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THE USE OF LABEL-FREE EXPRESSION PROFILING FOR THE DETECTION OF C-REACTIVE PROTEIN IN HUMAN SERUM

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INTRODUCTION

A common goal of proteomic experiments is the qualitative identification of proteins from biological samples. However, the study of protein expression levels between samples has become increasingly important and several approaches utilizing stable isotope labelling of samples in combination with separation and subsequent analysis by mass spectrometry, have been described [1,2]. In addition, a previous study using an oa-Tof mass spectrometer, in a parallel manner, provided enhanced protein identification in combination with relative protein quantitation via isotope labelling [3].

We have recently presented a concept for qualitative and quantitative protein profiling using LC/MS, without the use of isotope labelling [4]. This strategy allows the relative change in abundance of individual peptides, and proteins, in highly complex mixtures

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to be determined. A key requirement in the studies is retention time reproducibility of the Waters nanoACQUITY UPLC™ System, coupled with the elevated resolution and mass measurement accuracy afforded by the Micromass® Q-Tof Premier™ mass spectrometer, which provides the selectivity and specificity necessary to allow ions to be identified and correlated across identically prepared control and experimental sample sets.

Here we have used this analytical approach to monitor C-reactive protein (CRP) in normal human serum and in serum containing an elevated level of CRP.

C- reactive protein (CRP).

C-reactive protein is part of the pentraxin family; and is characterized by discoid assembly and Ca^{2+} dependant ligand binding. It is well recognised as a non-specific acute phase protein produced by the liver in response to tissue injury, infection, and inflammation. It is found at relatively low levels in normal serum, with the normal range for CRP typically 200 to 5000 µg/mL. However, in response to an acute event, circulating levels may increase up to 100 fold. [5]. The protein, first discovered over 70 years ago, is receiving attention as evidence mounts suggesting that elevated levels may indicate a number of diseases [5,6].

METHODS

An aliquot of normal control serum and elevated CRP serum were depleted of the six most abundant proteins (Albumin, IgG, anti-trypsin, IgA, transferrin and haptoglobulin) using the multiple affinity removal system (Agilent, Palo Alto, CA).

- 40µL of serum was diluted to 200µL and injected onto the depletion column using the Waters Alliance® Bioseparations system.
- The flow through fraction was collected, washed with 100mM NH_4HCO_3 and concentrated to 300µL.

Sample digestion

A 50µL aliquot of each depleted serum sample was reduced, alkylated, and subsequently digested using sequencing grade trypsin (Promega, Madison, WI) in the presence of RapiGest™ SF to a total volume of 70µL. The resulting CRP elevated serum was diluted 1:5 with 5 µL injected on-column. The normal serum was diluted 1:1 and 5 µL injected. Enolase and Alcohol Dehydrogenase (digests) were spiked into both samples as internal standards. Samples were analysed by nanoscale LC/MS as previously described [4].

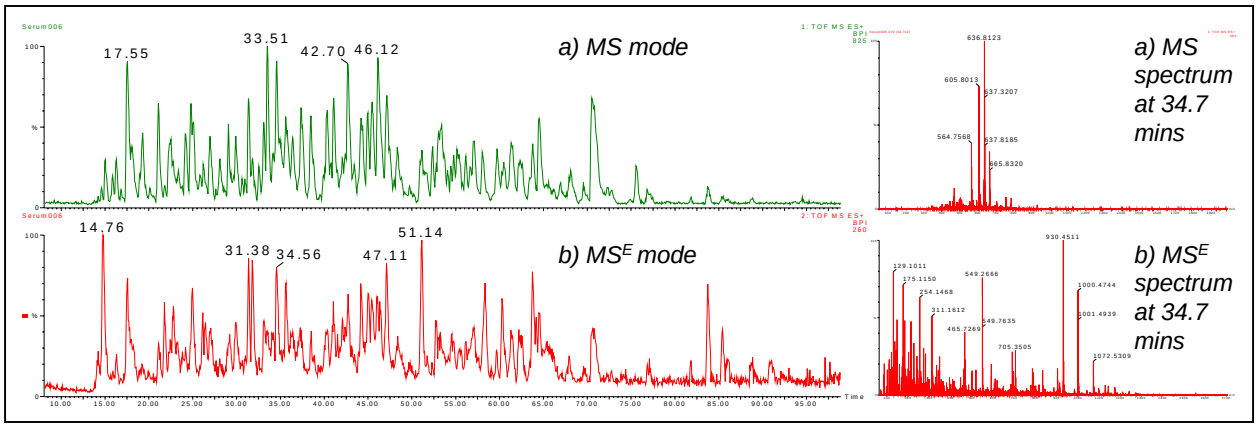

Figure 2. Nanoscale LC/MS analysis of a tryptic digest of depleted, CRP elevated, human serum

Mass Spectrometry

MS data was acquired using an alternating scan function, switching between low and elevated collision energy, during a single acquisition. The low collision energy was 8eV; whilst the elevated collision energy ramped from 23 – 33eV. An integration time of 1.5 seconds was used for each function and the lockmass reference channel was sampled every 30 seconds. Glu-Fibrinopeptide B (accurate mass of doubly charged species is 785.8426 amu) was infused in the enabled NanoLockSpray™ source. The TOF resolution, used in all experiments, was greater than 17,500 FWHM (W-Optics™).

Bioinformatics

Multiple data files per sample were processed using the Waters® Protein Expression System Informatics. The raw data was interrogated using an automated algorithm [4]. The de-isotoped monoisotopic exact mass, HPLC retention time and peak intensity/area for each detectable peptide was extracted.

The low energy data includes the exact mass of all detectable peptide molecular ions, whilst the parallel fragmentation data from the elevated energy scans provides information about the amino acid sequence for the peptide.

Results

Nanoscale LC/MS analysis of a depleted CRP elevated Human serum digest are shown in Figure 2. Chromatograms represent the low energy (2a) and elevated energy (2b) base peak intensity (BPI) plots. Inset is the mass spectrum obtained from each function at a time of 34.7 minutes. It can be seen that a number of ions -mainly doubly charged -are present in the source at one time. Examination of the corresponding elevated energy spectrum gives an indication of the complexity of the fragment ion data.

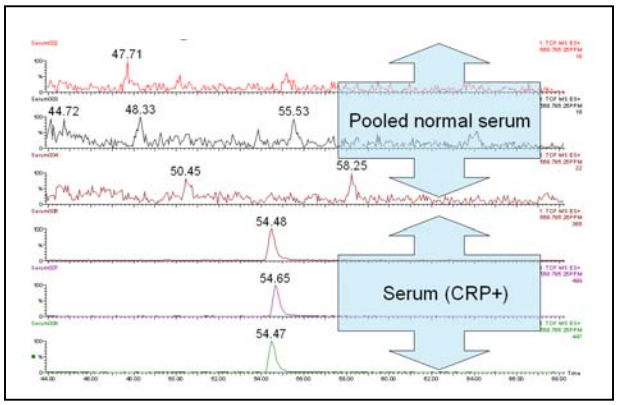

Figure 3. Exact mass chromatograms for m/z 568.785 (2+), from the analysis of the depleted normal (top) and CRP elevated serum (bottom).

Presented in Figure 3 are exact mass chromatograms for a single tryptic peptide, from the analysis of the depleted normal and depleted CRP elevated serum. In the CRP elevated serum the tryptic peptide GYSIFSYATK from the CRP sequence is present in all replicate

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experiments, with good signal to noise. In this case, the level of CRP in the serum was elevated to approximately 100 µg/mL.

Our preliminary experiments show that CRP can be detected in the range 1-100 µg/mL in human serum. Endogenous levels of CRP can be identified in the control serum with several tryptic peptides detected.

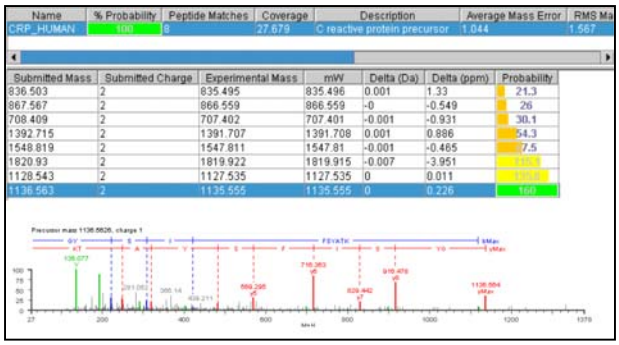

Figure 4. Identification of CRP from the LC/MS expression data

The browser picture, Figure 4**,** shows the identification of CRP from the LC/MS expression analysis of the elevated CRP serum. Eight peptides matched to human C-reactive protein precursor, corresponding to 27.7% sequence coverage, with an average mass error of 1.04 ppm. The matched fragment ions are highlighted in the display for the peptide GYSIFSYATK**.**

Peptide level quantification

The peptide ions (EMRT's) from replicate injections were clustered by their exact mass and retention time. These clustered EMRT pairs can be plotted to display the up and down regulation of peptides between samples. Subsequent databank searching of selected clusters leads to identification of the protein(s). An example is shown in Figure 5, where the mass of the peptide cluster (X-axis) is plotted against the natural log (ln) of the expression ratio (Y-axis). In this example we compare the normal control serum (sample 1) against the elevated CRP serum (sample 2). A total of 2,742 EMRT's are displayed in this plot. From this plot groups of EMRT's are selected by their 'fold change' (shown highlighted in blue) for databank searching. The result of this search identifies CRP, (Figure 5 inset).

In addition to the peptides (EMRT's) that indicate an up-regulation of CRP in the sample, several groups of EMRT's exhibited a significant down regulation. Selection of this group of clusters for databank searching identifies the proteins Complement C4 and apo-lipoprotein H. Interestingly Complement C4 is part of the acute-phase plasma protein response and its down regulation in this sample is unexpected.

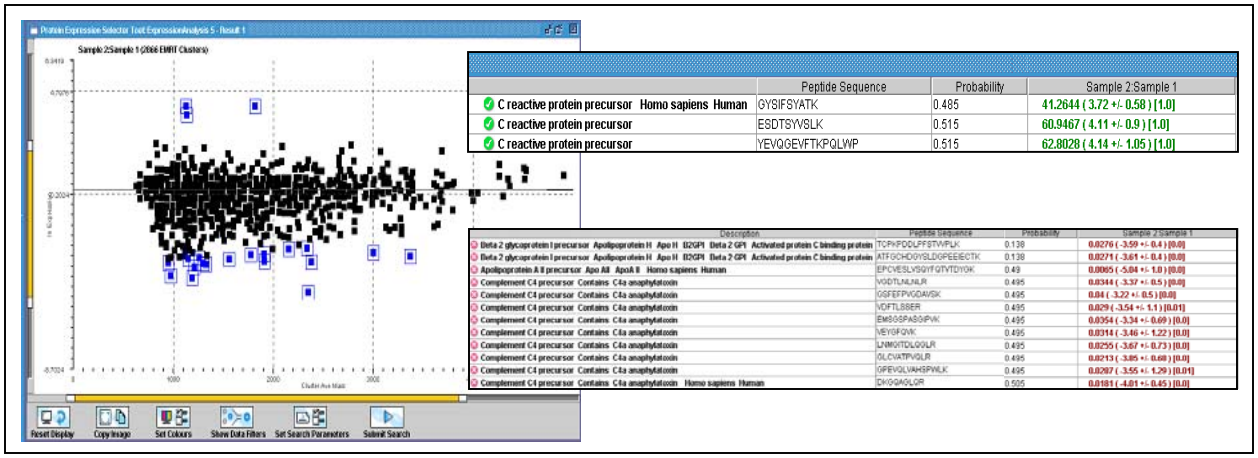

Figure 5. EMRT plot displaying cluster average mass vs expression ratio. Highlighted in blue are peptides whose expression ratios differ significantly between samples. The up-regulated peptides identify the CRP sequence when submitted to a databank search and the down-regulated peptides identified a number of other proteins, see inset panel.

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CONCLUSIONS

- Expression profiling of C-reactive protein (CRP) in depleted human serum has been shown using a label-free LC/MS based method
- The use of an affinity based depletion system was required to enable identification of CRP in both control and CRP elevated samples.
- Relative quantification of CRP could be made at the peptide level with the mean value of 55 calculated from 3 peptides.
- In addition to the up regulation of CRP, a number of further proteins were down regulated in the patient serum.
- This approach provides a comprehensive method of relative protein profiling suitable for time course or multi-patient studies

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