; Ivey, R. G.; Paulovich, A. G. *Journal of proteome research* **2015**, *14*, 4425.

(24) Kennedy, J. J.; Yan, P.; Zhao, L.; Ivey, R. G.; Voytovich, U. J.; Moore, H. D.; Lin, C.; Pogosova-Agadjanyan, E. L.; Stirewalt, D. L.; Reding, K. W. *Molecular & Cellular Proteomics* **2016**, *15*, 726.

(25) Li, Q.; Lü, C.; Liu, Z. *Journal of Chromatography A* **2013**, *1305*, 123.

(26) Schoenherr, R. M.; Zhao, L.; Ivey, R. G.; Voytovich, U. J.; Kennedy, J.; Yan, P.; Lin, C.; Whiteaker, J. R.; Paulovich, A. G. *Proteomics* **2016**, *16*, 2141.

(27) Rossetti, C.; Levernæs, M. C. S.; Reubsaet, L.; Halvorsen, T. G. *Journal of Chromatography A* **2016**, DOI 10.1016/j.chroma.2016.09.069.

(28) Berger, P.; Sturgeon, C.; Bidart, J.-M.; Paus, E.; Gerth, R.; Niang, M.; Bristow, A.; Birken, S.; Stenman, U.-H. *Tumor biology* **2002**, *23*, 1.