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Development of a novel sample preparation approach for bottom-up shotgun proteomics

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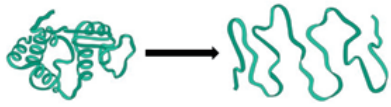
Introduction

Profiling of proteins and peptides represents a complex analytical problem due to high chemical variability, and aims to provide a better understanding of the function and implication of a species into a given physiological process. A protein profiling strategy, the so-called 'bottom up' shotgun approach, involves the proteolytic digestion of a solution of proteins followed by the analysis of the released peptides. When aiming to increase the chance for protein identification, the use of efficient methods of protein digestion becomes indispensable. The present work is based on the evaluation of a target with a modified functionalised surface (Tethis SpA), allowing all stages of sample processing to be performed on the same sample spot.

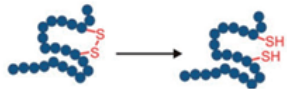
1. Denaturation

➡ ***i)*** **5 µL ProteaseMAX™ surfactant;** ***ii)*** **RT ,10 min;** ***iii)*** **remove reagent**



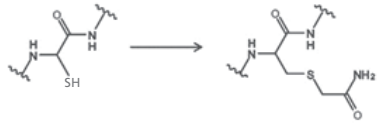
2. Reduction

➡ ***i)*** **10 µL 1,4-Dithiothreitol (DTT);** ***ii)*** **50°C, 15 min;** ***iii)*** **remove reagent**



3. Alkylation

➡ ***i)*** **10 µL iodoacetamide (IAA);** ***ii)*** **RT, dark, 15 min;** ***iii)*** **remove reagent**



4. On-chip clean-up

➡ ***i)*** **4 µL water**

5. On-chip trypsin digestion[a]/deglycosylation[b]

a ➡ ***i)*** **6 µL Trypsin in ammonium bicarbonate;** ***ii)*** **47°C, 30 min**

b ➡ ***i)*** **6 µL PNGase F in ammonium bicarbonate;** ***ii)*** **45°C, 4 hrs**

6. MALDI MS analysis

Figure 1. Sample preparation workflow.

Methods and Materials

All protein samples, reagents and matrices were purchased from Sigma-Aldrich (St. Louis, MO). During optimization of the on-target sample processing, a suitable amount of protein was loaded onto the target in order to keep the protein-to-enzyme ratio optimal. All the sample processing steps (denaturation, reduction, alkylation and trypsin/PNGase F digestion) have been carried out on-target (Figure 1). MALDI-MS analyses of the intact (native and post-reduction/alkylation) protein as well as of the tryptic and deglycosylation products were conducted on an AXIMA Performance MALDI-TOF-TOF mass spectrometer (Shimadzu, Manchester, UK; Figure 2a). Figure 2b shows an example of the exceptional volume capacity of the target which permits to perform an on-target sample processing.

a)

b)

Figure 2. a) AXIMA Performance MALDI-TOF-TOF mass spectrometer; b) Target's volume capacity (10 µL water applied).

Results

On-chip trypsin digestion

The success of a 'bottom-up' protein analysis is dependent on an efficient and complete digestion. Common digestion protocols using trypsin enzyme involve a step of reduction and alkylation (RCM, abbreviated) prior to digestion to improve the trypsin efficiency by allowing it to have easier access to the inner core of the protein. The overall process of a protein digestion can be tedious and time-consuming as each step has to be carefully optimized in order to maximize the digestion efficiency. The results shown in Figure 3 highlight the capability of the novel, functionalized target to perform on-target trypsin digestion with or without RCM (BSA digest in this example). Good Mascot scores can be appreciated even without conducting the RCM (red trace). Figure 4 shows the comparison between the BSA digest from on-target and in-solution (with and without RCM followed by on-target clean-up) digestion. Interestingly, the on-chip trypsin digestion (without RCM) gave a good, slightly better, score in a much shorter time (30 mins vs. overnight).

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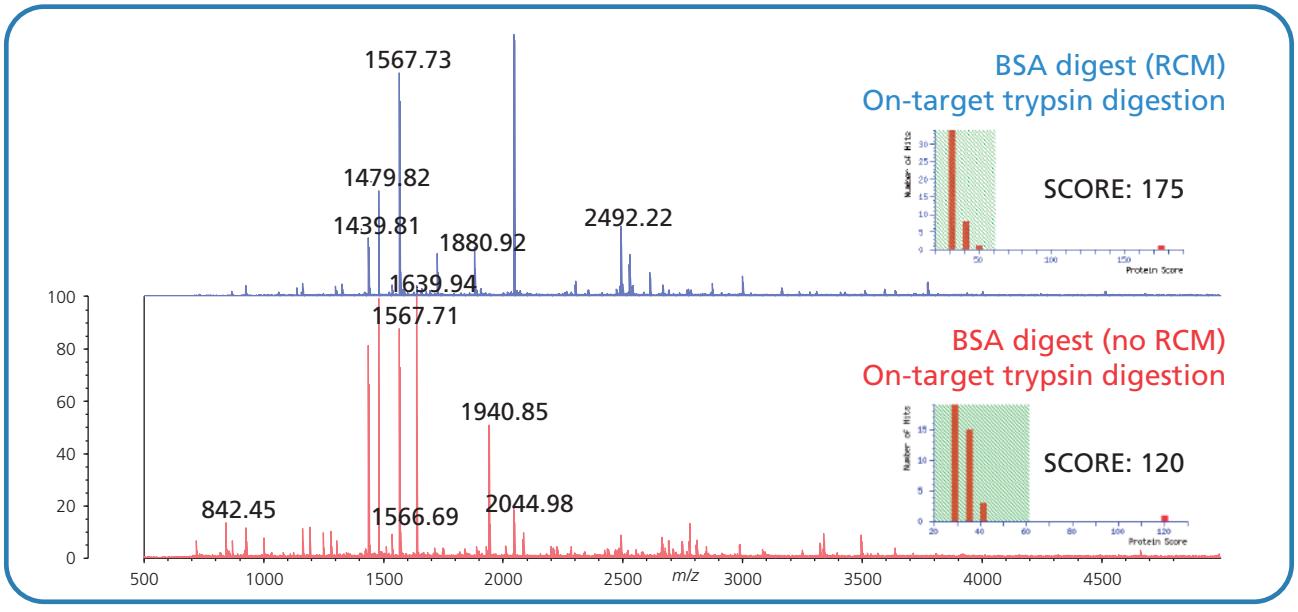

Figure 3. Comparison between the BSA on-target trypsin digestion with and without RCM.

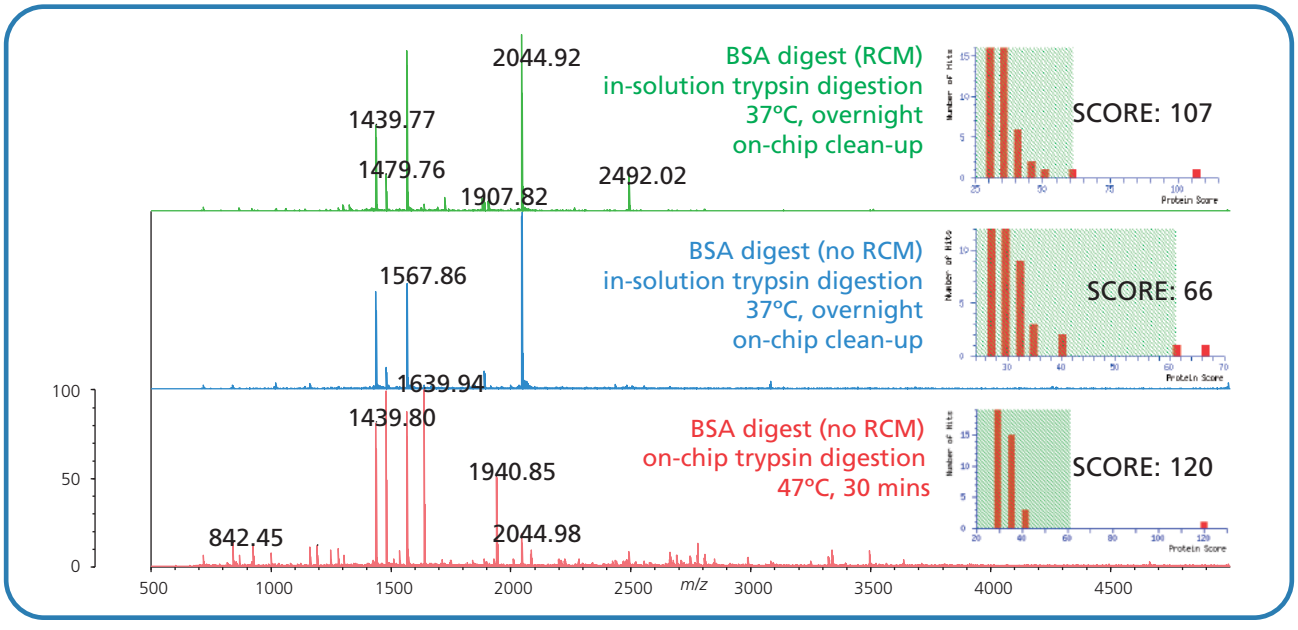

Figure 4. Comparison between the BSA on-target (no RCM) and in-solution (with and without RCM) trypsin digestion.

On-chip reduction/alkylation

The capability of the target to perform the processes of reduction and alkylation has been evaluated. Ovalbumin and haemoglobin proteins were tested. Figure 5a illustrates the intact mass check post-RCM of haemoglobin. Peaks with a mass shift of 57 Da with respect to the alpha-subunit (one cysteine) and beta-subunit (two cysteines) are observed as evidence of the carboxyamidomethylation that occurred. The result of the on-target RCM and trypsin digestion is shown in Figure 5b.

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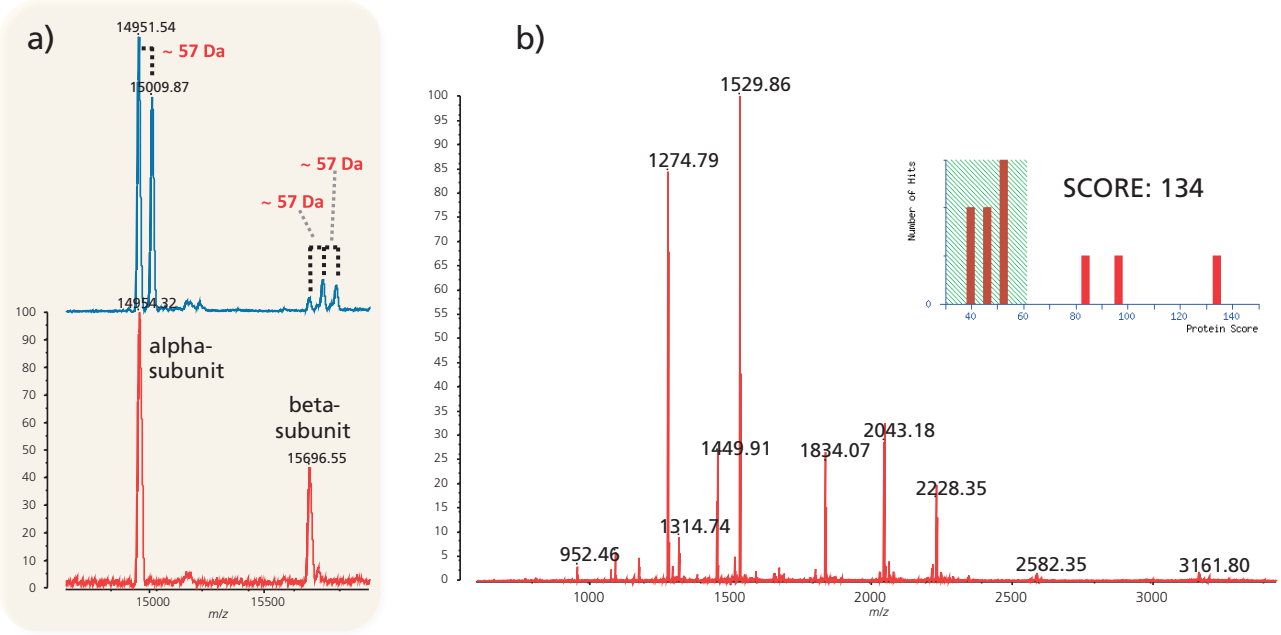

Figure 5. a) on-chip RCM of haemoglobin (red trace: native species; blue trace: post-RCM species) ; b) Haemoglobin on-target RCM and trypsin digest.

Figure 6a illustrates the intact mass check post-RCM of ovalbumin. A ΔM of 343 Da is expected and consistent with a total of six cysteines. However, such a relatively small delta-mass is difficult to be accurately determined at the low-resolution typical of the linear-mode MALDI systems. The spectrum of the ovalbumin post-RCM provides evidence of the mass-shift expected. The result of the on-target RCM and trypsin digestion highlights a peptide-rich spectrum and a high score (Figure 6b).

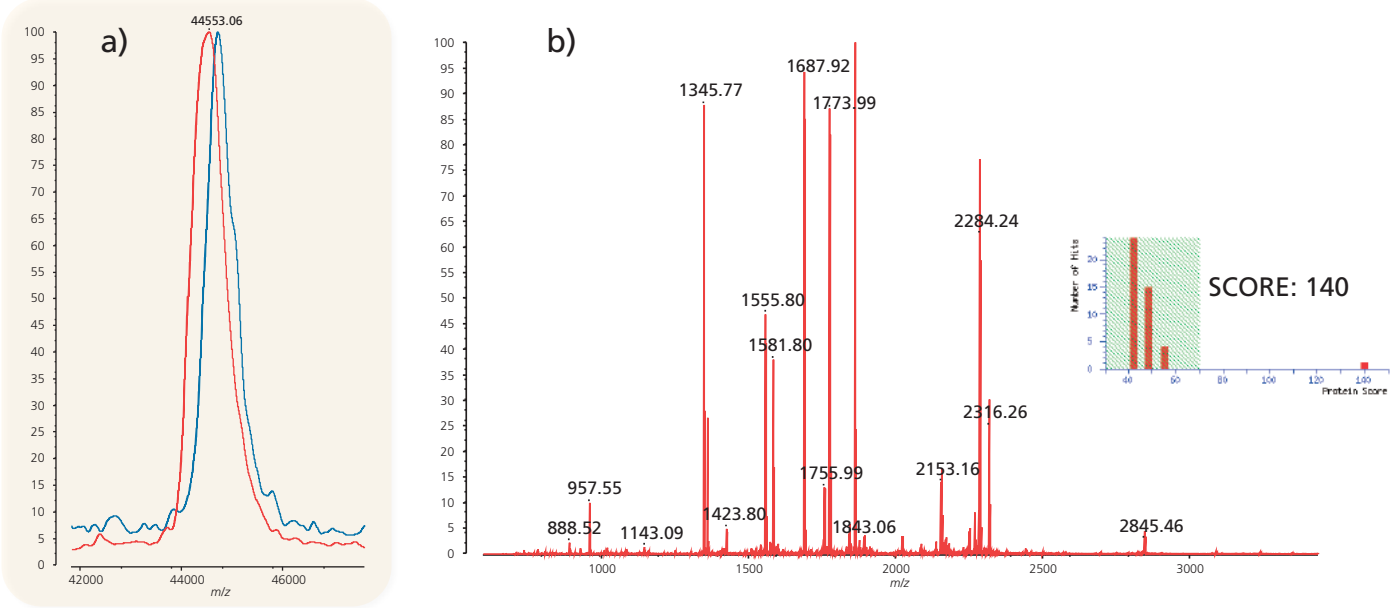

Figure 6. a) on-chip RCM of ovalbumin (red trace: native species; blue trace: post-RCM species) ; b) Ovalbumin on-target RCM and trypsin digest.

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On-chip deglycosylation

N-glycosylation of proteins is known to be a biologically important post-translational modification. The glycans are involved in many biological regulation and recognition processes, and can generate a high variability in composition and mass of the glycoprotein. The glycan removal is most commonly achieved through enzymatic cleavage (e.g. PNGase F). Figure 7a illustrates the stages of optimization of the on-target deglycosylation process. As can be observed, after a period of 4 hours at a temperature of 45°C a similar extent of glycan removal was observed to the in-solution method (green trace). This turned out being advantageous over the common overnight/37°C treatment (Figure 7b, blue trace). Moreover, the peak with a ΔM ~ 1.4 kDa (a common fucosylated biantennary glycan core accounts for 1,446 Da) with respect to the reduced/alkylated species is present as evidence of the successful deglycosylation.

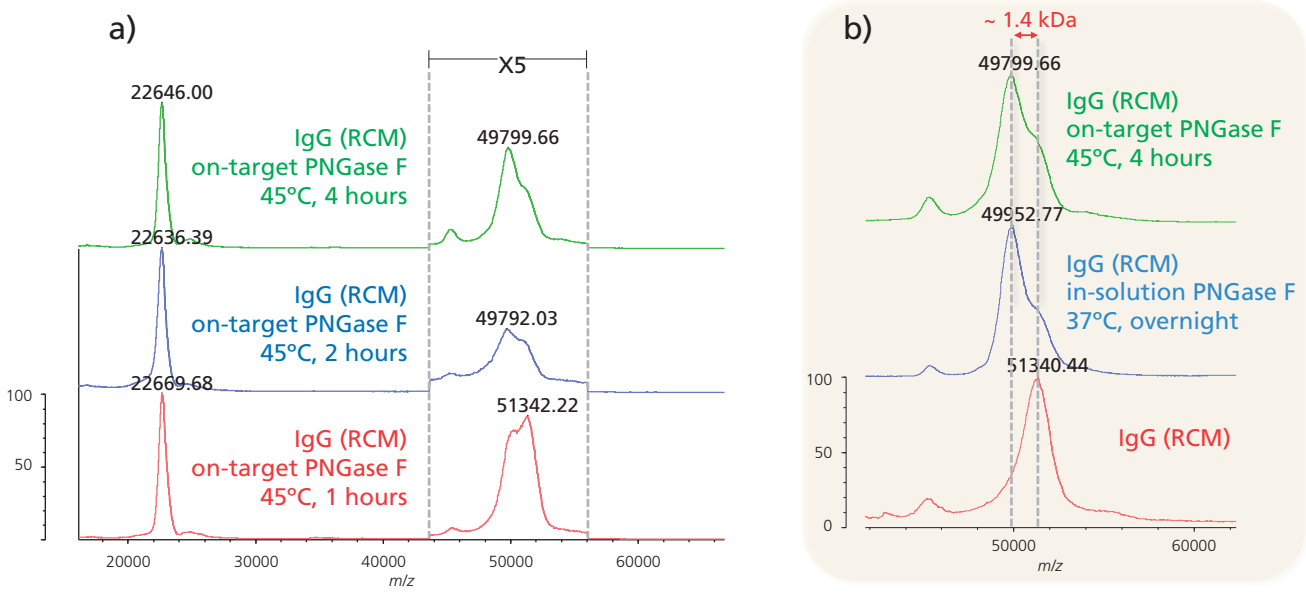

Figure 7. a) Optimization of the on-target deglycosylation;
b) Comparison between the on-target and in-solution deglycosylation.

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Figure 8 shows the results of the deglycosylation of ovalbumin. As can be seen, evidence of the completion of the deglycosylation process is given by the shift of the ovalbumin peak of around 1.4 kDa with respect to the reduced/alkylated form.

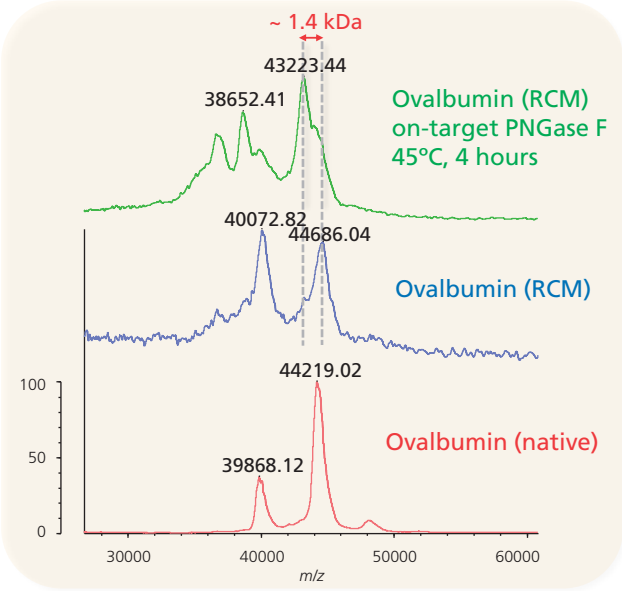

Figure 8. on-target ovalbumin deglycosylation.

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